

PhD Thesis

**The trafficking and signaling of HCMV encoded
chemokine receptors**

submitted by

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for the Academic Degree of

**Doctor of Philosophy
(Ph.D.)**

at the

Medical University of Graz

Institute of Experimental and Clinical Pharmacology

under Supervision of

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2010

Acknowledgements

I would like to thank my supervisor Maria Waldhoer for all the support, feedback and friendship. I remember those times, when nothing seemed to work out – but you always believed in me and my ability to accomplish my PhD. The preparation of this document would not have been possible without you.

I also want to express my gratitude to Jennifer Whistler whose expertise added considerably to this work. I really appreciate that you welcomed me into your group – I enjoyed working in San Francisco very much and learned a lot. Thank you for all your support and the continuous exchange of ideas. Lene - thank you for supervising and helping me during my stay in SF. You welcomed me into your home and your life – thank you for being such a great friend.

Research is teamwork – that is one important lesson I learned during the last three years. I want to thank all the lab members, co-workers and dear friends for their support, help and all the discussions – not only about science, but about life. Work would not have been that enjoyable and fun without you! My special thanks go to Veronika and Wolfi for all their help in the lab. You are the ones that are holding this group together. Lisi - thank you for the great teamwork on our joined project. At times, I would have been lost without you. I will truly miss you all and the fun times we had together.

I also want to express my gratitude to my committee members Akos Heinemann and Helmut Schaidler for their feedback. Thanks to Akos for the critical reading of my thesis.

Karin Osibow was the one person to go to, when experiencing any problems with the PhD curriculum. Thank you Karin – you really made life so much easier.

My special thanks go to my family for the unconditional support and continuous encouragements throughout my entire life. I am very grateful that you are always there for me – in good times and in difficult times. Thank you so much for believing in me!

Markus, I don't even have the words to tell you how much I thank you for being there for me every single day! I know, I can always count on you and that makes all the difference.

Table of contents

Table of contents.....	I
Abbreviations.....	III
Summary.....	V
Zusammenfassung	VII
1. Introduction.....	1
1.1. G protein coupled receptors.....	1
1.1.1. G protein mediated signaling.....	3
1.1.2. “Non-classical” signaling pathways of 7TM/GPCRs.....	5
1.1.3. Homo- and heteromerization of 7TM/GPCRs.....	6
1.1.4. Trafficking of 7TM/GPCRs.....	9
1.1.5. Post-endocytic sorting or 7TM/GPCRs	11
1.1.5.1. The recycling pathway.....	13
1.1.5.2. The lysosomal/degradative pathway.....	13
1.1.5.3. GASP-1	16
1.2. The chemokine system.....	18
1.3. The human cytomegalovirus.....	21
1.3.1. The HCMV encoded receptors US27 and UL78	23
1.3.2. The HCMV encoded receptor UL33	24
1.3.3. The HCMV encoded receptor US28.....	25
2. Results.....	32
2.1. Part I : The signaling and trafficking of US28.....	32
2.1.1. Background and specific aims	33
2.1.2. Protein-protein interaction of GASP-1 and US28.	33
2.1.3. The post-endocytic sorting of US28	34
2.1.4. GASP-1 mediated degradation of US28.....	44
2.1.5. Recycling of US28 in the absence of GASP-1	45
2.1.6. Summary – Aim I	47
2.1.7. US28 mediated IP accumulation	47
2.1.8. Transcription factors activation by US28	51
2.1.9. Summary - Aim 2	54

Introduction

2.2.	Part II: Heteromerization of HCMV encoded 7TM/GPCRs	56
2.2.1.	Background and specific aims	56
2.2.2.	Interaction of HCMV encoded 7TM/GPCRs	58
2.2.3.	Summary – Aim 1	64
2.2.4.	Co-localization and interaction of CCR5 and US28.....	65
2.2.5.	Co-localization of UL78, UL33 and CCR5	68
2.2.6.	Co-localization of US28 and H4R	71
2.2.7.	Summary- Aim 2	74
3.	Discussion.....	76
4.	Materials	83
4.1.	Reagents.....	83
4.2.	DNA constructs.....	84
4.3.	Equipment and Instrumentation.....	85
5.	Methods	86
5.1.	Cell culture.....	86
5.2.	Lentivirus production and knock-down of endogenous GASP-1	86
5.3.	Immunocytochemistry	86
5.4.	Recycling experiments.....	88
5.5.	Reporter gene assays.....	88
5.6.	ELISA (enzyme linked immunosorbent assay)	89
5.7.	Inositol Phosphate (IP) accumulation assay	89
5.8.	Isolation and culturing of Mouse Embryos Fibroblasts (MEFs)	90
5.9.	Stable transfection of HEK293 cells.....	91
5.10.	Statistical analysis.....	91
6.	Appendix.....	110

Abbreviations

7TM	Seven transmembrane
AC	Adenylylcyclase
AP-2	Adaptor protein-2
AT1	Angiotensin 1 receptor
B2-AR	Beta 2 adrenergic receptor
B2R	Bradykinin 2 receptor
BRET	Bioluminescence resonance energy transfer
CB1R	Cannabinoid receptor 1
CCR5	CC chemokine receptor 5
cGASP-1	C-terminally truncated GPCR associated sorting protein 1
COX-2	Cyclooxygenase-2
CREB	cyclic AMP responsive element binding protein
D5R	Dopamine 5 receptor
DAG	Diacylglycerol
DNA	Desoxyribonucleic acid
DOR	Delta opioid receptor
EBV	Epstein Barr virus
EEA.1	Early endosomal antigen 1
EGFR	Endothelial growth factor receptor
ER	Endoplasmatic reticulum
ERK	Extracellular signal regulated kinase
ESCRT	Endosomal sorting complex required for transport
FAK	Focal adhesion kinase
FRET	Fluorescence resonance energy transfer
GASP-1	G protein coupled receptor associated sorting protein 1
GPCR	G protein coupled receptor
GRK	G protein coupled receptor kinase
HCMV	Human cytomegalovirus
H4R	Histamine 4 receptor
HHV-5	Human herpesvirus -5
HIV	Human immunodeficiency virus
Hrs	Hepatocyte growth factor regulated tyrosine kinase substrate

Abbreviations

IP3	Innositol triphosphate
KO	Knock out
LAMP	Lysosomal associated membrane protein
LHR	Luteinizing hormone receptor
MAPK	Mitogen activated protein kinase
MCP-1	Monocyte chemotactic protein 1
MEF	Mouse embryonic fibroblast
MIEP	Major immediate early promoter
MIP-1 β	Macrophage inflammatory protein 1 beta
MOR	Mu opioid receptor
MVB	Multivesicular bodies
NFAT	Nuclear factor of activated T cells
NF- κ B	Nuclear factor kappa B
ORF74	Open reading frame 74
PDZ	Postsynaptic density 95/disc large/zonula occludens-1
PKC	Proteinkinase C
PLC	Phospholipase C
PM	Plasma membrane
rGASP-1	Rescue GPCR associated sorting protein 1
shGASP-1	Short hairpin RNA targeting GASP-1
shScr	Short hairpin RNA scrambled
SMC	Smooth muscle cells
SNX-1	Sorting nexin -1
SRF	Serum response factor
STAM	Signal transduction adapter molecule
T1R1	Taste receptor type 1
TLR4	Toll like receptor 4
TSG101	Tumor suppressor gene 101
UEV	Ubiquitin E2 variant
UL33	Unique long 33
UL78	Unique long 78
US28	Unique short 28
US27	Unique short 27
WT	Wild type

Summary

Human cytomegalovirus (HCMV) is a widespread pathogen that infects up to 80% of the human population. In healthy hosts, HCMV infection does not cause any disease, but in immunocompromised patients it is implicated in severe complications. After primary infection viral latency is established by an array of mechanisms to evade the immune response. HCMV expresses four viral 7 transmembrane (7TM)/G protein coupled receptors (GPCRs) – US28, US27, UL33 and UL78 – that show close homology to endogenous chemokine receptors. The HCMV encoded receptors US27, UL33 and UL78 are so called “orphan” receptors, whose functions in the viral life cycle have not yet been identified. On the other hand, US28 was shown to bind endogenous chemokines and to mediate ligand-dependent as well as ligand-independent functions. Due to the ability of US28 to activate multiple signaling cascades, the receptor was suggested to be implicated in the early reprogramming of newly infected cells and viral dissemination. US28 is predominantly localized intracellularly in multivesicular bodies and late endosomes/lysosomes. It is insinuated that this might be the place where US28 is incorporated into the viral envelope during the final stages of virus assembly.

In my thesis I demonstrate that the viral receptor US28 is targeted to late endosomes/lysosomes by a direct interaction with the GPCR-associated sorting protein (GASP-1). GASP-1 is known to be an important sorting protein that targets several 7TM/GPCRs to the lysosomal pathway for degradation. The prevention of the US28/GASP-1 interaction – e.g. by overexpression of a dominant negative competitor or by knock down of GASP-1 using shRNA - significantly delays degradation of US28 and promotes its recycling. Furthermore, the modification of endogenous GASP -1 levels is directly connected with an alteration of the constitutive signaling capacity of US28. When GASP-1 is overexpressed, the inositol phosphate (IP) accumulation and the activation of transcription factors NF- κ B (nuclear factor κ B) and CREB (cyclic AMP responsive element binding protein) are significantly increased. On the other hand, the disruption of the GASP-1/US28 interaction reduces the signaling capacity of US28. Hence, this study shows for the first time the existence of a direct connection between GASP-1 dependent receptor sorting and the signaling activity of the receptor.

Summary

In addition to the regulation through sorting processes, the trafficking and signaling properties of receptors can be altered through hetero-/oligomerization. I provide evidence that the signaling capacity of US28 is controlled by the interaction with other virally encoded 7TM/GPCRs. Co-expression of US28 with the orphan HCMV encoded receptors UL33 and UL78 decreases the activation of transcription factors by US28. In contrast, co-expression of US27 significantly enhances the signaling activity of US28. Hence, it seems that US27, UL33 and UL78 encoded by HCMV are able to differentially modulate the signaling of US28.

Further on, I demonstrate that US28 is able to interact with endogenous 7TM/GPCRs, such as histamine 4 receptor (H4R) or CC chemokine receptor 5 (CCR5). Co-expression of US28 reduces the overall expression level of H4R and both receptors co-localize in the endoplasmatic reticulum (ER). Hence, the heteromerization with US28 might inhibit the export of H4R to the plasma membrane. Interestingly, the internalization-deficient US28 Δ 300 does not alter the surface expression of H4R, suggesting that the constitutive endocytosis/trafficking of US28 is involved in the ER retention of H4R. Moreover, US28 is also able to heteromerize with the endogenous CC chemokine receptor 5 (CCR5). Transient expression of US28 in stable transfected CCR5 cells decreases the surface expression of CCR5 and leads to its retention in the ER. Finally, the interaction with CCR5 also significantly reduces the activation of the transcription factor NF- κ B by US28.

Summarized, I provide evidence that the activity of US28 is tightly regulated by various processes, such as (i) the direct interaction with GASP-1 and (ii) the heteromerization with HCMV-encoded and endogenous 7TM/GPCRs. US28 is thought to be implicated in HCMV-inflicted pathogenesis, the development of vascular disease and cancer. Hence, preventing the US28/GASP-1 interaction or targeting US28-containing heteromers with selective ligands might be clinically relevant and guide future drug design.

Zusammenfassung

Das humane Cytomegalovirus (HCMV) stellt ein weitverbreitetes Pathogen dar, das große Teile der Bevölkerung infiziert und bei Individuen mit unterdrücktem Immunsystem zu ernsthaften Komplikationen führen kann. Eine Infektion mit HCMV bringt eine lebenslange Latenz mit sich, die durch verschiedene Mechanismen zur Umgehung einer Immunantwort gewährleistet ist. HCMV exprimiert beispielsweise vier G Protein gekoppelte Rezeptoren (GPCRs) – US28, US27, UL33 und UL78 - die eine ausgeprägte Homologie mit endogenen Chemokinrezeptoren aufweisen. Die Funktionen von US27, UL33 und UL78 sind bislang noch nicht geklärt. Für US28 wurden andererseits schon einige interessante Eigenschaften identifiziert, wie die Fähigkeit zur konstitutiven Endozytose oder die konstitutive Signalaktivität. Weiters wurde gezeigt, dass US28 verschiedene endogene CC-Chemokine und Fraktalkin binden kann. Basierend auf der Vielzahl von Signal-Kaskaden die durch US28 aktiviert werden können, wird über eine Rolle des Rezeptors in der Reprogrammierung von neuinfizierten Zellen sowie der Verbreitung des Virus im Körper spekuliert. Ermöglicht werden diese Funktionen eventuell durch die Anwesenheit von US28 auf den Hüllen der HCMV Virionen. Da der Rezeptor hauptsächlich in den intrazellulären Kompartimenten lokalisiert ist, wird vermutet, dass US28 dort in die viralen Hüllen eingebaut wird.

Ich habe im Zuge dieser Arbeit einen Mechanismus identifiziert, durch den der Rezeptor US28 gezielt zu diesen Kompartimenten transportiert wird. Das GPCR Assoziierte Sorting Protein 1 (GASP-1) bewirkt durch eine direkte Interaktion mit dem cytoplasmatischen Terminus des Rezeptors eine Sortierung zu den Lysosomen. Wird diese Interaktion verhindert – z.B. durch Überexpressierung eines dominant negativen Proteins oder durch den „knock down“ von GASP-1 mittels shRNA – wird einerseits der proteolytische Abbau verzögert und andererseits der Rücktransport zur Zellmembran, das „Recycling“ verstärkt. Interessanterweise hat die Manipulation des endogenen GASP-1 Gehaltes nicht nur Einfluss auf die Sortierung, sondern auch eine veränderte Signalaktivität des Rezeptors zur Folge. Die Überexpressierung von GASP-1 führt zu einer gesteigerten Aktivität, wobei die Blockade der Interaktion mit GASP-1 eine signifikant reduzierte Signaltransduktion durch US28 bewirkt. Ich zeige hiermit zum ersten Mal auf, dass die Interaktion eines GPCRs mit GASP-1 nicht nur zu einer

Zusammenfassung

veränderten Sortierung führt, sondern auch direkten Einfluss auf die Aktivität des Rezeptors hat.

Die Aktivierung von Signalwegen kann jedoch nicht nur durch eine differentielle Sortierung des Rezeptors zwischen Recycling und Degradierung kontrolliert werden, sondern auch durch Heteromerisierung mit anderen Rezeptoren. HCMV exprimiert neben US28 noch drei weitere GPCRs, deren Funktionen noch nicht geklärt sind. Bei der Untersuchung einer möglichen Interaktion dieser Rezeptoren hat sich gezeigt, dass die Signaltransduktion von US28 durch Heteromerisierung beeinflusst wird. Interessanterweise bewirkten UL33 und UL78 eine reduzierte, und US27 eine gesteigerte Aktivität von US28. Diese gegensätzliche Modulation von US28 stellt möglicherweise einen Mechanismus dar, um je nach Beschaffenheit optimale Bedingungen für HCMV zu schaffen.

Weiters konnte ich zeigen, dass US28 nicht nur mit HCMV Rezeptoren, sondern auch mit zellulären Rezeptoren, wie beispielsweise dem CC Chemokinrezeptor 5 (CCR5) oder dem Histaminrezeptor 4 (H4R) interagiert. Interessanterweise vermindert US28 den Export von CCR5 und H4R aus dem Endoplasmatischen Retikulum und verursachte damit eine reduzierte Expression dieser Rezeptoren an der Zelloberfläche. Zusätzlich hat die Interaktion zwischen US28 und CCR5 auch funktionelle Auswirkungen auf die Signaltransduktion von US28 und eine verminderte Aktivierung von Transkriptionsfaktoren zur Folge. Durch die beeinträchtigte Oberflächenexpression von CCR5 und H4R in der Gegenwart von US28 ist eine Hemmung der Funktionalität und Signaltransduktion dieser Rezeptoren möglich. Dies würde einen zusätzlichen Weg zur Immunmodulation durch HCMV darstellen und erfordert daher eine weitere Aufklärung.

Zusammenfassend konnte ich aufzeigen, dass die konstitutive Aktivität von US28 durch mehrere Mechanismen, wie (i) die GASP-1 abhängige Sortierung oder (ii) die Heteromerisierung mit viralen und zellulären Rezeptoren reguliert wird. Diese Erkenntnisse zeigen neue Möglichkeiten auf, um US28-abhängige Funktionen zu beeinflussen und ebnen den Weg für neue therapeutische Ansätze.

1. Introduction

1.1. G protein coupled receptors

G-protein coupled receptors (GPCRs) are often referred to as seven transmembrane (7TM) receptors and comprise the largest superfamily of membrane proteins. These receptors are implicated in translating extracellular signals into intracellular messages that ultimately lead to cellular responses. Although belonging to one superfamily, 7TM/GPCRs can be classified into 5 subfamilies on the basis of sequence homology and/or pharmacological characteristics (Fredriksson *et al.*, 2003; Kristiansen, 2004). The main subfamilies comprise the rhodopsin-like receptors (family A), the secretin-like receptors (family B) and the glutamate receptor-like GPCRs (family C) (Pierce *et al.*, 2002). The adhesion family and the frizzled/smoothed receptor family make up the minor subfamilies (Gether, 2000; Horn *et al.*, 1998).

The largest and most diverse subfamily of 7TM/GPCRs is formed by the rhodopsin-like/family A receptors. The basic structural features of these receptors were identified with the use of two dimensional models of rhodopsin (Schertler *et al.*, 1993). However, important advances were made by studying the three dimensional crystal structure of rhodopsin (Palczewski *et al.*, 2000) and the β 2-adrenergic receptor (β 2-AR) (Rasmussen *et al.*, 2007), which for example revealed a cytoplasmic eighth helix. Additionally, these studies showed that some motifs among family A receptors, such as the E/DRY motif on the transmembrane domain 3 as well as the NPxxY_(5,6) motif on the transmembrane domain 7 are highly conserved (Fritze *et al.*, 2003; Gether, 2000). The highest variability was found in the carboxyl terminus, the amino terminus and the intracellular loop spanning transmembrane domain 5 and 6. Comparison of the structures of rhodopsin and β 2-AR showed that there are substantial alterations in their TM helices, which might account for their different activation states (see Fig. 1). Furthermore, it was observed that both receptors show major differences on their extracellular termini (Rasmussen *et al.*, 2007; Cherezov *et al.*, 2007; Rosenbaum *et al.*, 2007). Nevertheless, rhodopsin and β 2-AR were also reported to share some features, such as a network of hydrogen bonds ranging from their ligand binding pocket to their cytoplasmic side, which was proposed to be involved in favouring activating

Introduction

conformational changes ((Li *et al.*, 2004;Pardo *et al.*, 2007); reviewed in (Weis & Kobilka, 2008)).

Figure 1

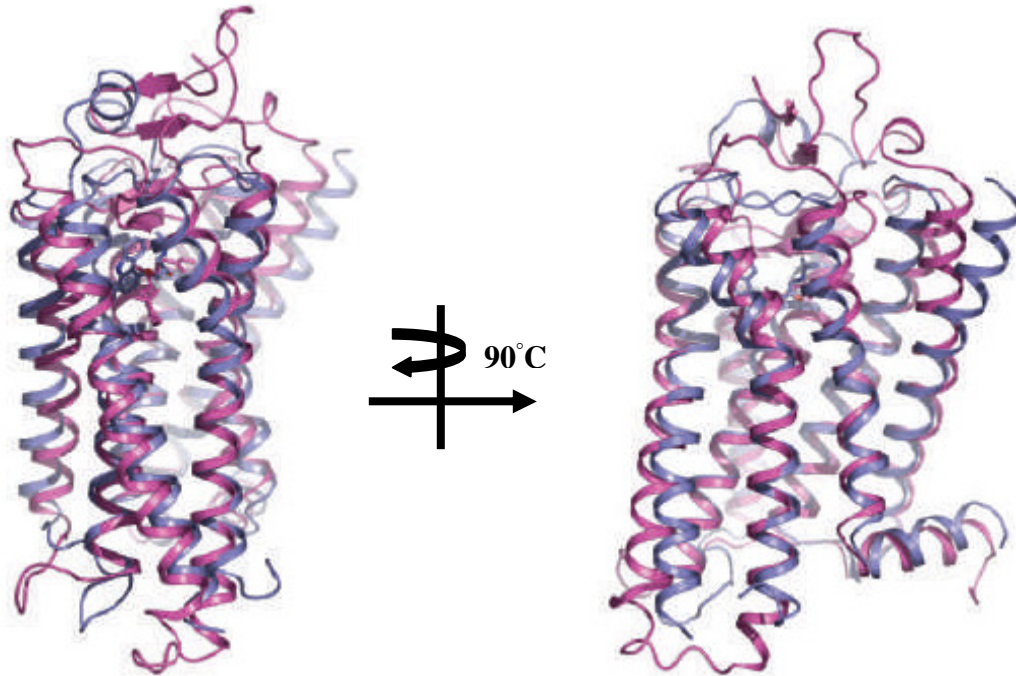


Figure 1 Superimposed crystal structures of *rhodopsin* and *β2-AR* (adapted from (Rosenbaum *et al.*, 2009)).

7TM/GPCRs can be activated by a wide range of ligands including neurotransmitters, hormones, photons and odorants (Klabunde & Hessler, 2002;Gether, 2000). On the other hand, despite the efforts to identify ligands for all the known 7TM/GPCRs, there are still non-machted “orphan” receptors whose functions are still to be determined. Most 7TM/GPCRs analyzed to date were shown to possess a baseline level of constitutive, agonist independent activity, which was suggested to be caused by their state of conformational equilibrium. Agonists and partial agonists are believed to shift the equilibrium towards a more active conformation and thus lead to an increased signaling activity. In contrast to this, inverse agonists change the conformation to a more inactive state, hence inhibiting the basal activity (see Fig. 2). Antagonists do not affect the baseline level of activity of the receptor, but sterically block the binding/activation through other ligands (for reviews see (Kobilka & Deupi, 2007;Rosenbaum *et al.*, 2009)).

Figure 2

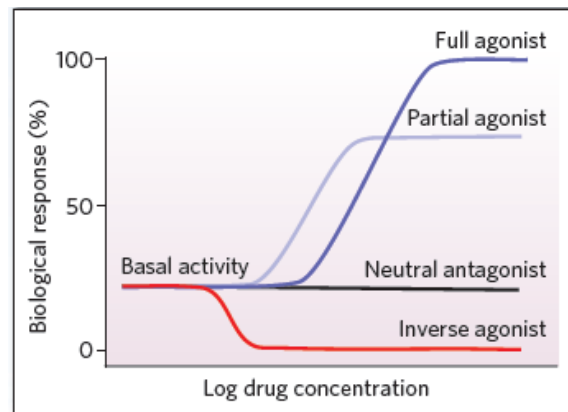


Figure 2 Efficiency of different ligands (adapted from (Rosenbaum et al., 2009)). Full agonists lead to a maximal activation, whereas partial agonists do not induce the full biological response. Neutral antagonists do not change the basal activity of receptors. In contrast, inverse agonists have an inhibitory effect on constitutive receptor activity.

1.1.1. G protein mediated signaling

One way in which 7TM/GPCRs transmit their signals from the cell surface to intracellular effectors is via coupling to heterotrimeric G proteins. G proteins consist of three subunits, a GDP (guanine diphosphate) binding α -subunit as well as a β - and γ -subunit. Activation of 7TM/GPCRs by an agonist induces a conformational change of the receptor, which then catalyzes the exchange of guanine nucleotides bound to the α -subunit. Following the activation of the G-protein, the GTP-bound α -subunit dissociates from the stable $\beta\gamma$ dimer and subsequently the signal transduction is initiated. Termination of the G-protein mediated signalling can be achieved by the hydrolysis of GTP to GDP catalyzed by specific GTPases. To date, 16 different mammalian $G\alpha$ -subunits have been identified, the most important four classes that mediate different signal transduction pathways being: $G_{\alpha s}$, $G_{\alpha i/o}$, $G_{\alpha q}$ and $G_{\alpha 12/13}$ (Cabrera-Vera et al., 2003). Therefore, the selectivity of 7TM/GPCRs for certain $G\alpha$ -subunits is often used to classify their primary signaling pathways (see Fig. 3).

Coupling of 7TM/GPCRs to $G_{\alpha s}$ -proteins leads to the activation of adenylyl cyclase (AC) and thus to an increased accumulation of cAMP (cyclic adenosine 3', 5'-monophosphate) (Fields & Casey, 1997). Elevated levels of cAMP activate the enzyme protein kinase A (PKA). Subsequently, one of the four PKA subunits dissociates and translocates to the nucleus, where it phosphorylates the cAMP responsive element binding

Introduction

protein (CREB) and consequently regulates gene expression (Montminy & Bilezikjian, 1987). Cholera toxin, a bacterial toxin produced by *Vibrio cholerae*, catalyses the irreversible ADP-ribosylation of G α s-subunits and thus leads to a constitutive activation of AC and increased cAMP levels ((De Haan & Hirst, 2004); for review see (Vanden *et al.*, 2007)). In contrast to this signaling cascade, coupling of receptors to Gai/o-proteins has opposing effects on AC and thus lowers the intracellular cAMP levels. Pertussis toxin is widely used to selectively inhibit the signal transduction via Gai/o-proteins. Its mechanism of action involves the ribosylation of the G α -subunit with adenosine diphosphate (ADP), which irreversibly prevents its interaction with the receptor (Katada & Ui, 1982; Murayama & Ui, 1983). The G α q mediated signaling pathway involves the activation of the enzyme phospholipase C- β (PLC- β) and the subsequent production of the second messengers IP3 (inositol triphosphate) and DAG (diacylglycerol). IP3 binds to specific receptors on intracellular Ca²⁺ stores and hence induces the increase of Ca²⁺ levels, whereas DAG leads to the recruitment and activation of protein kinase C- β (PKC- β) (Taylor *et al.*, 1991). Activation of G α 12/13-proteins was reported to yield the interaction with various RhoGEFs and the subsequent stimulation of RhoA dependent signaling cascades (Buhl *et al.*, 1995; Gohla *et al.*, 1998).

Several 7TM/GPCRs show selectivity for specific classes of G α -subunits, whereas others were shown to couple promiscuously to different G α -proteins. The endogenous chemokine receptor CCR5 for example couples only to Gai/o-proteins (Zhao *et al.*, 1998), whereas the virally encoded chemokine receptor US28 was reported to transmit signals via Gai/o, G α 16 and G α q proteins (Billstrom *et al.*, 1998; Casarosa *et al.*, 2001; Waldhoer *et al.*, 2002; Moepps *et al.*, 2008). In addition to the signal transduction mediated by the G α -subunits, many downstream effectors and second messengers can also be activated via the stable $\beta\gamma$ dimer. For instance, it was reported that $\beta\gamma$ complexes induce the MAPK (mitogen activated protein kinase) cascade as well as Ras dependent signaling pathways (Clapham & Neer, 1997; Crespo *et al.*, 1994).

Intriguingly, not only 7TM/GPCRs seem to be able to couple to and transmit signals through heterotrimeric G proteins. Recently, Gong *et al.* reported that the platelet integrin $\alpha_{IIb}\beta_3$ was able to bind to G α 13 proteins upon ligand stimulation. Furthermore, this interaction was suggested to represent a dynamic mechanism for regulating the activity of RhoA (Gong *et al.*, 2010).

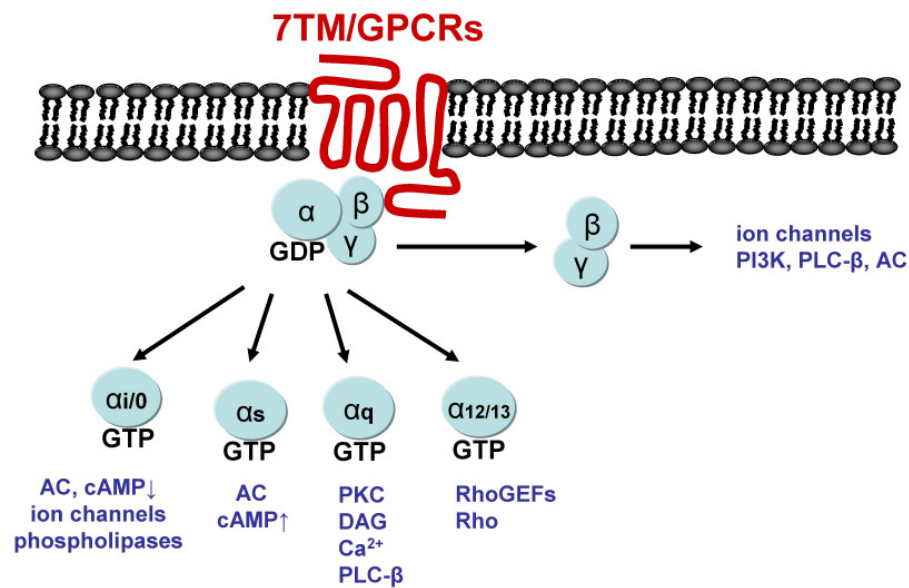
Figure 3

Figure 3 Major G-protein mediated signaling pathways of 7TM/GPCRs. Coupling of receptors to $G_{\alpha i/o}$ proteins inhibits adenyly cyclase (AC), ion channels and phospholipases, whereas coupling to $G_{\alpha s}$ stimulates these effectors. Activation of $G_{\alpha q}$ causes the activation of protein kinase c (PKC) and phospholipase C (PLC). Receptors that couple to $G_{\alpha 12/13}$ are known to induce RhoGEFs (guanine nucleotide exchange factor) and Rho activation. Furthermore, the stable $\beta\gamma$ dimer that dissociates upon activation of the G protein also stimulates effectors, such as PLC, AC or phosphoinositol 3 kinase (PI3K).

1.1.2. “Non-classical” signaling pathways of 7TM/GPCRs

In addition to the “classical” way in which 7TM/GPCRs transmit their signals from the cell surface via coupling to G proteins, new mechanisms of signal transduction have emerged (for review see (Calebiro *et al.*, 2010)). Over the last couple of years, accumulating evidence highlighted the capability of 7TM/GPCRs to signal from intracellular compartments. The first evidence came from Daaka *et al.* who reported that endocytosis of the $\beta 2$ -adrenergic receptor is a prerequisite for the activation of the extracellular signal regulated kinase (ERK) (Daaka *et al.*, 1998). Additionally, β -arrestins were found to anchor activated ERK to the endosomal membranes, facilitating endosomal ERK signaling (DeFea *et al.*, 2000; Terrillon & Bouvier, 2004). The assumption that 7TM/GPCRs transmit their signals only at the cell surface is

furthermore challenged by the finding that the yeast GPCR Ste2 activates PI3K and MAPK at the endosomes (Slessareva *et al.*, 2006). Recently, the thyroid stimulating hormone receptor (TSHR) was reported to increase cAMP production after internalization. Intriguingly, the same study showed that the signaling from the plasma membrane was distinguishable from the signaling response induced at the endosomes (Calebiro *et al.*, 2009). This study thus provides the first evidence that 7TM/GPCR signaling from endosomal compartments is not only possible, but can also be quantitatively different from their signaling induced at the cell surface.

1.1.3. Homo- and heteromerization of 7TM/GPCRs

It is suggested that the activity of 7TM/GPCRs is determined not only by their interactions with various ligands that alter their conformational states, but also through intermolecular interactions with other receptors– e.g. dimerization or oligomerization. To date, it is widely accepted that most, if not all, 7TM/GPCRs are organized in dimeric/oligomeric complexes. Receptor oligomers may be composed of identical (= homomers) or 7TM/GPCRs of different heritage (= heteromers). The first evidence for receptor heteromerization was provided by simple western blot and immunoprecipitation experiments. Nowadays, heteromeric complexes are identified by FRET (fluorescence resonance energy transfer) or BRET (bioluminescence resonance energy transfer) assays. In this approach, one receptor is tagged with a donor molecule (e.g. Renilla luciferase), whereas the other interaction partner is linked to an acceptor molecule (e.g. yellow fluorescence protein). In case both receptors interact and thus are in close proximity (1-10nm), energy from the donor is transferred to the acceptor molecule and the resulting emission can be measured (for review see (Ferre *et al.*, 2009)).

One of the first examples demonstrating the importance of receptor heteromerization was given by the taste receptor type 1 (T1R1) and taste receptor type 3 (T1R3). It was shown that both receptors need to heteromerize to be functional (Nelson *et al.*, 2002). The heteromerization of T1R2 and T1R3 was reported to result in the formation of the sweet receptors (Nelson *et al.*, 2001). Another intriguing example represents the metabotropic GABA_B receptor, which consists of the two isoforms GABA_BR1 and GABA_BR2. It was established that the heteromerization of both isoforms are essential for the cell surface expression of the functional receptor. GABA_BR2 was suggested to

Introduction

function as a molecular chaperone that is important for the proper folding of the other isoform and also guides its transport to the plasma membrane (Galvez *et al.*, 2001). Additionally, the formation of the GABA_BR1/GABA_BR2 dimer is a prerequisite for receptor mediated signaling (reviewed in (Bouvier, 2001)).

The importance of 7TM/GPCR oligomerization is most convincingly illustrated by the functional alterations induced when two different 7TM/GPCRs associate to form heteromeric complexes. Indeed, modifications in pharmacological profiles, signaling activities and trafficking properties have been observed for many 7TM/GPCRs when engaged into heteromeric complexes (Neote *et al.*, 1993; Milligan, 2004; Ferre *et al.*, 2009). Sartania and co-workers showed that the wild-type β 2-AR and a mutant β 2-AR form dimers and that agonist binding to only one of the receptors is sufficient to cause endocytosis (Sartania *et al.*, 2007). Additionally, Cao *et al.* observed that co-expression of a recycling deficient β 2-AR inhibited the recycling of the wild-type β 2-AR and yielded its targeting to the lysosomes (Cao *et al.*, 2005).

It was suggested that the presence of receptor dimers in the absence of activating ligands might account for the constitutive level of basal activity shown for several 7TM/GPCRs ((Kroeger *et al.*, 2001); reviewed in (Bouvier, 2001)). The observation that the formation of heteromeric structures can significantly alter the signal transduction of the involved receptors highlights the importance of heteromer selective ligands. One example for such a ligand is the 6'-guanidinoaltrindole (6'-GNTI), that was shown to selectively bind to the DOR (delta opioid receptor)/KOR (kappa opioid receptor) heteromer (Waldhoer *et al.*, 2005).

Interestingly, it was reported that 7TM/GPCRs can also form heteromeric complexes with other classes of proteins, such as ion channel receptors. The dopamine 5 receptor (D5R) for example was shown to interact with the GABA operated chloride channel GABA_A receptor. Agonist stimulation was reported to lead to a crosstalk between both proteins and to an altered signaling activity that was facilitated via direct physical interaction (Liu *et al.*, 2000). 7TM/GPCRs and ion-channel receptors represent the major regulators of neurotransmission and hence their mutual regulation might represent a new mechanism in controlling synaptic signaling. The studies of receptor heteromerization have also been extended towards orphan 7TM/GPCRs, which may

Introduction

associate with non-orphan receptors, thus influencing their functions. The orphan 7TM/GPCR GPR50 for example was found to modulate the signaling activity of the melatonin MT1 receptor (Levoye *et al.*, 2006). The heteromerization of GPR50 with MT1 was shown to inhibit MT1 mediated signaling, which is facilitated via prevention of agonist binding and agonist-induced G protein coupling (Levoye *et al.*, 2006). Hence, it is tempting to speculate that the function of certain orphan receptors might be given through the modulation of other proteins via the formation of heteromeric complexes.

Interestingly, it was also reported that virally encoded GPCRs are able to form heteromeric complexes with human receptors. The G protein coupled receptor BILF1 encoded by Epstein-Barr virus (EBV) for example was shown to heteromerize with various endogenous CC and CXC chemokine receptors (Vischer *et al.*, 2008). The functional consequences of the interaction between the orphan viral receptor BILF1 and the host chemokine receptors are still unknown. However, the authors speculate that heteromerization might represent a way to hijacking human GPCRs, thus influencing their signaling for the benefit of the virus.

Recently, the *in vivo* relevance of 7TM/GPCR heteromerization was confirmed in an animal model, where the luteinizing hormone receptor (LHR) was used as a model for family A 7TM/GPCRs. In a LHR knock out mouse model the authors co-expressed a binding- and a signaling- deficient LHR mutant and showed that intermolecular interactions between these receptors were able to rescue LHR function (Rivero-Muller *et al.*, 2010).

Given the multiple functional consequences of receptor heteromerization, ranging from controlling their biogenesis, trafficking and signaling capacities, it becomes clear that any deregulation might be implicated in disease development. Indeed, several examples can be given that support this hypothesis. An alteration of the relative amount of heteromers of the angiotensin 1 AT1 receptor and the bradykinin B2R receptor were suggested to be associated with preeclampsia and experimental hypertension (bdAlla *et al.*, 2001;bdAlla *et al.*, 2005). In addition, the mutation of the Ca²⁺ sensing receptor, which leads to its retention in the ER, was associated with familial hypocalciuric hypercalcaemia and neonatal hyperthyroidism ((Pidacheva *et al.*, 2006); reviewed in (Milligan, 2008)).

Introduction

Hence, it can be concluded that the principle of receptor heteromerization has important implications for drug design. Furthermore, the discovery of differential or ligand biased signal transduction from receptors involved in heteromeric complexes might also be able to explain some unanticipated side effects of currently marketed drugs. Unraveling the molecular mechanisms and functional consequences of receptor heteromerization might thus represent the basis for new therapeutic applications and guide novel ways in drug design (reviewed in (Maggio *et al.*, 2005; Prinster *et al.*, 2005)).

1.1.4. Trafficking of 7TM/GPCRs

Transport of 7TM/GPCRs to the cells surface

Receptor trafficking involves the delivery of mature receptors from the endoplasmatic reticulum (ER) to the plasma membrane, their removal from the cell surface upon agonist stimulation and the subsequent sorting between recycling and degradative fates. 7TM/GPCRs are extensively regulated under normal conditions.

Cell surface targeting of 7TM/GPCRs

One of the ways in which receptor-mediated signaling is modulated is by regulating their expression level on the plasma membrane (PM). The ER is believed to be an important site that ensures the quality control and facilitates that only functional receptors reach the cell surface. Molecular chaperones were suggested to be implicated in this process by interacting with receptors to assist their proper folding. The maturation and trafficking of the toll-like receptor 4 (TLR4) from the ER to the plasma membrane for example was shown to be regulated by molecular chaperones (Saitoh, 2009). Furthermore, it was reported that membrane-permeable opioid ligands can act as pharmacological chaperones and facilitate the maturation and export of opioid receptors (Petaja-Repo *et al.*, 2002). The function of these pharmacological chaperones was also shown to be independent of the signaling activity of these ligands (Petaja-Repo *et al.*, 2002). Additionally, it was reported that β -adrenergic ligands acted as pharmacological chaperones, which promoted the dimerization and ER export of the β 1-adrenoreceptor (Kobayashi *et al.*, 2009). Recently, endoplasmatic reticulum (ER) export/retention signals were identified that can control the trafficking of various 7TM/GPCRs to the

Introduction

cell surface. The $\alpha 2$ -adrenergic receptor, which shows only limited expression on the PM, but is extensively retained in the ER, was reported to possess a conserved sequence in its extracellular terminal region that regulates its export. Consequently, removal or mutation of the sequence was shown to decrease the retention in the ER and increase its expression on the plasma membrane (Angelotti *et al.*, 2010). Moreover, the importance of ER dependent quality control can be assessed from various diseases that result from any deregulation, such cystic fibrosis or nephrogenic diabetes insipidus (Kim & Arvan, 1998; Morello & Bichet, 2001).

Endocytosis – removal of 7TM/GPCRs from the cell surface

In addition to controlling the trafficking of receptors from the ER to the plasma membrane, 7TM/GPCRs can also be removed from the cell surface by a process called endocytosis. Therein, agonist-activated receptors are rapidly and reversibly silenced by their removal from the PM to an endocytic compartment. This process appears to regulate signaling from nearly all 7TM/GPCRs analyzed to date (Bohm *et al.*, 1997) and can furthermore be differentially regulated by various ligands (Keith *et al.*, 1998; Keith *et al.*, 1996). Endocytosis is typically facilitated by phosphorylation of the receptor by G protein coupled receptor kinase (GRK) and the subsequent interaction with (beta) arrestins. In general, receptors can be endocytosed via clathrin coated pits, caveolae or uncoated pits (Claing *et al.*, 2002). Most cell surface receptors are internalized by clathrin mediated endocytosis (see Fig. 4). The invagination and pit assembly are supported by clathrin as well as adaptor proteins (e.g. AP-2) and accessory proteins (Hinrichsen *et al.*, 2006). (reviewed in (Wolfe & Trejo, 2007)).

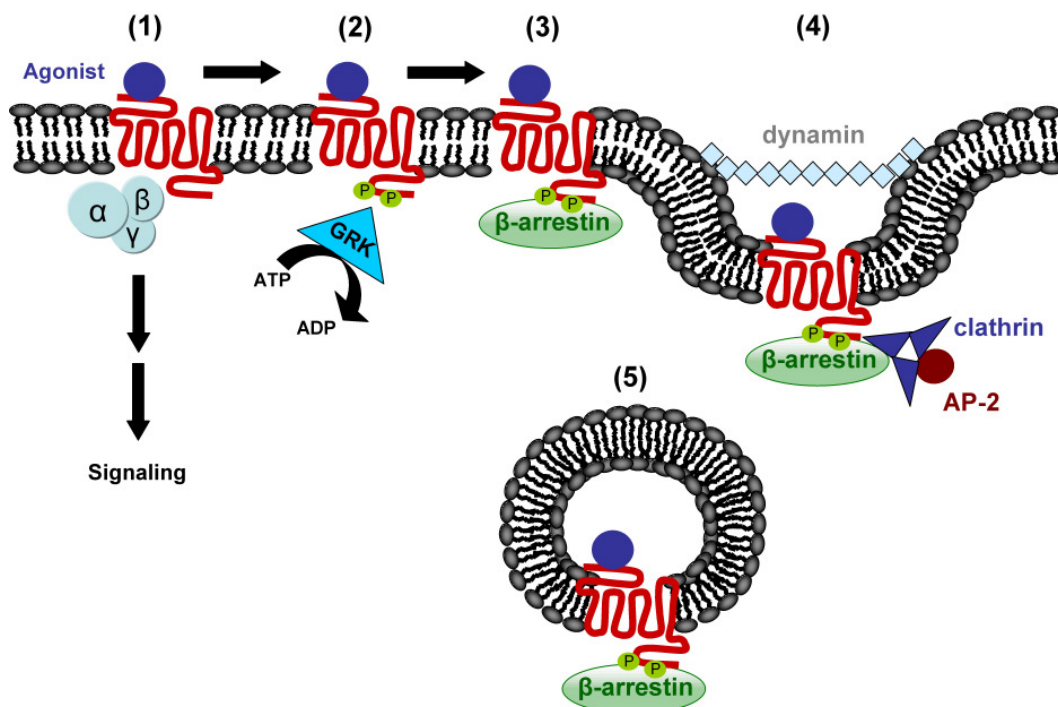
Figure 4

Figure 4. β -arrestin/clathrin-dependent endocytosis of 7TM/GPCRs. Agonist binding to 7TM/GPCRs leads to activation and G protein mediated signal transduction (1). Desensitization of receptors is then facilitated by phosphorylation through GPCR kinases (GRK) (2) and subsequent recruitment of arrestins (3). Endocytosis is finally mediated by clathrin and adaptor protein complex 2 (AP-2) (4). The vesicle abscission is facilitated by dynamin and leads to the formation of cargo containing endocytic vesicles (5).

1.1.5. Post-endocytic sorting of 7TM/GPCRs

Following endocytosis, individual receptors can be sorted differentially between recycling endosomes and lysosomes (Tsao *et al.*, 2001; Whistler *et al.*, 2002). This sorting mechanism can contribute to receptor regulation in two ways that have opposing effects on cell signaling. First, endocytosis can serve as a mechanism for receptor resensitization by delivering internalized receptors to endosomes, from where they are recycled to the plasma membrane in a fully active state. Alternatively, rapid internalization can serve as a first step toward receptor downregulation by delivering the receptors to endosomes, from which they are sent to lysosomes for degradation (see Fig. 5). Most membrane proteins are rapidly recycled, presumably by default (Mayor *et al.*, 1993). Therefore, it is likely that membrane receptors that are rapidly degraded

Introduction

following their endocytosis do so through a specific targeting mechanism. The sorting of individual receptors between recycling and degradative fates is biochemically specific and, not surprisingly given the importance of sorting for signal transduction, this process appears to be highly regulated.

The cascade of events that are globally referred to as “receptor sorting” is thought to be initiated already at the plasma membrane, where receptors are ‘tagged’ – e.g. phosphorylated or ubiquitinated - to determine their endocytic and post-endocytic fate. Receptor phosphorylation can occur in response to activation by an agonist and is often necessary for receptor endocytosis (for reviews see (Premont & Gainetdinov, 2007; Reiter & Lefkowitz, 2006; Ferguson *et al.*, 1998)). However, it has also been suggested that receptor phosphorylation not only influences endocytosis, but also determines the post-endocytic fate of a receptor by regulating interactions with sorting proteins or by inducing other posttranslational modifications (Cao *et al.*, 1999; Marchese & Benovic, 2001). Ubiquitination of 7TM/GPCRs involves the covalent attachment of ubiquitin to lysine residues of the cytoplasmic tail. Indeed, it has, in some cases, been shown to act as a ‘sorting signal’ that promotes endocytosis and/or lysosomal targeting (Mukhopadhyay & Riezman, 2007; Traub & Lukacs, 2007; Lefkowitz *et al.*, 1998).

Figure 5

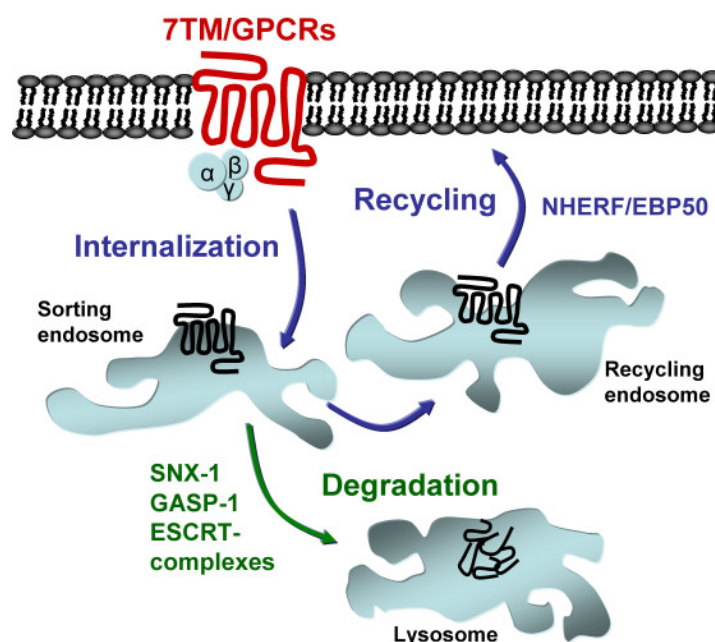


Figure 5. Post-endocytic sorting of 7TM/GPCRs. Following endocytosis, receptors are differentially sorted between recycling and degradative fates. Recycling of receptors

Introduction

can be mediated by interaction with PDZ (postsynaptic density 95/disc large/zonula occludens-1) domain containing proteins or other sorting proteins such as NHERF (Na^+/H^+ exchanger regulatory factor)/EBP50 (Erizin-radixin-moesin binding phosphoprotein 50). Interaction with the ESCRT (endosomal sorting complex required for transport) machinery, SNX-1 (sorting nexin 1) or GASP-1 (GPCR associated sorting protein 1) leads to targeting to the lysosomal pathway for degradation.

1.1.5.1. The recycling pathway

Traditionally, recycling of receptors – e.g. their trafficking from endosomal compartments back to the cell surface is thought to occur by default. This hypothesis is based on the observations that (i) membrane itself is continuously recycled and (ii) that disruption of lysosomal sorting often results in enhanced recycling of receptors (Bilodeau *et al.*, 2002; Babst *et al.*, 2000; Maxfield & McGraw, 2004). Nevertheless, this simple model is not able to explain the recycling of certain 7TM/GPCRs. Additionally, recent findings support a model of regulated recycling of 7TM/GPCRs that is mediated through specific recycling sequences contained in their cytoplasmic tails (reviewed in (Hanyaloglu & Von Zastrow, 2008)). Until now, a remarkable variety of recycling promoting cytoplasmic sequences were identified (Gage *et al.*, 2005; Tanowitz & von, 2003; He *et al.*, 2006). One example for sequence directed recycling involves the β_2 adrenergic receptor (β_2 -AR), which contains a distal sequence (DSLL) that was shown to be important for its efficient recycling via interactions with PDZ (postsynaptic density 95/disc large/zonula occludens-1) domain-containing proteins from the NHERF (Na^+/H^+ exchanger regulatory factor)/EBP50 (Erizin-radixin-moesin binding phosphoprotein 50) family (Gage *et al.*, 2005).

1.1.5.2. The lysosomal/degradative pathway

There are multiple pathways and interactions with various sorting proteins/complexes that regulate the targeting of 7TM/GPCRs to the lysosomal pathway for degradation. The most important sorting proteins involved in this process are summarized below.

ESCRT sorting machinery

The sorting of receptors to the lysosomal pathway is facilitated by the interaction with an array of sorting proteins. The highly conserved ESCRT (endosomal sorting complex required for transport) machinery directs the transport of ubiquitinated cargo for degradation via a compartment termed as multivesicular body (MVB) (see Fig. 6; reviewed in (Hanyaloglu & von Zastrow, 2008)). The sorting process is initiated by a protein complex (often referred to as ESCRT-0) consisting of the hepatocyte growth factor regulated tyrosine kinase substrate (Hrs) and the signal transduction adapter molecule (STAM), which recognize ubiquitinated proteins with their ubiquitin-interaction motifs (UIMs). By interaction of Hrs with the ubiquitin E2 variant (UEV) domain of the tumor suppressor gene-101 (TSG101), the binding of ESCRT -I (endosomal sorting complex required for transport) complex is initiated. Subsequently, ESCRT-II and -III complexes are recruited to the endosomal membrane, which deliver the cargo to the lysosomes for degradation (Saksena *et al.*, 2007; Hurley & Emr, 2006; Slagsvold *et al.*, 2006). Intriguingly, ubiquitination of at least 7TM/GPCRs does not appear to be required for degradation, but the ESCRT machinery is required. Together these data suggest that the ESCRT machinery can recognize select non-ubiquitinated receptors and transport them for degradation.

Sorting nexin-1

Sorting nexin-1 (SNX-1) is another protein that is involved in targeting 7TM/GPCRs to the lysosomal pathway. SNX-1 was shown to bind to Hrs, thus promoting the targeting of the epidermal growth factor (EGF) receptor to the degradative pathway (Chin *et al.*, 2001). Additionally, it was also reported that SNX-1 is able to interact with the c-tails of various other 7TM/GPCRs, such as the protease-activated receptor-1 (PAR1) (Wang *et al.*, 2002), virally encoded chemokine receptor US28 or the delta opioid receptor (DOR) (Heydorn *et al.*, 2004). Although some of the receptors that bind to SNX-1 are targeted for degradation, others were shown to recycle. The muscarinic receptors M1 and M4 for example were reported to interact with SNX-1 (Heydorn *et al.*, 2004), but are still efficiently recycled (van Koppen, 2001; Krudewig *et al.*, 2000).

Dysbindin

Recently, dysbindin, a cytoplasmic protein encoded by DTNBP1 (Benson *et al.*, 2001), was suggested to be involved in targeting 7TM/GPCRs to the lysosomes. Marley *et al.*

Introduction

showed that dysbidin was able to regulate the post-endocytic sorting of dopamine 2 receptors and delta opioid receptors to the degradative pathway, however, the proteolysis of the endothelial growth factor receptor (EGFR) was not affected (Marley *et al.*, 2010). Furthermore, dysbidin was shown to co-immunoprecipitate with GASP-1 and Hrs, a component of the ESCRT-0 complex (Marley & von, 2010).

Figure 6

Cytosol

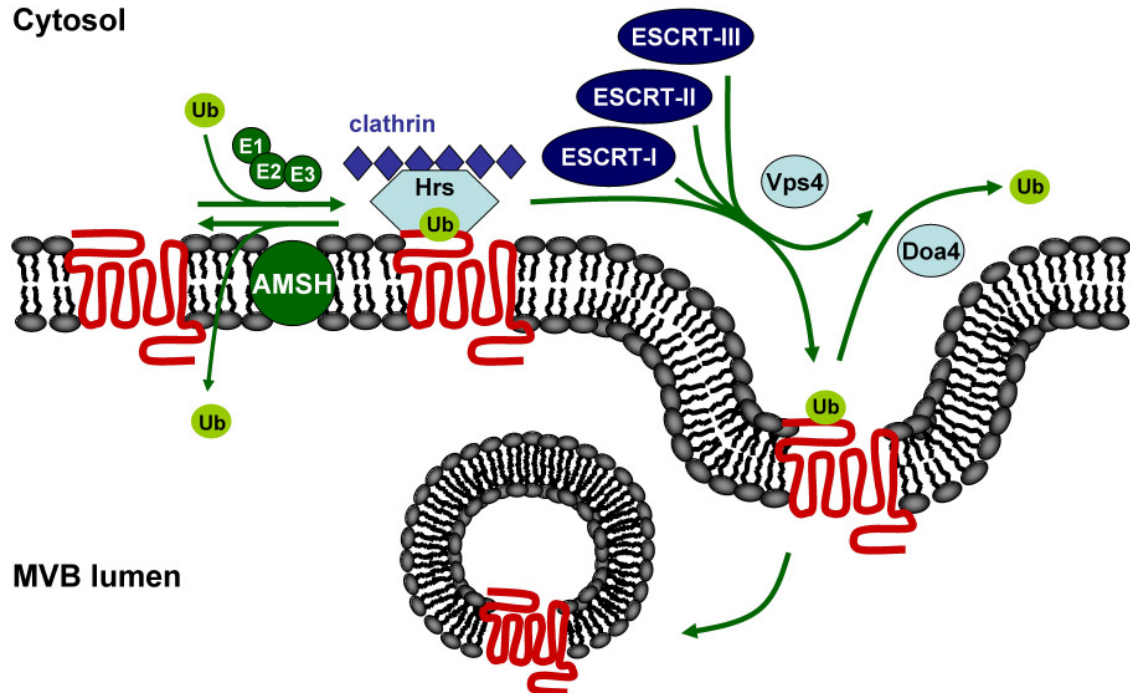


Figure 6: ESCRT dependent sorting of 7TM/GPCRs. Cargo ubiquitination (Ub) is mediated by the sequential action of the enzymes E1, E2 and E3. The removal of ubiquitin is catalyzed by deubiquitinating enzymes, such as AMSH (associated molecularae with the SH3 domain of STAM). In the first step Hrs (hepatocyte growth factor regulated tyrosine kinase substrate) is binding to the ubiquitinated cargo protein and subsequently ESCRT-I, -II and -III complexes are recruited. The invagination and formation of MVB (multivesicular bodies) is mediated by a clathrin-dependent mechanism. Cytoplasmic ubiquitin can be recycled via deubiquitination enzymes, such as Doa4 (degradation of alpha 4) and the ATP-ase Vps4 (vacuolar protein sorting gene 4) then leads to the disassembly of the ESCRT complexes.

1.1.5.3. GASP-1

One sorting protein that specifically targets 7TM/GPCRs to the lysosomal/degradative pathway is the GPCR-associated sorting protein (GASP-1) (Whistler *et al.*, 2002; for review see (Moser *et al.*, 2010)). GASP-1 is a large acidic protein of 1394 amino acids that is expressed at high levels throughout the brain and the kidneys (as shown by RT-PCR, see <http://www.kazusa.or.jp/huge/gfpage/KIAA0443>). The carboxyl terminus of GASP-1 is involved in binding the cytoplasmic tail of G-protein coupled receptors from subfamilies A and B. Although a total of 10 family members of GASP-1 (GASP-2 to GASP-10) has since been suggested (Simonin *et al.*, 2004), no functional evidence has been provided that these ‘new’ family members are involved in the sorting of G-protein coupled receptors. GASP-2, the closest homologue of GASP1, shows 35 % amino acids similarity. Interestingly, the COOH terminal 497 amino acids of GASP-1, also called cGASP-1, shares 62 % sequence identity with the c-terminal region of GASP-2.

GASP-1 dependent sorting of 7TM/GPCRs

Several 7TM/GPCRs that interact with GASP-1 were shown to be targeted for degradation after endocytosis (Whistler *et al.*, 2002; Simonin *et al.*, 2004; Bartlett *et al.*, 2005; Martini *et al.*, 2007; Tappe-Theodor *et al.*, 2007; Thompson *et al.*, 2007; Enquist *et al.*, 2007; Tschische *et al.*, 2010). Interestingly, GASP-1 was also reported to show selectivity for individual members of 7TM/GPCR families. The delta opioid receptor (DOR) and the bradykinin 1 receptor (B1R) were shown to interact with GASP-1 and are thus targeted to the lysosomes for degradation. In contrast to this, the respective family members mu opioid receptor (MOR) and bradykinin 2 receptor did not bind to GASP-1 and were hence efficiently recycled after endocytosis (Whistler *et al.*, 2002; Enquist *et al.*, 2007). Another example can be given by the dopamine receptor family. Upon prolonged agonist stimulation, the D2 receptor was shown to be significantly degraded, whereas the D1 receptor was rapidly recycled (Bartlett *et al.*, 2005). The dopamine 2 receptor was reported to directly interact with GASP-1 both *in vitro* and in rat brain extracts. In contrast, D1R did not bind to GASP-1. Moreover, upon blocking of the GASP-1/D2R interaction with the dominant negative cGASP-1, D2 receptor degradation was delayed. Additionally, the inability of D2R in neurons of the rat VTA (ventral tegmental area) to resensitize after agonist treatment was shown to be facilitated by the interaction with GASP-1. Disruption of the binding of GASP-1 to

Introduction

D2R by treatment with an anti GASP-1 antibody, enhanced the recovery of functional D2 receptors (Bartlett *et al.*, 2005).

A study by Thompson and co-workers (Thompson *et al.*, 2007) showed that the affinity of individual receptors for GASP-1 controls their post-endocytic sorting. For instance, GASP-1 bound to the c-tail of wild type β 2AR in GST-pull down assays. In contrast to this, no direct protein-protein interaction of β 2AR with GASP-1 was detected in HEK293 cells, which thus resulted in an efficient recycling of the receptor. Hence, it seems that recycling proteins - such as NHERF/EBP50 and N-ethylmaleimide-sensitive factor (NSF) - successfully compete with GASP-1 for binding to this receptor. Indeed, when the PDZ domain of the β 2AR was disrupted, thus preventing the interaction with recycling proteins, mutant β 2AR associated with GASP-1 and was targeted for degradation (Thompson *et al.*, 2007). Another example is the MOR, where structural sequence determinants contained in the c-terminus of the receptor seem to prevent the binding to GASP-1. Indeed, the truncation of the last 17 amino acids of the receptor (Tanowitz & Von Zastrow, 2003) was sufficient to enhance its interaction with GASP-1, thus leading to its lysosomal targeting (Thompson *et al.*, 2007). On the other hand, the deletion of a small motif within the c-tail of D1R prevented the recycling of the receptor, but had no effect on the affinity for the sorting protein GASP-1. Hence, this mutant D1R was neither recycled, nor degraded. In conclusion, disruption of the interaction of 7TM/GPCRs with recycling proteins *per se* does not promote lysosomal targeting, unless the receptor shows affinity for degrading sorting proteins such as GASP-1 (Thompson *et al.*, 2007).

GASP-1 dependent functions in vivo

Recently, the *in vivo* relevance of GASP-1 dependent sorting of 7TM/GPCRs was also established in animal models. Tappe-Theodor *et al.* showed that CB1R is downregulated in primary cultured rat spinal neurons after prolonged agonist treatment. Furthermore, the degradation of CB1R was mediated by the sorting protein GASP-1 (Tappe-Theodor *et al.*, 2007). The development of tolerance to the synthetic cannabinoid agonist WIN55,212-2 was selectively analysed in the superficial spinal laminae, where GASP-1 and CB1R were found to colocalize. Intriguingly, they found that mice overexpressing the dominant negative competitor cGASP-1 did not develop tolerance to the effects of WIN55,212-2. Furthermore, they showed that the reduced

Introduction

tolerance was connected with a significantly reduced downregulation of the receptor in these mice (Tappe-Theodor *et al.*, 2007). Recently, Martini *et al.* confirmed the importance of GASP-1 mediated downregulation of CB1R in the development of behavioural tolerance to the effects of WIN55,212-2 (Martini *et al.*, 2010). Interestingly, GASP-1 knock out (KO) mice showed significantly reduced tolerance after chronic application of WIN55,212-2 in comparison to the WT littermates (Martini *et al.*, 2010).

In addition, Boeuf *et al.* investigated the role of GASP-1 dependent receptor sorting on the effects of chronic application of cocaine. Therefore, they used GASP-1 knock out (KO) mice and analysed the reinforcing properties of cocaine in self administration experiments, as well as sensitization to its locomotor effects. As a result, GASP-1 KO animals showed reduced locomotor response to a prolonged application of cocaine and less sensitization in comparison to wild type (WT) mice (Boeuf *et al.*, 2009). Additionally, a recent study by Thompson and co-workers showed that GASP-1 knock-out mice developed significantly reduced locomotor activity and sensitization after repeated administration of cocaine. WT littermates showed significantly higher stereotypic behaviours induced by chronic application cocaine in comparison to GASP-1 KO animals (Thompson *et al.*, 2010). The authors hypothesize that the changed sensitization to cocaine might be due to an altered balance between the stimulatory D1R and the inhibitory D2R levels.

The *in vivo* relevance of GASP-1 dependent sorting of 7TM/GPCRs including the development of tolerance or the sensitization to drugs might guide new therapeutic approaches. However, more studies are needed to unravel the complex processes and the involved proteins and mechanisms controlling the sorting of 7TM/GPCRs.

1.2. The chemokine system

The chemokine system comprises chemokines (chemotactic cytokines), which bind to and activate their cognate receptors, named chemokine receptors. Chemokine receptors are predominantly Gai-coupled 7TM/GPCRs that are expressed on many different cell types – e.g. endothelial cells, smooth muscle cells, leukocytes, epithelial cells, neural cells and stroma cells (Murphy *et al.*, 2000). Chemokines are small, mostly basic proteins

Introduction

with a size of around 8-10 kDa that are specialized in cell-cell signaling in the immune system. These structurally related peptides can be classified into four groups, depending on the number and sequential relation of the first two of four conserved cystein residues: the C-, CC-, CXC- or CX3C-chemokines (Zlotnik & Yoshie, 2000). Several chemokines were shown to exclusively bind to one receptor, whereas other chemokines can activate multiple chemokine receptors (for review see (Bonecchi *et al.*, 2009)). The expression of certain chemokines, especially during inflammatory conditions, is mostly regulated by inducible transcription. Additionally, the activity of chemokines, such as CXCL16 and CX3CL1, can be regulated by enzymatic cleavage (for review see (Ransohoff, 2009)).

The chemokine system is well known for its important role in the immune and nervous system. Chemokines were first identified to regulate leukocyte extravasation. In this process, chemokine receptor signaling mediates the initial cell arrest as well as the clustering and conformational change of integrins, which leads to a high affinity interaction of leukocytes and vascular endothelial cells (Alon *et al.*, 2003; Schreiber *et al.*, 2007). Furthermore, the chemokine system was suggested to be involved in developmental processes and organogenesis (Forster *et al.*, 1996). CXCR4 and its respective chemokine CXCL12 were found to be expressed at a very early stage during embryogenesis. Moreover, the receptor and its cognate ligand were suggested to be important in developmental processes of the nervous, urogenital, cardiovascular and hematopoietic system in mice (for review see (Ransohoff, 2009)). The importance of CXCR4 and CXCR7 in generating chemokine gradients for neuronal cell migration was also confirmed in zebrafish (Boldajipour *et al.*, 2008; Tiveron & Cremer, 2008). Another intriguing feature of CXCR4 and CCR5 is their co-receptor function for HIV-1 (human immunodeficiency virus type 1). It was shown that some HIV-1 variants only use CCR5 (R5 variant) or CXCR4 (X4 variant) as co-receptors, whereas other variants take advantage of both chemokine receptors (e.g. R5X4 variant) (Berger *et al.*, 1998). Interestingly, a co-receptor switch from R5 HIV-1 virus to R5X4 or X4 strains is observed in about 50% of the patients and leads to an enhanced progression of the disease (Koot *et al.*, 1993). The binding of HIV-1 virus to the chemokine co-receptors facilitates membrane fusion and induces signal transduction. Recently, a critical function of CXCR4 mediated G α i-dependent signaling was found to enable HIV infection of resting CD4 T cells (Yoder *et al.*, 2008). These results point toward an important role of endogenous chemokine receptors in HIV infection and its inflicted pathogenesis.

Additionally, due to the multiple functions of the chemokine system, any deregulation is implicated in disease states, such as cancer, atherosclerosis and neuroinflammatory processes (Biber *et al.*, 2006; Zlotnik, 2006; Zernecke & Weber, 2010).

1.2.1. Virally encoded chemokines and chemokine receptors

Several herpesviruses encode 7TM/GPCRs with high homology to endogenous chemokine receptors. Although the exact functions are not known, their involvement in viral replication (Beisser *et al.*, 1998), evasion of the host immune system by chemokine scavenging (Bodaghi *et al.*, 1998), viral entry (Pleskoff *et al.*, 1998), viral dissemination (Pleskoff *et al.*, 1998; Streblow *et al.*, 1999) or reactivation of latent virus (Lee *et al.*, 2003; Pleskoff *et al.*, 1998) have been suggested. The human herpesvirus 8 (HHV8), also known as Kaposi's sarcoma associated herpesvirus (KSHV), encodes the 7TM/GPCR ORF74 (open reading frame 74). ORF74 shares the highest homology with the endogenous chemokine receptor CXCR2 (Cesarman *et al.*, 1996; Guo *et al.*, 1997) and was shown to be constitutively active (Arvanitakis *et al.*, 1997). Furthermore, a causative link between ORF74 and the development of Kaposi's Sarcoma was established (Sodhi *et al.*, 2004). HHV-6 was also reported to encode two 7TM/GPCRs, U12 and U51, that show homology to human chemokine receptors (Milne *et al.*, 2000). U51 was shown to bind endogenous chemokines, such as RANTES, eotaxin as well as MCP-1,-2 and-3. Additionally, it was established that this virally encoded receptor is able to modulate endogenous RANTES levels (Milne *et al.*, 2000). HHV-7 also encodes a 7TM/GPCR, namely U12, which was found to mediate cell migration towards a gradient of EB11-ligand chemokine (ELC) (Tadagaki *et al.*, 2005).

In addition to the use of chemokine receptor homologues for immune evasion, some viruses also express their own chemokines. HHV-8, for example, expresses the three chemokines vMIP-I, vMIP-II and vMIP-III that were shown to bind to endogenous chemokine receptors (Kledal *et al.*, 1997; Fernandez *et al.*, 2000; Nicholas *et al.*, 1997; Dairaghi *et al.*, 1999). Additionally, the viral chemokine homologue U83 (vCCL4) expressed by the HHV-6 was identified as an agonist of the endogenous chemokine receptor CCR2 (Zou P *et al.*, 1999; Lutichau *et al.*, 2003). Finally, also the human cytomegalovirus (HCMV) expresses the viral chemokine UL146 (vCXCL1), which

functions as an agonist after binding to the cellular chemokine receptor CXCR2 (Penfold *et al.*, 1999).

1.3. The human cytomegalovirus

Human cytomegalovirus (HCMV), also known as human herpesvirus 5 (HH5), is a large and highly species-specific virus that belongs to the family of beta-herpesviruses. The virion is build up by a double-stranded DNA core in an icosahedral nucleocapsid, which is furthermore enveloped by a proteinaceous matrix and a lipid bilayer containing viral glycoproteins (Chen *et al.*, 1999;Crough *et al.*, 2007). Depending on the geographical region, the seroprevalence of HCMV ranges between 30% and 90% (Staras *et al.*, 2006;Britt, 2008). Transmission of HCMV can be facilitated via sexual contact, placental transfer, saliva, blood transfusion or organ transplantation and hematopoietic stem cells (Kutza *et al.*, 1998). In general, infection with HCMV is asymptomatic and rarely causes complications in immunocompetent hosts. In contrast, in immunocompromised hosts, such as transplant recipients or HIV infected individuals, fetuses and newborns, HCMV infection causes severe complications, such as graft rejection or systemic infections (Gandhi & Khanna, 2004;Humar & Michaels, 2006;Ross & Boppana, 2005;Griffiths, 2006).

After primary infection of cells that are susceptible to HCMV, viral replication takes place in a variety of cell types, such as endothelial cells, macrophages, dendritic cells, epithelial cells, fibroblasts, smooth muscle cells, neuronal cells and hepatocytes (Sinzger *et al.*, 2008;Sinzger *et al.*, 1995;Britt, 2008). During productive infection, viral genes are expressed in a coordinated and temporal cascade. First, immediate early (IE) genes are expressed under the control of the major immediate early promoter (MIEP) region. The MIEP region possesses a powerful transcriptional enhancer and was shown to contain binding sites for transcription factors such as the nuclear factor kappa B (NF- κ B), Elk-1 (ets-like gene-1), SRF-1 (serum response factor) or the cyclic AMP responsive element binding protein (CREB). The role of the four NF- κ B binding sites within the MIEP region is not completely established. In some studies inhibition of NF- κ B signaling yielded an impaired viral replication (DeMeritt *et al.*, 2004;Caposio *et al.*, 2007), whereas other reports show that the deletion of the binding sites within the MIEP region had no effects (Benedict *et al.*, 2004;Gustems *et al.*, 2006). A possible

Introduction

explanation for these controversial results might be given through the dependence on the proliferative state of the cell (Caposio *et al.*, 2007). Recently, Caposio and co-workers reported that the combination of different MIEP binding sites is important for the optimization of IE gene expression. The lack of NF- κ B-induced MIEP transactivation could be compensated by an increased binding of SRF and Elk-1 in proliferating cells (Caposio *et al.*, 2010). In agreement with other reports, the CREB binding site was also shown to cooperatively interact with other transcription factor binding sites and thus enhanced gene transcription by the MIEP region (Lashmit *et al.*, 2009).

The IE gene products are important for the regulation of the subsequent expression of cellular genes and viral early (E) and late (L) genes (Stenberg, 1996; Colberg-Poley, 1996). Early genes are thought to encode functions associated with the replication of viral DNA, whereas late gene products encode structural proteins. Later on in the viral life cycle, latency is established primarily in cells of the myeloid lineage (Sinclair & Sissons, 2006). Infection of monocytes was shown to induce a proinflammatory phenotype that promotes viral spread and shows a high level of chemokinesis (Smith *et al.*, 2007; Smith *et al.*, 2004; Bentz *et al.*, 2006). Recently, it was reported that the epidermal growth factor receptor (EGFR) mediates the viral entry of HCMV into monocytes (Chan *et al.*, 2009). Furthermore, the activation of EGFR was also suggested to be essential in mediating the monocyte motility and transendothelial migration (Chan *et al.*, 2009).

Another interesting property of HCMV is its ability to persist lifelong in its host. Viral latency is defined by the presence of viral genome, but the absence of infectious virion production. The strategy of the establishment of viral latency is also used by other herpesviruses, such as EBV (Amon & Farrell, 2005) or herpes simplex virus type 1 (Halford & Schaffer, 2001). Sporadic reactivation of HCMV can occur in immunocompetent hosts, but usually does not cause any severe complications. In contrast to this, in immunocompromised individuals, reactivation of HCMV often leads to higher mortality. In HIV-1 positive individuals for example, productive HCMV infection is thought to correlate with an enhanced progression of the disease (Palella, Jr. *et al.*, 1998; Spector *et al.*, 1998). The exact mechanism of HCMV reactivation is not fully understood. However, it is suggested that the CREB site in the MIE enhancer

Introduction

might be of importance for the reactivation of the virus (for review see (Stinski & Isomura, 2008)).

HCMV infection and reactivation were shown to yield the enhancement of cellular responses, including the activation of transcription factors, accumulation of cAMP, inositol phosphate hydrolysis and the metabolism of arachidonic acid (Evers *et al.*, 2004; Challacombe *et al.*, 2004; Zhu *et al.*, 1998). 7TM/GPCRs are thought to be key regulators of cellular signal transduction. Intriguingly, HCMV also encodes four viral GPCRs (v-GPCRs), namely UL33 (unique long 33), UL78, US27 (unique short 27) and US28 that show high homology to human chemokine receptors (for review see (Landolfo *et al.*, 2003)). It is suggested that these “pirated” receptors are used by HCMV to evade the immune system and redirect cellular signaling cascades to its benefits (for review see (Vischer *et al.*, 2006)).

1.3.1. The HCMV encoded receptors US27 and UL78

US27 and UL78 are the least characterized 7TM/GPCRs encoded by HCMV. US27 shares about 23% sequence homology with the endogenous chemokine receptor CX3CR3 (Vischer *et al.*, 2006). However, until now US27 is still termed an orphan receptor, meaning that no ligands were identified that bind to the receptor or induce any signaling pathways (Bodaghi *et al.*, 1998). Upon HCMV infection, US27 was shown to be expressed during late infection states and was furthermore found to be present on HCMV virions (Margulies & Gibson, 2007; Fraile-Ramos *et al.*, 2002). To date no functions for US27 in the HCMV lifecycle or its inflicted pathologies have been identified. Interestingly, US27 was also shown to share about 40% sequence homology with the HCMV encoded receptor US28. Furthermore, it was shown that US27 is predominately localized in lysosomal compartments and co-localizes with US28 (Fraile-Ramos *et al.*, 2002).

The UL78 gene family is conserved among different β -herpesviruses, including rat CVM (R78), mouse CMV (M78), HHV-6A and B (U51) and HHV-7 (U51) (Beisser *et al.*, 1999; Dominguez G. *et al.*, 1999; Nicholas, 1996; Nicholas, 1996; Rawlinson *et al.*, 1996; Vink *et al.*, 2000). In a cell culture model, both M78- and R78-deleted viruses showed a decreased production of virus progeny. The same study indicated that animals

infected with M78- or R78-disrupted viruses had reduced mortality rates (Oliveira & Shenk, 2001; Beisser *et al.*, 1999). HCMV encoded UL78 is similar to US27, an orphan receptor that shares only very limited homology with endogenous chemokine receptors (Vischer *et al.*, 2006). Although UL78 is expressed during the early stage of HCMV infection (Vischer *et al.*, 2006), the receptor is not necessary for viral replication (Michel *et al.*, 2005).

1.3.2. The HCMV encoded receptor UL33

Similar to UL78, UL33-like genes are also conserved among β -herpesviruses, such as rat CVM (R33), mouse CMV (M33), HHV-6A and B (U12) and HHV-7 (U12) (Beisser *et al.*, 1999; Dominguez G. *et al.*, 1999; Nicholas, 1996; Nicholas, 1996; Rawlinson *et al.*, 1996; Vink *et al.*, 2000). Although the CMV encoded UL33 gene products share some homology to chemokine receptors, so far no endogenous chemokines or any other ligands were identified to bind/activate these receptors. Interestingly, CMV-encoded UL33 receptors were reported to possess constitutive signaling capacity (Waldhoer *et al.*, 2002). R33 for example activates phospholipase C via coupling to G α q and G α i/o, inhibits CRE-induced transcription and activates the transcription factors SRE (serum responsive element) and NF- κ B (Gruijthuisen *et al.*, 2002). The mouse homologue M33 activates PLC as well as the NF- κ B and CRE-driven transcription in a ligand independent manner (Waldhoer *et al.*, 2002). R33 and M33 have also been studied *in vivo*: infection with R33-deleted virus for example yielded a significantly lower mortality in comparison to the wild type virus infected rats (Beisser *et al.*, 1998). Furthermore, after infection, M33- or R33-disrupted viruses were not detectable in the salivary glands – one of the major sites of CMV replication and persistence (Beisser *et al.*, 1998; Davis-Poynter *et al.*, 1997). HCMV-encoded UL33 shows the highest homology with the endogenous chemokine receptor CCR10 (Vischer *et al.*, 2006b). Similar to US27 and UL78, UL33 is also referred to as an orphan receptor and neither binds any known chemokines nor any other ligands. It was reported that UL33 is transcribed during the early phase of HCMV infection (Bodaghi *et al.*, 1998), but its function in the viral lifecycle is still unclear. Recently, it was reported that UL33 co-localizes with the recycling endosomal marker TfR (transferrin receptor) and the early endosomal markers EEA1 (early endosomal antigen 1) in HCMV-infected cells (Cepeda *et al.*, 2009). In cell lines, UL33 was shown to be predominantly located

Introduction

intracellularly and it also co-localizes with the lysosomal marker LAMP (lysosome-associated membrane protein) (Fraile-Ramos *et al.*, 2002). Furthermore, it was reported that UL33 shows some constitutive- e.g. ligand independent-activity. When expressed in COS-7 cells, UL33 was shown to induce inositol phosphate (IP) accumulation via coupling with Gαq and Gai/o-proteins (Casarosa *et al.*, 2003;Waldhoer *et al.*, 2002). The activation of the downstream transcription factor CREB by UL33 is mediated via Gαq, Gas and the stable βγ-dimer as well as by activation of p38 MAP kinase (Casarosa *et al.*, 2003;Waldhoer *et al.*, 2002).

1.3.3. The HCMV encoded receptor US28

The 7TM/GPCR US28 (unique short 28) is the by far best characterized receptor encoded by HCMV and is transcribed as an early gene, just shortly after the IE genes and as early as two hours after infection (Zipeto *et al.*, 1999). In addition, US28 was also shown to be expressed at later time points during infection as well as in fully lytic and latent infection states (Billstrom *et al.*, 1998;Beisser *et al.*, 2001). The presence of US28 on the virions of HCMV was shown to be dispensable for viral replication, but was suggested to be important for the viral life cycle as it might enhance cell-cell fusion, thus promoting viral spread (Fraile-Ramos *et al.*, 2002;Cepeda *et al.*, 2009;Kledal *et al.*, 1998;Pleskoff *et al.*, 1998). Furthermore, US28 was reported to activate the immediate early HCMV promoter, which might lead to the subsequent transactivation of other viral genes (Boomker *et al.*, 2006).

Ligand independent signaling activity

Several virally encoded 7TM/GPCRs, including ORF74 encoded by human herpesvirus 8, were reported to possess constitutive, e.g. ligand-independent signaling activity. US28 activates the Gαq/PLC signaling cascade in a constitutive manner, thus activating NF-κB and the nuclear factor of activated T cells (NFAT) (Casarosa *et al.*, 2001;McLean *et al.*, 2004) (see Fig. 7). In addition, it was shown that the stable βγ dimer is important for the activation of the transcription factor NF- κB (Casarosa *et al.*, 2001;McLean *et al.*, 2004). The transcription factor CREB was also reported to be activated, which was suggested to involve p38 kinase (Waldhoer *et al.*, 2002) (see Fig. 7). Recently, Wen *et al.* showed that the constitutively activated CREB can bind to the

Introduction

promoter of the major immediate early (MIE) gene of HCMV and thus enhances their transcription (Wen *et al.*, 2009). This finding suggests an involvement of US28 in the early transcriptional activity of HCMV. The activation of the serum response factor SRF was found to be mediated via coupling of US28 with Gαq and the subsequent activation of RhoA (Moepps *et al.*, 2008). Surprisingly, the activation of SRF was reported to be independent of PLC-β, but modulated by constitutively active Gα16 subunits, which were suggested to compete with Gαq for coupling with US28 (Moepps *et al.*, 2008). Interestingly, US28 was also shown to up-regulate several proteins involved in oncogenesis, especially cyclooxygenase-2 (COX-2), a transcriptional target of NF-κB, suggesting a possible role for US28 in tumorigenesis (Maussang *et al.*, 2009).

Recently it was shown that recombinant HCMV containing the c-terminally truncated US28/1-314 mutant (deletion of amino acids 315-354) exhibited an enhanced activation of PLC-β in comparison to the wild type receptor. The same study indicated that infection with HCMV expressing the mutant US28/1-314 also yielded a prolonged Ca²⁺ release upon stimulation with CCL5 (Stropes *et al.*, 2009). These findings suggest that the c-terminal region of US28 plays an important role in controlling agonist-independent, as well as agonist-dependent, signaling activity. In addition, Stropes *et al.* also reported that CX3CL1 can act (i) as an inverse agonist that induces PLC activation and (ii) as an agonist for US28-mediated Ca²⁺ release (Stropes *et al.*, 2009).

Figure 7

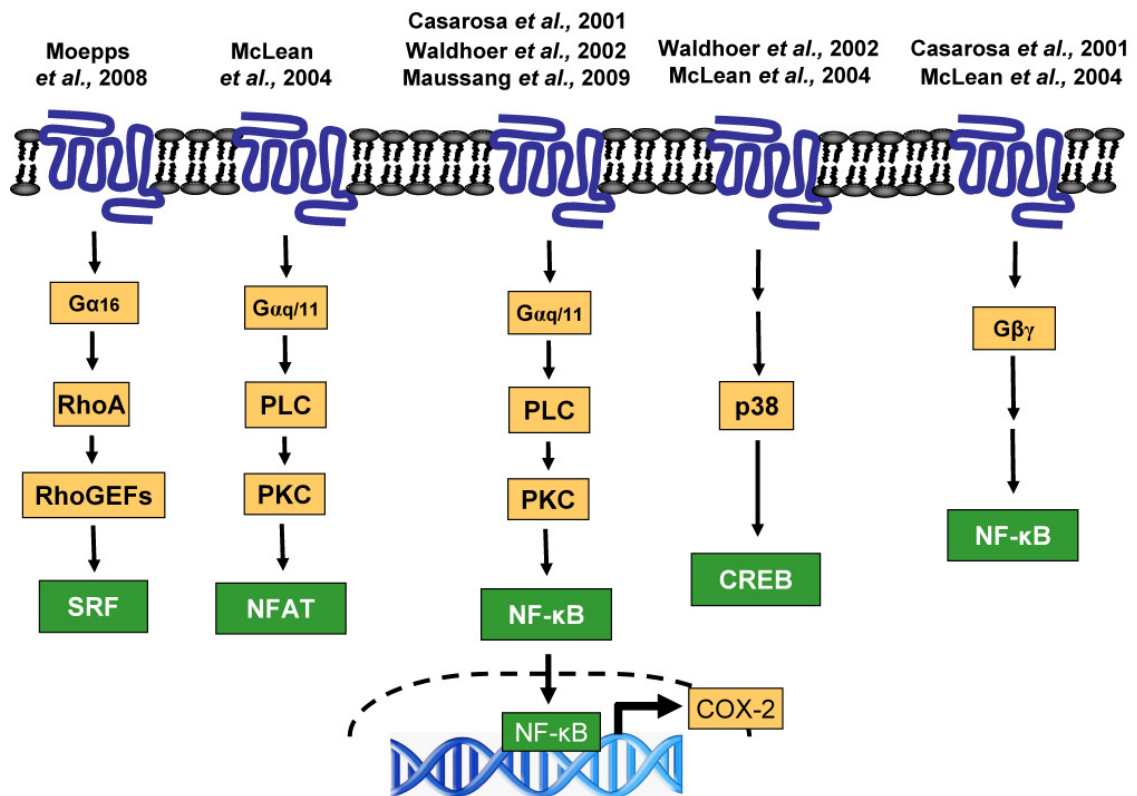


Figure 7. Constitutive signaling cascades activated by the HCMV encoded receptor US28. US28 can activate the transcription factors NF-κB (nuclear factor kappa B) and NFAT (nuclear factor of activated T cells) via Gaq/ PLC (phospholipase). NF-κB can also be activated via the stable βγ dimer. Subsequently, NF-κB can translocate to the nucleus and enhance the transcription of COX-2. The transcription factor CREB (cyclic AMP responsive element binding protein) is activated via p38 MAP kinase. SRF (serum response factor) is activated via coupling with Ga16 proteins and involves RhoA.

Ligand-dependent signaling of US28

Interestingly, the virally encoded receptor US28 shows not only high sequence homology with the endogenous chemokine receptors CCR1 and CX3CR1 (Vischer *et al.*, 2006), but is furthermore able to bind a broad spectrum of endogenous CC and CX3C chemokines, such as CCL2 (MCP-1), CCL3 (MIP-1β), CCL4 (MIP-1α), CCL5 (RANTES) and CX3CL1 (fractalkine) (Kuhn *et al.*, 1995; Kledal *et al.*, 1998). It was reported that the CC chemokines CCL2 (Gao & Murphy, 1994), CCL5 and CCL7 (Billstrom *et al.*, 1998) are able to induce Ca²⁺ release in US28-expressing cells.

Introduction

Stimulation of US28 with CCL5 was reported to induce Ca^{2+} signaling and activation of the ERK via coupling with G α i and G α 16 proteins, whereas CCL7 induced Ca^{2+} release is mediated only via G α 16 proteins (Billstrom *et al.*, 1998). Furthermore, the reported increase in intracellular Ca^{2+} in HCMV infected cells upon stimulation with CCL5 was shown to be dependent on US28 (Vieira *et al.*, 1998). Other CC chemokines that bind to US28 with high affinity, such as CCL3 and CCL4, do not induce Ca^{2+} flux or any other signaling cascades (Gao & Murphy, 1994; Kuhn *et al.*, 1995). CX3CL1 (fractalkine) was described to act as a partial inverse agonist in connection with US28-mediated IP turnover and its activation of transcription factors (Waldhoer *et al.*, 2002). Intriguingly, it was also observed that fractalkine causes a concentration-dependent increase of the signaling capacity of the endocytosis-deficient US28 mutant (US28 Δ 300) (Waldhoer *et al.*, 2003). Hence the agonist properties of CX3CL1 might be masked by the rapid internalization of US28 and are thus not detectable with the wild-type receptor. Recently, some synthetic compounds were identified that act as inverse agonists on US28. These small, non-peptide compounds (VUF2274) were derived from antagonists of the endogenous chemokine CCR1 (Hulshof *et al.*, 2005). VUF2274 was shown to prevent the binding of CCL5 to US28 and furthermore the US28-induced activation of PLC (Casarosa *et al.*, 2003). Importantly, it was also reported that these inverse agonists block the co-receptor function of US28 for HIV-1 entry (Casarosa *et al.*, 2003).

Another interesting property of US28 is its ability to mediate the migration of vascular smooth muscle cells (SMC) towards CCL2 and CCL5 (Streblow *et al.*, 1999) (see Fig. 8). This cell migration was found to be induced by coupling of US28 to G α 12/13-proteins (Melnychuk *et al.*, 2004) and subsequent activation of the non-receptor tyrosine kinase Src that phosphorylates the FAK (focal adhesion kinase) (Streblow *et al.*, 2003). In addition, Vomaske *et al.* demonstrated that SMC migration induced by US28 is ligand- and cell type-specific (Vomaske *et al.*, 2009). CCL5, CCL2 and CX3CL1 were equally able to stimulate US28-dependent migration of SMC; however, in macrophages CX3CL1 produced the most potent effects. The same study showed that CCL5-stimulated migration occurs via activation of G α 12/13-proteins, whereas CX3CL1 facilitates coupling of US28 to G α q proteins (Vomaske *et al.*, 2009).

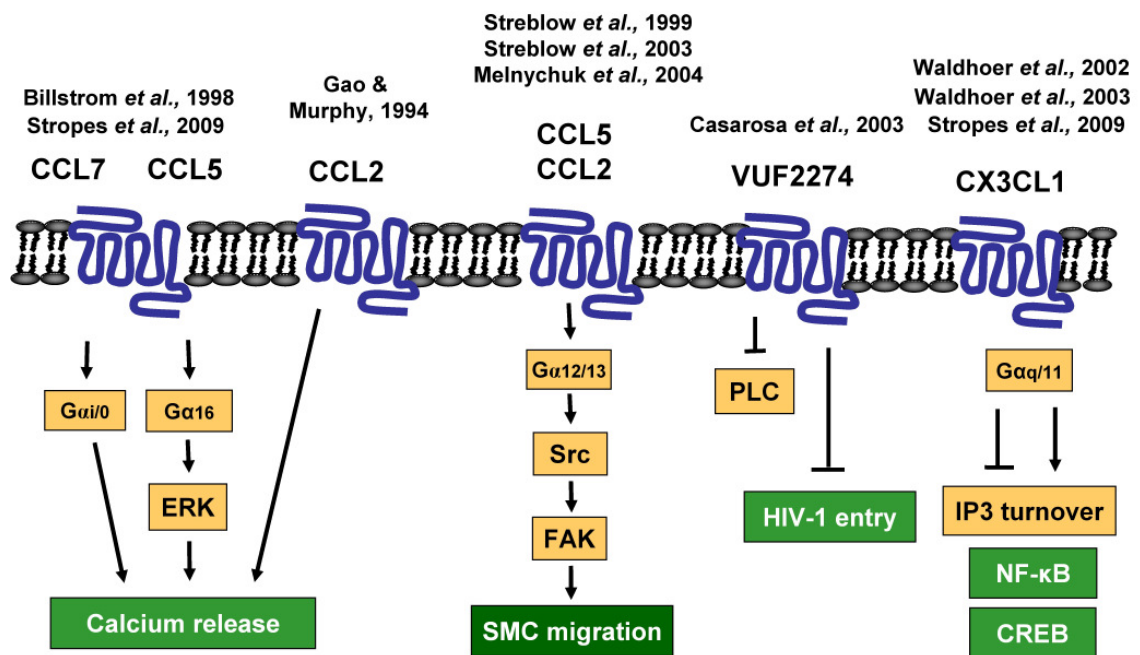
Figure 8

Figure 8. Ligand dependent signaling pathways activated by US28. Upon stimulation with CC-chemokines CCL2 and CCL5, US28 mediates smooth muscle cell (SMC) migration via coupling to Gα12/13 and activation of non-receptor tyrosine kinase (Src) and focal adhesion kinase (FAK). CCL2, CCL5 and CCL7 lead to US28-mediated Ca²⁺ release. The inverse agonist VUF2274 inhibits US28-induced PLC activation and the co-receptor function of US28 for HIV-1 entry. CX3CL1 reduces the IP3 turnover and the activation of transcription factors by US28.

Trafficking of US28

Another interesting property of US28 is its constitutive endocytic activity, which results in the localization of the receptor in the membranes of intracellular organelles, especially late endosomes/lysosomes and multi-vesicular bodies (MVBs) (Waldhoer *et al.*, 2002; Fraile-Ramos *et al.*, 2001; Tschische *et al.*, 2010). On the other hand only a minor fraction of US28 is found on the cell surface (Waldhoer *et al.*, 2003; Fraile-Ramos *et al.*, 2001; Tschische *et al.*, 2010). Furthermore, it has been suggested that the virions of HCMV may be assembled in these MVBs, whereby the viral receptor is incorporated into the viral membranes during the final stages of virus assembly (Fraile-Ramos *et al.*, 2002; Cepeda *et al.*, 2009).

Introduction

It was shown that US28 constitutively internalizes through multiple mechanisms, including endocytosis via clathrin coated pits, and to a lesser extent via caveolae or lipid rafts (Droese *et al.*, 2004). In addition, it was reported that although US28 may utilize β -arrestin for internalization (Miller *et al.*, 2003), it can be endocytosed in β -arrestin-deficient cells (Fraile-Ramos *et al.*, 2003). The ability of US28 to undergo constitutive endocytosis was shown to be controlled by its cytoplasmic tail (c-tail). Interestingly, this property was found to be transferable to other 7TM/GPCRs. The exchange of the c-tail of the tachykinin (NK1) receptor with the US28 c-tails resulted in the constitutive endocytosis of the NK1 receptor (Waldhoer *et al.*, 2003). Additionally, truncation of the US28 c-terminus (US28 Δ 300 and US28 Δ 317) prevented the constitutive endocytosis and yielded the surface expression of the receptor (Waldhoer *et al.*, 2003). By employing a signalling-mute US28, where the arginine of its DRY motif was substituted with an alanine (US28 R129A), Waldhoer *et al.* also established that the constitutive endocytosis is independent of its constitutive signalling activity (Waldhoer *et al.*, 2003). Another way to regulate the trafficking of 7TM/GPCRs is thought to occur via post-translational modifications. The constitutive phosphorylation of the c-tail of US28 was reported to be of importance, since mutation of all phosphorylation sites yielded an increased surface expression of US28 (Mokros *et al.*, 2002). In agreement with this study, Waldhoer and co-workers also used a c-terminally truncated mutant of US28 (US28 Δ 300) that showed increased surface expression and also increased constitutive signaling activity (Waldhoer *et al.*, 2003).

US28 in HCMV inflicted pathogenesis

The ability of US28 to mediate SMC migration was suggested to be the causative link between HCMV infection and the accelerated progression of vascular diseases (Streblow *et al.*, 2003). The up-regulation of COX-2 in NIH3T3 cells by US28 (Maussang *et al.*, 2009), in addition to its ability to induce tumours in an NIH3T3 nude mouse model (Maussang *et al.*, 2006), point toward a possible role of US28 in tumorigenesis. Indeed, human cytomegalovirus has been shown to be present in various malignancies, including breast cancer (Soderberg-Naucler, 2008), colon cancer (Harkins *et al.*, 2002) or malignant glioblastoma (Sabatier *et al.*, 2005). Furthermore, US28 was also shown to function as a co-receptor for HIV-1 infection in CD4+ cells (Pleskoff *et al.*, 1997). The inverse agonist VUF2274 is able to decrease US28-mediated HIV-1

Introduction

entry (Casarosa *et al.*, 2003). Summarized, the functional involvement of the HCMV encoded receptor US28 in various disease states might represent a possibility for therapeutic interventions.

2. Results

2.1.Part I : The signaling and trafficking of US28*

*recently published in:

Pia Tschische, Elisabeth Moser, Dawn Thompson, Henry F. Vischer, Gerald P. Parzmair, Veronika Pommer, Wolfgang Platzer, Thomas Schwarzbraun, Helmut Schaidler, Martine J. Smit, Lene Martini, Jennifer L. Whistler & Maria Waldhoer. The G protein-coupled receptor associated sorting protein GASP-1 regulates the signaling and trafficking of the HCMV encoded chemokine receptor US28. *Traffic* (2010); 11: 660-674 (see Appendix 1)

2.1.1. Background and specific aims

The HCMV encoded chemokine receptor US28 is constitutively endocytosed and thus predominantly located in intracellular compartments including lysosomes and multivesicular bodies (MVB). This is of particular interest, since it was suggested that the virions of HCMV may be assembled in these MVBs, whereby US28 is incorporated into the viral membranes. However, the mechanism and sorting proteins targeting the receptor to these intracellular compartments were not known. Furthermore, US28 constitutively activates different signaling cascades and was thus suggested to be involved in the early reprogramming of the host cells, immune evasion and viral dissemination.

I aimed to identify the mechanisms by which US28 is targeted to the intracellular compartments. The G protein coupled receptor-associated sorting protein (GASP-1) was shown to target several 7TM/GPCRs to the lysosomal pathway and was thus chosen as a possible candidate involved in the sorting of US28. Furthermore, the post-endocytic trafficking is a fundamental process that controls the signaling activity of 7TM/GPCRs. Hence, I also determined the signaling capacity of US28 in dependence of its post-endocytic fate.

The major project aims were as following:

AIM 1: Evaluate the role of GASP-1 in the post-endocytic sorting of US28

AIM 2: Assess the role of GASP-1 in the signaling capacity of US28

2.1.2. Protein-protein interaction of GASP-1 and US28.

GASP-1 interacts with the cytoplasmatic portions of many 7TM/GPCRs (Heydorn *et al.*, 2004). For members of the opioid (Whistler *et al.*, 2002), dopamine (Bartlett *et al.*, 2005;Thompson *et al.*, 2007), bradykinin (Enquist *et al.*, 2007) and cannabinoid (Martini *et al.*, 2007;Tappe-Theodor *et al.*, 2007) receptor families, GASP-1 has been shown to not only bind, but also regulate the trafficking of these receptors, suggesting that interaction with GASP-1 could facilitate degradation of diverse 7TM/GPCRs.

Since US28 has been reported to constitutively endocytose and sort to vesicles of the classical degradation pathway, i.e. late endosomes and lysosomes (Waldhoer *et al.*,

2003; Fraile-Ramos *et al.*, 2001), we examined whether degradation of the virally encoded receptor US28 was modulated by GASPs.

First, I examined whether GASP-1 and GASP-2 interacted with a GST-fusion protein containing the 59 amino acid carboxyterminus (c-terminus) of US28. *In vitro*, both GASP-1 and its homologue GASP-2 bound to the cytoplasmic tail of US28, as did the ‘degrading’ δ opioid receptor (DOR), which served as positive control (see (Tschische *et al.*, 2010) Fig. 1A and 1B). In contrast, a ‘recycling’ receptor, the μ opioid receptor (MOR), did not bind to either GASP-1 or GASP-2 (Whistler *et al.*, 2002). Consistent with its ability to interact with GASP-1 and GASP-2 *in vitro*, US28 co-immunoprecipitated endogenous GASP-1 and GASP-2 in HEK293 cells (see (Tschische *et al.*, 2010) Fig. 1C).

Summarized, these data suggest that US28 directly interacts with GASP-1 and GASP-2 via its carboxy-terminus.

AIM 1: Evaluate the role of GASP-1 in the post-endocytic sorting of US28

Since we were able to show that GASP-1 interacts with the c-tail of US28, we next sought to elucidate the role of GASP-1 in the post-endocytic trafficking of US28. Since GASP-1 has been reported to be a sorting protein that targets various 7TM/GPCRs to the lysosomal pathway, we tested whether preventing the interaction of US28 and GASP-1 could alter the post-endocytic localization of US28. We approached this question using two independent techniques. We examined the effects of (i) dominant negative cGASP-1 and of (ii) shRNA knock-down of GASP-1 on the localization of US28.

2.1.3. The post-endocytic sorting of US28

In previous studies (Whistler *et al.*, 2002; Martini *et al.*, 2007), the c-terminal fragment of GASP-1, corresponding to the COOH-terminal 497 residues of GASP-1 (cGASP-1), has been used as a dominant negative to compete with GASP-1 for receptor-binding, thus changing the sorting of receptors targeted for degradation by GASP-1. We were able to show that dominant negative cGASP-1 binds to the c-tail of US28 and competes for binding to the US28 c-tail with full length GASP-1 (see (Tschische *et al.*, 2010) Fig. 2A and 2B, conducted by Parzmair G., Medical University of Graz). To examine whether overexpression of cGASP-1 altered the trafficking of US28, we generated a cell

line that stably expressed GFP-cGASP at ~100 fold over endogenous GASP-1 (see (Tschische *et al.*, 2010) Suppl. Fig. 1 conducted by Moser E., Medical University of Graz). Cells overexpressing GFP-cGASP-1 and untransfected HEK293 cells were transiently transfected with Flag-US28 and ‘fed’ antibody to the extracellular Flag-tag of US28 for 45 minutes to monitor the constitutive endocytosis and trafficking of US28 in live cells (Whistler *et al.*, 2002; Martini *et al.*, 2007). As has been shown previously (Waldhoer *et al.*, 2003), and in Figure 9A, US28 is predominantly found in lysosomes after 45 minutes in HEK293 cells, as evidenced by its co-localization with the lysosomal markers LAMP1 and LAMP2. In contrast, in the presence of GFP-cGASP-1, US28 undergoes a dramatic redistribution to vesicles close to the cell membrane that do not colocalize with the lysosomal markers (Fig. 9B, see arrows) (conducted in collaboration with Moser E., Medical University of Graz).

Figure 9

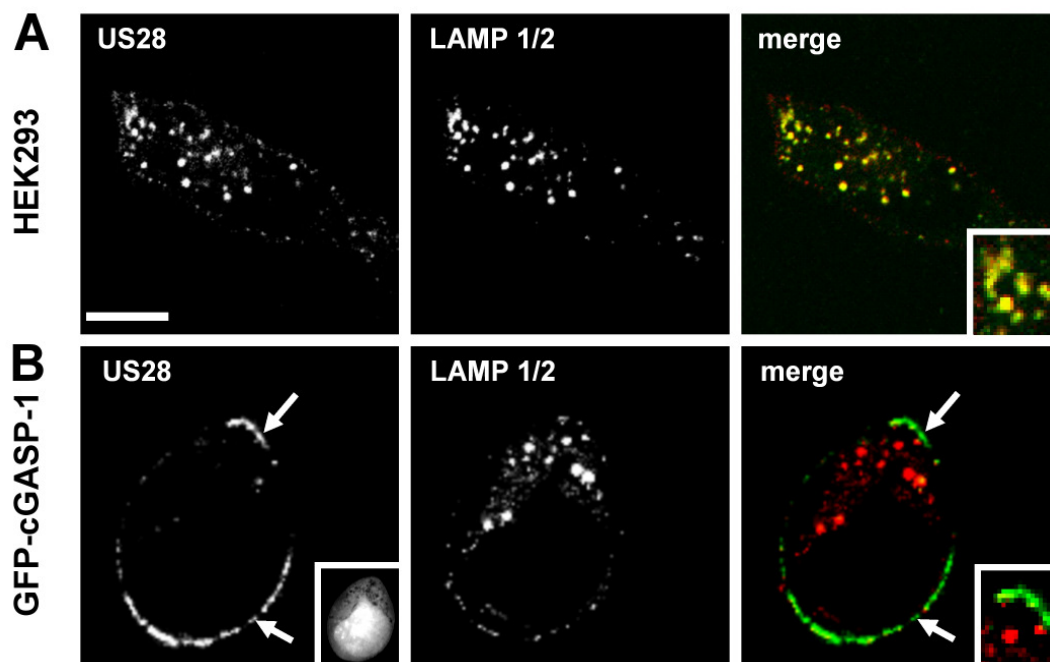


Figure 9. A. US28 is sorted to lysosomes. HEK293 cells transiently transfected with Flag-US28 were ‘fed’ antibody to the extracellular Flag-tag for 45 minutes, then fixed and permeabilized. Receptors (green) were analysed for co-localization with the lysosomal markers LAMP1 and LAMP2 (red), merge: yellow. (These experiments were done in collaboration with Moser E.) **B. cGASP-1 alters the localization of US28.** Antibody feeding experiments in HEK293 cells stably expressing GFP-cGASP-1 (see

Results Part I

insert) and transiently expressing Flag-US28 revealed a redistribution of the receptor to vesicles close to the cell surface (see arrows). Green: Flag-US28, red: LAMP1/2, merge: yellow, (These experiments were done in collaboration with Moser E.)

To further address the role of GASP-1 in the lysosomal targeting of US28, we developed shRNAs that knock-down the expression of GASP-1 (shRNAs were designed by Thompson D., Ernest Gallo Clinic and Research Center, University of California San Francisco). Lentiviral infection of HEK293 cells with shRNA targeting GASP-1 (Fig. 10A, shGASP-1) for 48 hours showed an efficient knock-down of GASP-1 protein levels in western blot, while scrambled shRNA virus had no effect on GASP-1 levels (Fig. 10A, shScr). In both, shScr and shGASP-1 infected cells, GASP-2 levels remained unchanged (Fig. 10A, see 130 kDa band). In immunofluorescence microscopy US28 co-localized with LAMP-positive endosomes in cells infected with shScr virus (Fig. 10B). In contrast, in cells infected with the shGASP-1 virus, US28 showed a prominent redistribution to vesicles close to the cell membrane (Fig. 9B, white arrows), with a close resemblance to the receptor localization observed in cells overexpressing cGASP-1 (compare Figs. 9B and 10C, respectively), and no co-localization with lysosomes (Fig. 10C, red) (experiments were conducted in collaboration with Moser E., Medical University of Graz.).

Figure 10

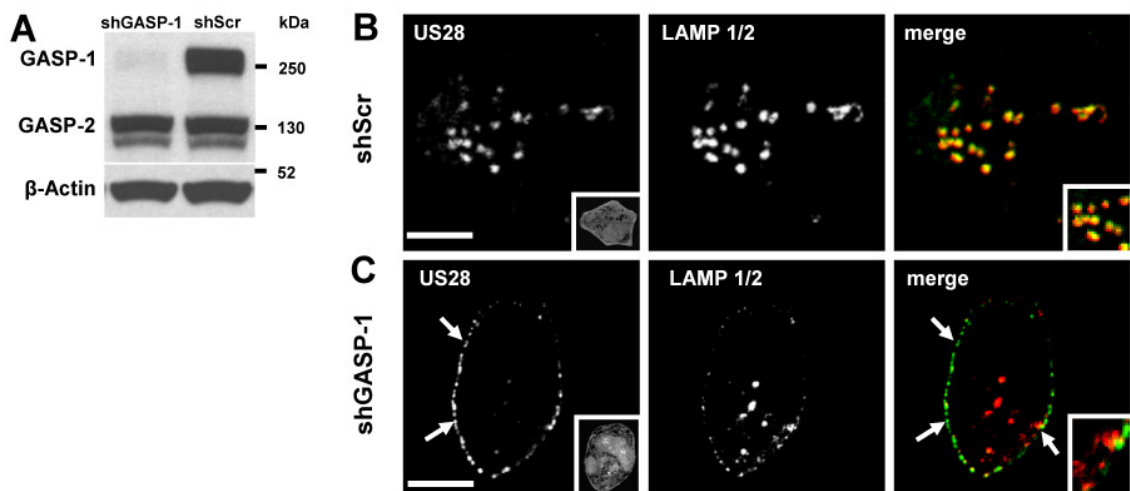


Figure 10. A. Knock-down of endogenous GASP-1 in HEK293 cells using a shRNA-lentivirus. HEK293 cells were infected with either shGASP-1 or shScr virus. 48 hours post-infection, cells were lysed, separated by SDS/PAGE and immunoblotted for GASP-1. β -Actin staining served as loading control (lower panel). GASP-2 levels remained unchanged in both shScr and shGASP-1 infected cells (see 130 kDa band). **B & C. shRNA knock-down of GASP-1 prevents lysosomal targeting of US28.** HEK293 cells were infected with either shScr virus (**B**) or shGASP-1 virus (**C**) and transfected with Flag-US28. Antibody feeding experiments showed that US28 co-localizes with LAMP1 and LAMP2 in cells infected with scrambled virus (**B**), whereas the receptor is redistributed to vesicles close to the cell surface in cells infected with shGASP-1 (**C**, see arrows). Green: Flag-US28, red: LAMP1/2, merge: yellow, insert: EGFP-shRNA-virus. Scale bars = 10 μ m. (These experiments were done in collaboration with Moser E.)

To rule out the possibility that shRNA knock down of GASP-1 exerts off-target effects on lysosomal function *per se*, we examined whether ligand-induced proteolysis of the epidermal growth factor receptor (EGFR) was intact in cells infected with shScr or shGASP-1 virus. Indeed, the degradation of EGFR was not impaired in cells depleted of GASP-1 by shRNA (see (Tschische *et al.*, 2010) Fig. 2F).

In order to identify the vesicles to which US28 is redistributed in cells overexpressing cGASP-1 (Fig. 9B), and in shGASP-1 infected cells (Fig. 10C) I tested whether these vesicles belonged to the early endosomes, defined by colocalization with the early endosomal antigen 1 (EEA.1) or to the recycling endosomal pathway defined as colocalization with transferrin. EEA.1 is a protein with the size of about 180 kDa that is associated with tubulovesicular early endosomes (Mu *et al.*, 1995). It was found to be present in the cytosol as well as in the membrane fraction and co-localizes with transferrin and Rab 5 (marker for early endosomes), but not with Rab7 (marker for late endosomes/lysosomes) (Mu *et al.*, 1995).

US28 partially co-localizes with EEA.1 in HEK293 cells (Fig. 11A, see insert). However, in cells overexpressing GFP-cGASP-1 (Fig. 11B, insert), no co-localization with EEA.1 was detectable in the US28-containing vesicles (Fig. 11B, arrows). Similarly, US28 co-localizes with EEA1 in shScr infected cells (Fig. 11C, insert), whereas US28 vesicles did not overlap with EEA.1 in shGASP-1 infected cells (Fig.

11D, arrows). Hence, the small US28 receptor pool detectable in early endosomes (Figs. 11A and 11C) seems to be dependent on the presence of GASP-1.

Figure 11

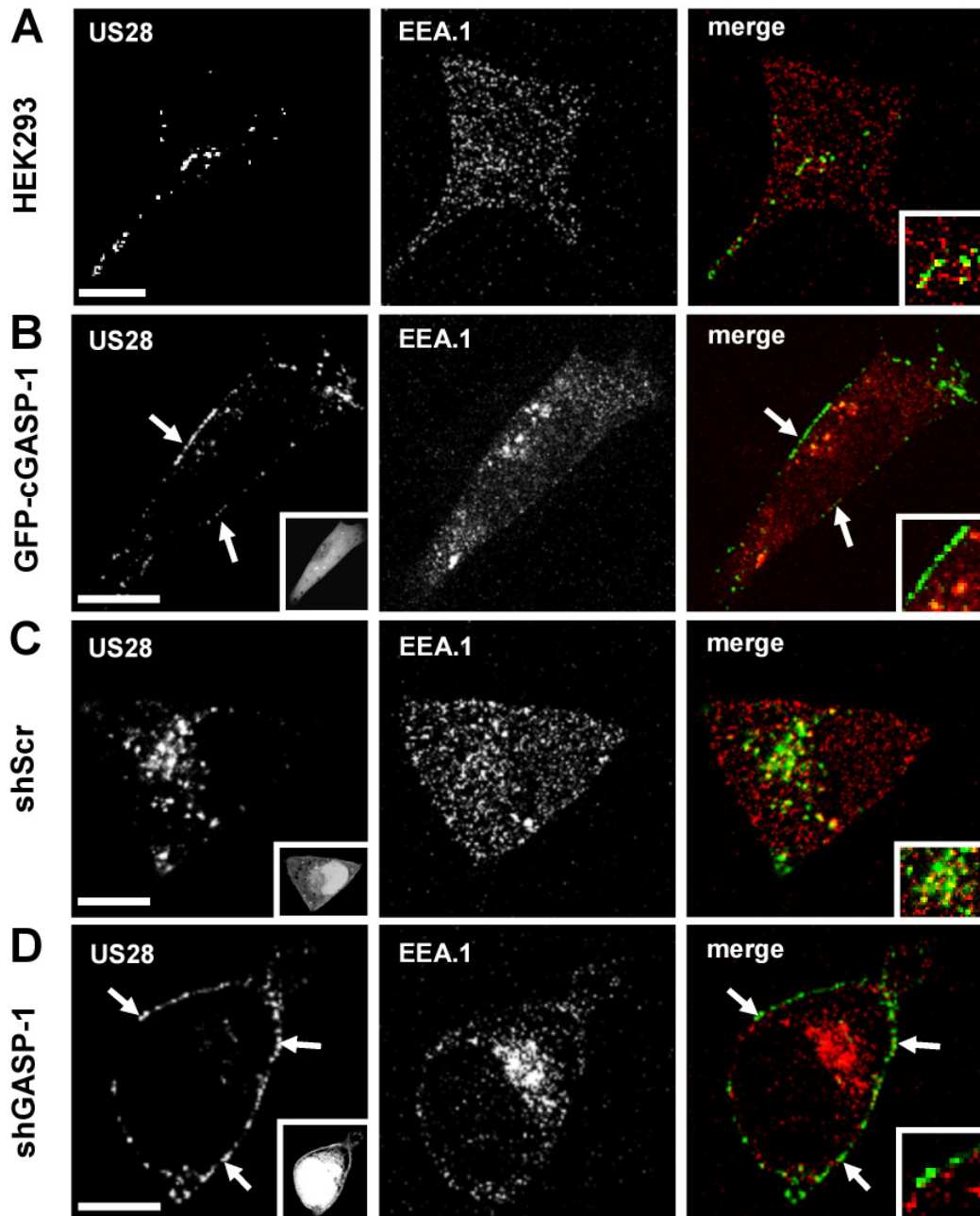


Figure 11. Co-localization of US28 with the endosomal markers EEA.1 and transferrin. A. Co-localization of US28 with EEA.1. HEK293 cells transiently transfected with Flag-US28 were ‘fed’ antibody to the extracellular Flag-tag for 45 minutes, then fixed and permeabilized. Receptors (green) were analysed for co-

Results Part I

localization with the early endosomal marker EEA.1 (red), merge: yellow. **B. US28 does not co-localize with EEA.1 in cGASP-1 overexpressing cells.** Antibody feeding experiments in HEK293 cells stably expressing GFP-cGASP-1 and transiently expressing Flag-US28 revealed no co-localization of the receptors with EEA.1. Green: Flag-US28, red: EEA.1, insert: GFP-cGASP-1. **C & D. Co-localization of US28 with EEA.1 in shScr and shGASP-1 infected cells.** HEK293 cells were infected with either shScr virus (C) or the shGASP-1 virus (D) and transfected with Flag-US28. Antibody feeding experiments showed that US28 partially co-localizes with EEA.1 in cells infected with scrambled shRNA (C) whereas there was no co-localization detectable in cells infected with shGASP-1 (D). Green: Flag-US28, red: EEA.1, merge: yellow, insert: EGFP-shRNA-virus.

It was previously reported that a fraction of the US28 receptor pool is found in early recycling endosomes ((Waldhoer *et al.*, 2003; Fraile-Ramos *et al.*, 2001) and Figs. 12A and 12C). Hence, I assessed whether the US28-containing vesicles in cells overexpressing GFP-cGASP-1 or devoid of GASP-1 co-localize with the recycling endosomal marker transferrin. Transferrin is a 77 kDa glycoprotein that belongs to the family of iron binding proteins. It is routinely used as a marker for the recycling pathway since it was shown to be rapidly recycled through a series of endosomal compartments (Jing *et al.*, 1990; Kim *et al.*, 2006).

Indeed, we observe a co-localization with transferrin in both, GFP-cGASP1 (12B, arrows and insert) and shGASP-1 (12D, arrows and insert) infected cells.

Figure 12

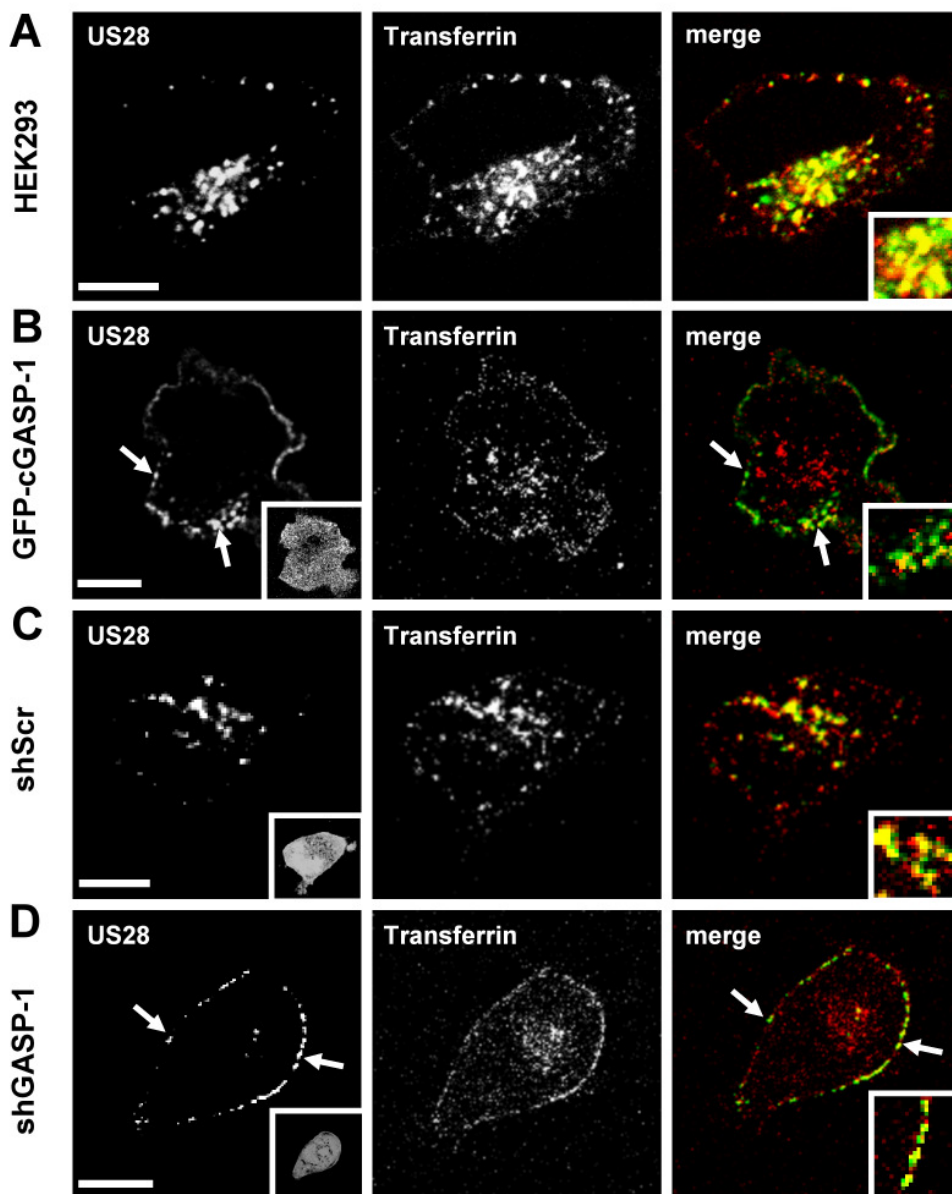


Figure 12 A. US28 co-localizes with the endosomal marker transferrin. Antibody feeding experiments in HEK293 cells transiently transfected with Flag-US28 were conducted in the presence of AlexaFluor 594-conjugated transferrin (for details see methods). Receptors (green) were analysed for co-localization with the endosomal marker transferrin (red), merge: yellow. **B. US28 co-localizes with transferrin in GFP-cGASP-1 overexpressing cells.** Antibody feeding experiments in HEK293 cells stably expressing GFP-cGASP-1 and transiently expressing Flag-US28. Green: Flag-US28, red: transferrin, merge: yellow, insert: GFP-cGASP-1. **C & D. Co-localization of US28 with transferrin in shScr and shGASP-1 infected cells.** HEK293 cells were transduced with either shScr virus (C) or the shGASP-1 virus (D) and transfected with Flag-US28.

Results Part I

Antibody feeding experiments showed that US28 partially co-localizes with transferrin in cells infected with scrambled virus (C) as well as in cells infected with shGASP-1 (D). Green: Flag-US28, red: transferrin, merge: yellow, insert: EGFP-shRNA-virus. Scale bars = 10 μ m.

In addition to the knock-down experiments in HEK293 cells, the importance of GASP-1 in targeting US28 to the lysosomal pathway was verified in GASP-1 knock out (KO) mouse embryonic fibroblasts (MEFs). Recently, GASP-1 KO, conditional GASP-1 KO mice as well as GASP-2 KO mice were established in Jennifer Whistlers laboratory at the Ernest Gallo Clinic and Research Center, UCSF (Martini *et al.*, 2010; Thompson *et al.*, 2010). Conditional GASP-1 knock out mice and the GASP-2 KO mice were used to generate immortalized MEF cell lines. Therefore, pregnant mice anaesthetized with isoflurane were sacrificed between day 13 and 17 post coitus. The uterine horns were dissected and the individual embryos were separated (see Fig. 13). Then, the tissue was minced and the cells were plated at the same inoculation density (for details see methods). The cells were kept on a three-day transfer schedule for about three months, which finally yielded immortalized mouse embryonic fibroblast cell lines.

Figure 13



Figure 13. Generation of mouse embryonic fibroblasts (MEFs). Deeply anaesthetized mice were sacrificed by cervical dislocation. Uterine horns were dissected and the individual embryos were separated.

In the conditional GASP-1 KO mice, the GASP-1 coding region is flanked (“floxed”) by two loxP sites. The addition of the enzyme Cre-recombinase, that catalyses a homologous recombination between the two loxP sites, yields the knock out of GASP-1 (see Fig. 14).

Figure 14

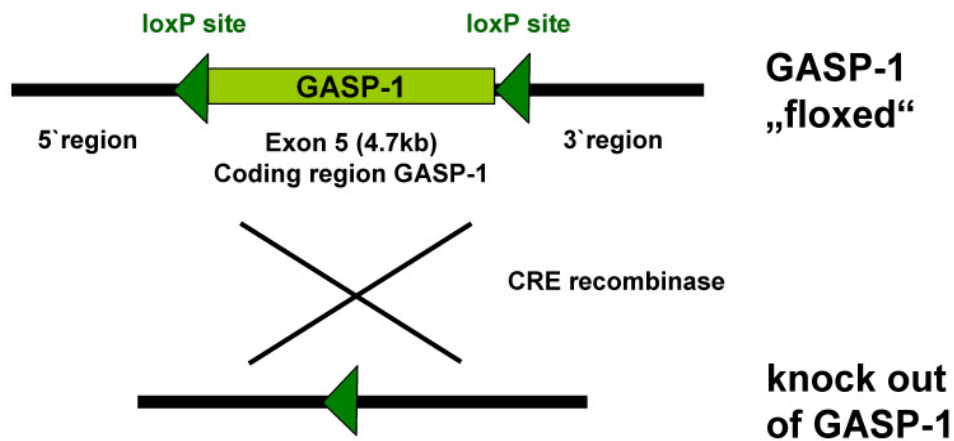


Figure 14. Knock out of GASP-1 in GASP-1 “floxed” MEFs. The GASP-1 coding region is flanked by two loxP sites (GASP-1 “floxed”). Cre recombinase catalyses the homologues recombination and yields the knock out of GASP-1.

The infection of GASP-1 “floxed” MEF cells with 800 multiplicities of infection (MOI) adenovirus encoding for Cre recombinase (Ad-CRE) yielded a significant knock down of GASP-1 in comparison to the cells infected with the control virus (Ad-GFP) (Fig. 15; compare lane 1 vs. lane 3, GASP-1 bands are at ~ 250 kDa) (see also (Martini *et al.*, 2010)).

Figure 15

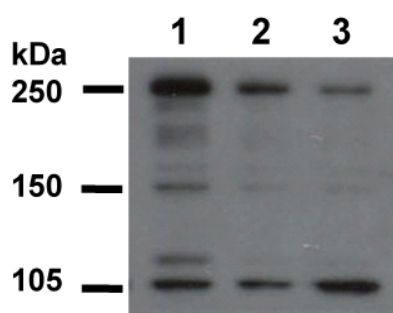


Figure 15. Knock down of GASP-1. GASP-1 floxed MEF cells were infected with Ad-CRE or Ad-GFP. After 48h, cells were lysed, separated by SDS-PAGE and blotted with α -GASP antibody (1:1000) and goat- α -rabbit HRP (1:4000). **Lane 1:** infection with Ad-GFP (800 MOI); **lane 2:** infection with Ad-CRE (200 MOI); **lane 3:** infection with Ad-CRE (800 MOI).

Results Part I

To determine the role of GASP-1 and GASP-2 in the trafficking of US28, antibody feeding experiments were conducted in GASP-1 “floxed” (wild type cells with intact GASP-1), GASP-1 KO and GASP-2 KO MEFs. Hence, MEF cells were transiently transfected with Flag-US28. 24h later, antibody feeding experiments were conducted. Then, the cells were fixed, stained under permeabilizing conditions and analyzed by fluorescence microscopy. In the Ad-GFP infected GASP-1 “floxed” cells (= control cells) US28 was predominantly found in intracellular compartments (Fig. 16A). A similar distribution of US28 was also observed in GASP-2 KO cells, suggesting that GASP-2 is not implicated in the post-endocytic sorting of US28 (Fig.16C). In contrast, in the absence of GASP-1, US28 was localized in vesicles close to the cell surface (Ad-Cre infected cells) (see Fig. 16B). These findings verify the important role of GASP-1 in the lysosomal sorting of US28.

Figure 16

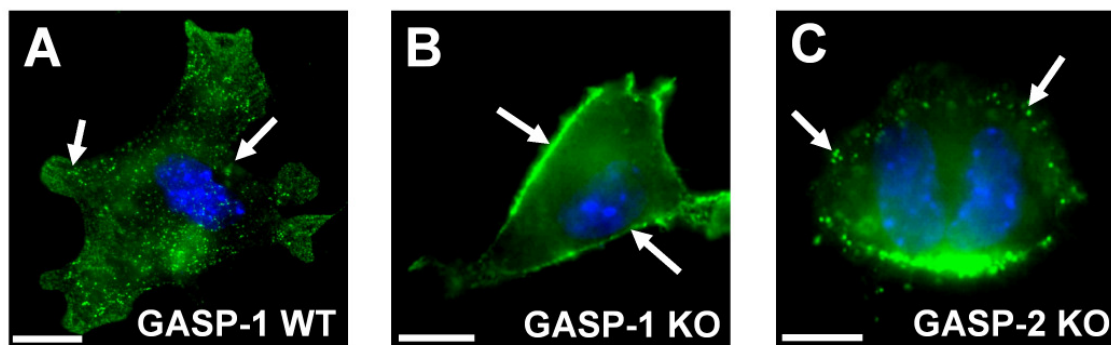


Figure 16. Localization of US28wt in MEF cells. *GASP-1* wild type (WT) “floxed” MEFs (A), *GASP-1* knock out (KO) MEFs (B) and *GASP-2* KO MEFs (C) were transiently transfected with Flag-US28. 24h later, antibody feeding experiments were conducted with monoclonal anti-Flag M1 for 45 minutes. After fixation, the cells were stained with IgG2b 488nm Alexa dyes. In *GASP-1* WT (A) and in *GASP-2* KO cells (C) US28 (green) is localized mainly in vesicles in the cell, whereas in the *GASP-1* knock out MEFs (B) the receptor is localized on the cell surface. Scale bars = 10 µm.

2.1.4. GASP-1 mediated degradation of US28

In order to quantitatively monitor the endocytic and post-endocytic fate of US28 in the absence or presence of GASP-1, we next performed biotin protection and degradation assays. This assay allows selective monitoring of the endocytic- and post-endocytic fate of a pool of receptors that reaches the cell surface and is subsequently endocytosed and either recycled or degraded (see also (Whistler *et al.*, 2002; Martini *et al.*, 2007; Bartlett *et al.*, 2005)).

Although the antibody feeding experiments suggest that US28 constitutively endocytoses in the absence of GASP-1, we wanted to test whether GASP-1 alters the kinetics of US28 internalization in either GFP-cGASP-1 overexpressing or in shGASP-1 transfected cells. These experiments showed that the amount of internalized receptor is increasing during a time course from 0 to 45 minutes in HEK293 cells, and cells stably overexpressing GFP-cGASP-1 and in cells transfected with shScr vs shGASP-1 (see (Tschische *et al.*, 2010); Fig. 4A & B; experiments were conducted by Moser E., Medical University of Graz). Hence, it seems that GASP-1 is not important for the internalization kinetics of US28. However, a higher amount of biotinylated surface receptor was consistently observed in cells overexpressing GFP-cGASP-1 and in shGASP-1 cells (compare 100% lanes in Figs. 4A and 4B in (Tschische *et al.*, 2010)).

In case GASP-1 is mediating the degradation of US28, one would expect that either the overexpression of cGASP-1 or the knock-down of endogenous GASP-1 levels could delay or even inhibit receptor degradation. Hence, the stability of the biotinylated and constitutively endocytosed US28 receptor pool was monitored. In cells overexpressing GFP-cGASP-1, the degradation of biotinylated and internalized US28 was delayed (see (Tschische *et al.*, 2010) Fig. 4C; conducted by Moser E., Medical University of Graz) when compared to control cells. Likewise, in HEK293 cells infected shGASP-1 lentivirus, internalized and biotinylated US28 receptor was also significantly more stable than in cells infected with shGASP-1 but not with shScr lentivirus (see (Tschische *et al.*, 2010) Fig. 4D; conducted by Moser E., Medical University of Graz).

Taken together, these results indicate that the interaction between US28 and GASP-1 is crucial for the sorting of the receptor to the degradative pathway.

2.1.5. Recycling of US28 in the absence of GASP-1

US28 has been reported to constitutively internalize and also to constitutively recycle in some circumstances ((Fraile-Ramos *et al.*, 2001) and Fig. 17). Next, I examined whether disruption of the US28 / GASP-1 interaction not only blocked receptor degradation, but also facilitated recycling.

To assess recycling, cells transiently transfected with Flag-US28 alone (Fig. 17A) and over-expressing GFP-cGASP-1 (Fig. 17B) were fed M1-antibody for 45 minutes to allow for receptor internalization. Then cells were stripped of the M1-antibody (Figs. 17A and 17B, strip) and resurfacing (recycling) of the receptors was monitored for 15 and 30 minutes. Cells were then fixed and recycled receptor was detected by staining with a fluorescent antibody targeting the M1-antibody under non-permeabilizing conditions. Control cells show weak surface staining of US28 (Figs. 17A and 17C, 45 min), which is consistent with the approx. 20% of surface expression of the total US28 receptor pool reported previously (Waldhoer *et al.*, 2003; Fraile-Ramos *et al.*, 2001). Nevertheless, recycling US28 was detected as early as 15 min after stripping (Figs. 17A and 17C, +15min panel). However, in cells over-expressing cGASP-GFP-1 (Fig. 17B, 45 min) or shGASP-1 cells (Fig. 17D, 45 min), significantly more US28 surface staining was visible than in control cells. Similarly to the control cells, recycling US28 was detectable in cells as early as 15 minutes after stripping (Figs. 17B and 17D, +15min panels) and US28 surface staining was detectable up to +30 minutes.

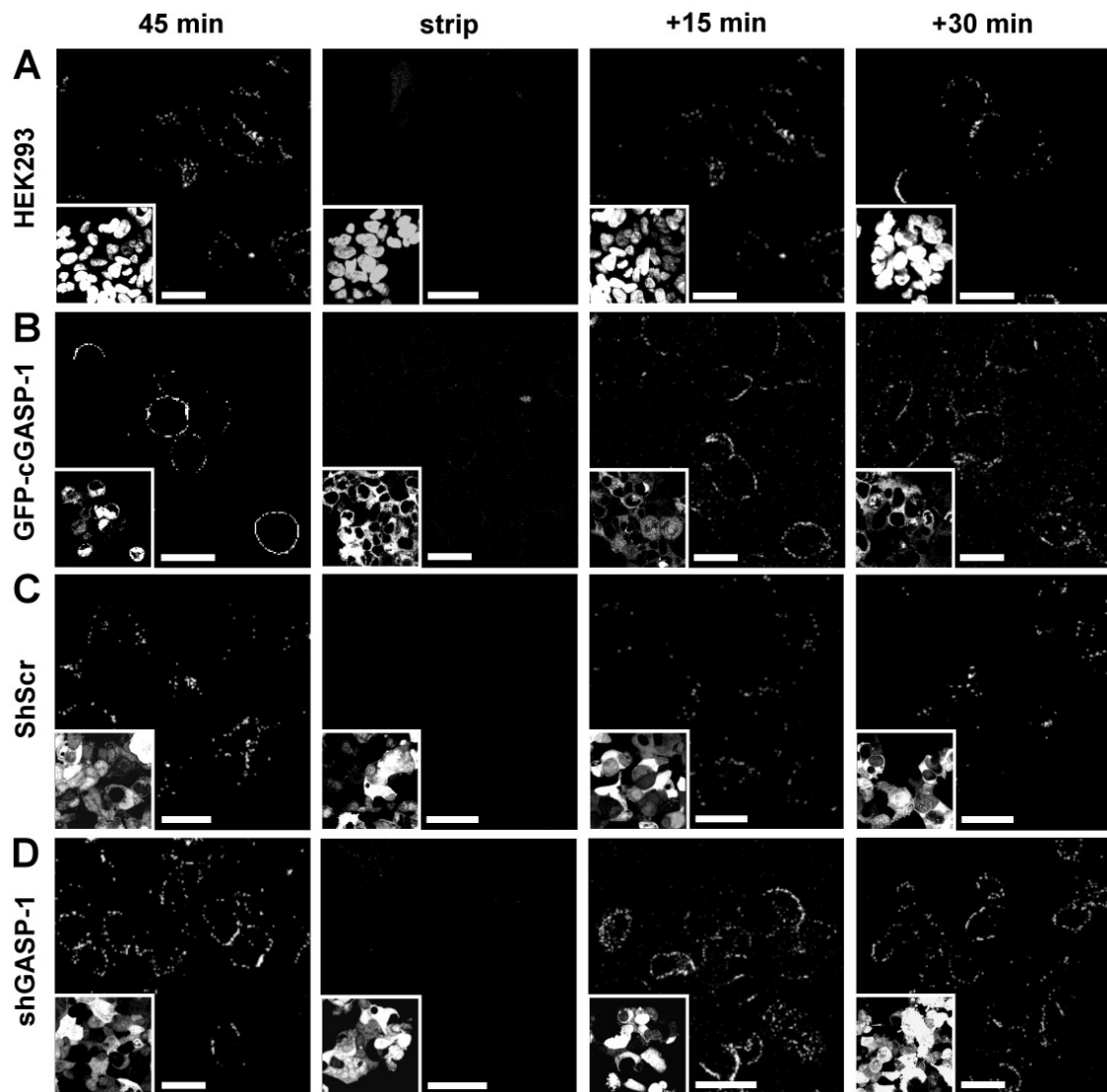
Figure 17

Figure 17. Constitutive recycling of US28 is not dependent on GASP-1. A & B Recycling of US28 is not impaired in cells overexpressing GFP-cGASP-1. HEK293 cells (A) or cells stably expressing GFP-cGASP-1 (B) transiently transfected with Flag-US28 were ‘fed’ a Ca^{2+} sensitive antibody to the extracellular Flag-tag for 45 minutes. Then the cells were stripped with PBS-EDTA (strip) and warm growth medium was added. After 15 or 30 minutes the cells were fixed and the recycled receptor was stained under non-permeabilizing conditions. Inserts: DAPI (A) and GFP-cGASP-1 (B). **C & D** Recycling of US28 is not impaired in shScr or shGASP-1 infected cells. HEK293 cells infected with shScr virus (C) or shGASP-1 virus (D) were transiently transfected with Flag-US28. Recycling experiments were performed as in A & B. inserts: GFP-shRNA lentivirus. Scale bars = 20 μm .

2.1.6. Summary – Aim I

From the experiments conducted in Aim 1, it can be concluded that the sorting protein GASP-1 is an important cellular determinant in regulating the post-endocytic trafficking of US28. US28 is targeted for degradation via a direct protein-protein interaction with the sorting protein GASP-1 (see (Tschische *et al.*, 2010) Fig. 1C). Disruption of this process results in a vesicular redistribution of US28 close to the plasma membrane (Figs. 10B and 11C). Furthermore, these vesicles were identified to co-localize with the recycling endosomal marker transferrin (Figs. 13B and 13D), but did not co-stain for the early endosomal marker EEA.1 (Figs. 12B and 12D). The importance of GASP-1 was also confirmed in GASP-1 knock out mouse embryonic fibroblast cells. Once again, a major redistribution of US28 to the cell surface was observed in these cells (Fig. 16B) when compared to the wild type MEFs (Fig. 16A). In addition, it was determined that GASP-2 is not involved in the regulation of the post-endocytic trafficking of US28. In GASP-2 KO MEF cells, US28 was found to be similarly distributed as in the WT cells (Fig. 16C). Furthermore, the degradation of US28 was significantly delayed in GASP-1 knock-down and cGASP-1 expressing cells (see (Tschische *et al.*, 2010) Fig. 4, conducted by Moser E., Medical University of Graz). In addition, constitutive recycling of US28 was enhanced when the US28-GASP-1 interaction is disrupted (Fig. 17) and results in a higher surface expression of US28 in these cells.

AIM 2: Assess the role of GASP-1 in the signaling capacity of US28

2.1.7. US28 mediated IP accumulation

US28 has been shown to regulate the $G_{\alpha q}$ /phospholipase C (PLC) pathway both constitutively and in a ligand-dependent manner (Casarosa *et al.*, 2001; Waldhoer *et al.*, 2002; Minisini *et al.*, 2003; Stropes *et al.*, 2009). Hence, I aimed to examine the ability of US28 to mediate the $G_{\alpha q}$ /PLC dependent accumulation of inositol phosphate (IP) in cells where the interaction with GASP-1 had been altered. Since GASP-1 was found to facilitate the degradation of US28, I anticipated that disruption of the US28/GASP-1

interaction might enhance constitutive signaling of US28. Surprisingly, a ~ 1.5 fold overexpression of GASP-1 (Fig. 18B, upper panel) significantly increased the stimulation of IP turnover by US28 (Fig. 18A; compare black vs. striped bars). In contrast, overexpression of cGASP-1 (Fig. 18B lower panel) significantly reduced the IP turnover (Fig. 18A; compare black vs. grey bars). These effects were not attributable to different total expression levels of US28 in the respective assays, as verified by ELISA (Fig. 18C).

Figure 18

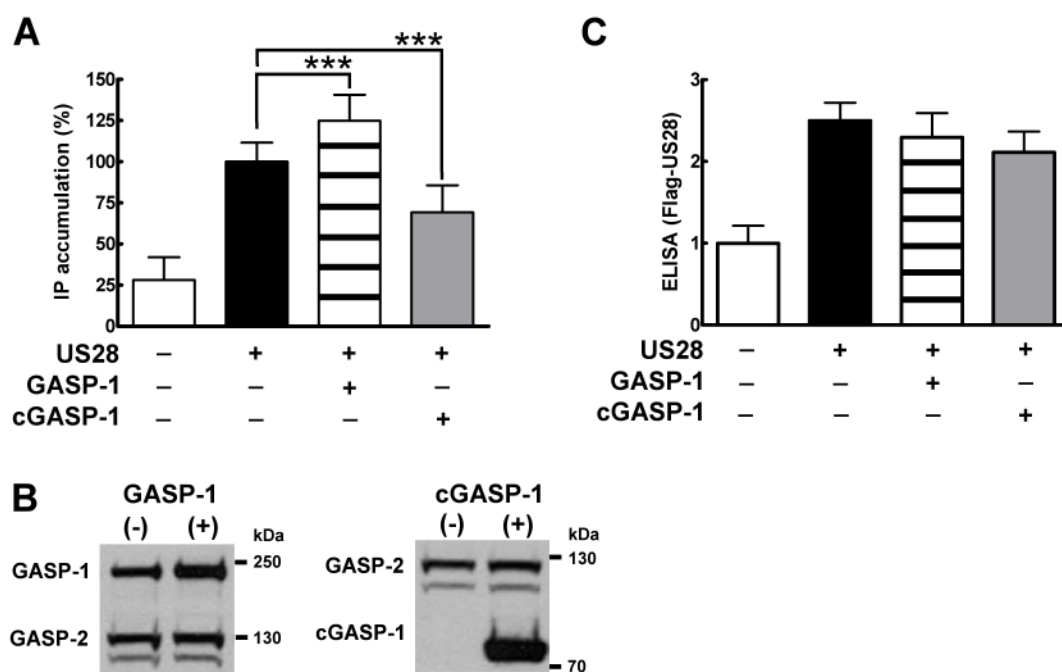


Figure 18. GASP-1 modulates US28-mediated inositol phosphate (IP) accumulation.

A. Overexpression of GASP-1 and cGASP-1 alters US28-mediated IP turnover.

HEK293 cells were transiently transfected with Flag-US28 (75 ng/well) and 100 ng/well of either pcDNA3.1 (black bar), HA-GASP-1 (striped bar) or HA-cGASP-1 (grey bar). Controls were transfected with pcDNA3.1 (175 ng/well, white bar). IP production mediated by US28 was allowed to accumulate for 45 minutes. Values were normalized to the cell number and 100 % corresponds to the basal activity of US28 (black bar). Data are means of 6 experiments $\pm$ SD carried out in quadruplicates, *** $p < 0.001$.

B. Expression levels of HA-GASP-1 and HA-cGASP-1 in HEK293-US28 cells. HEK293 cells were transiently transfected with Flag-US28 in combination

Results Part I

with pcDNA3.1 and HA-GASP-1 (upper panel) or with HA-cGASP-1 (lower panel). 24 hours later, the cells were lysed, separated by SDS/PAGE and blotted with GASP antibody. Overexpression of HA-GASP-1 or HA-cGASP-1 did not alter GASP-2 levels.

C. US28 levels in HEK293 cells overexpressing GASP-1 and cGASP-1. *HEK293 cells were transfected as in A. The expression levels of US28 were assayed by ELISA against the Flag epitope tag of US28. Values were normalized to the cell number. Data are means $\pm$ SD of one representative experiment carried out in quadruplicates. The control (white bar) was set to one and the other values were normalized accordingly.*

Next, I assessed the IP turnover in cells in which GASP-1 has been knocked down with the shGASP-1 virus. In agreement with my previous findings, the IP turnover was significantly reduced in shGASP-1 infected cells (Fig. 19A, compare black vs. striped bar).

To confirm that the decreased signaling in shGASP-1 infected cells was due to the knock down of GASP-1, I conducted rescue experiments with a shRNA-insensitive r(escue)GASP-1 (rGASP-1 was cloned by Schwarzbraun T., Medical University of Graz). Transfection of shGASP-1 infected cells with rGASP-1 resulted in a replenished GASP-1 pool (Fig. 19B). Furthermore, we confirmed that rGASP-1 bound to the c-terminus of US28 *in vitro* (see (Tschische *et al.*, 2010), Suppl.Fig. 3, conducted by Parzmair G., Medical University of Graz). Indeed, as Fig. 19A (compare striped vs. grey bars) shows, the decreased IP turnover in shGASP-1 infected cells could be restored by the re-expression of rGASP-1. US28 receptor levels were comparable in all assays (Fig. 19C).

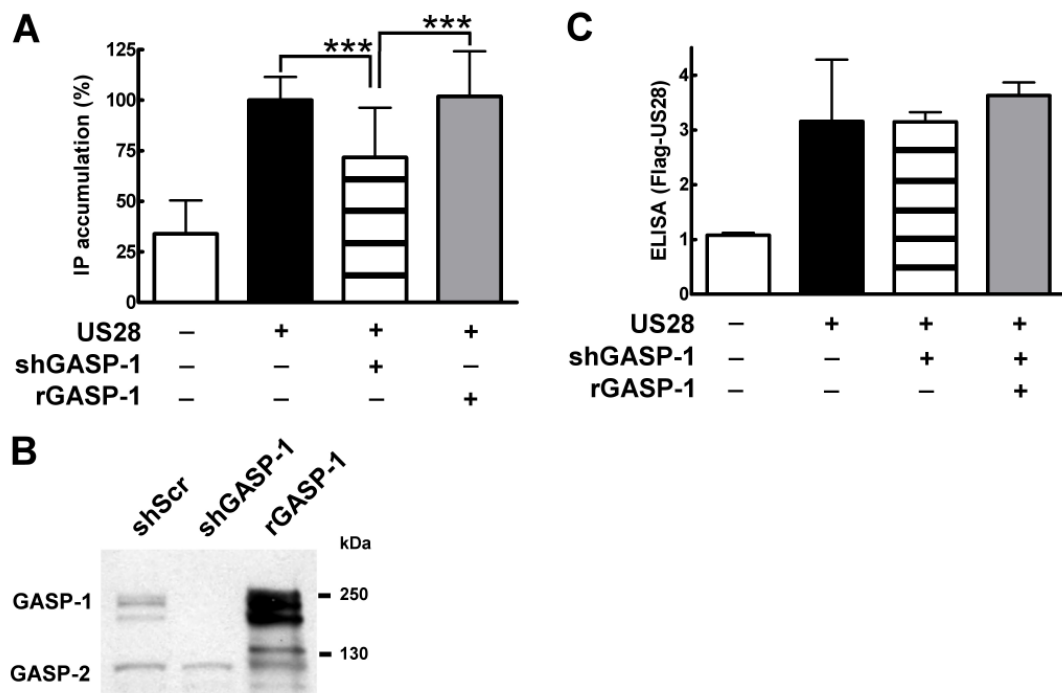
Figure 19

Figure 19A. Reduced IP accumulation in shGASP-1 infected cells is restored by overexpression of rGASP-1. HEK293 cells were infected shScr (black bar) or the shGASP-1 virus (striped bar and grey bar). Then cells were transiently transfected with Flag-US28 (75 ng/well) and either 100 ng/well pcDNA3.1 (black and striped bars) or 100 ng/well HA-rGASP-1 (grey bar). Controls were transfected with pcDNA3.1 (175 ng/well, white bar). The IP accumulation assay was conducted as described in A. Values were normalized to the cell number and 100 % corresponds to the basal activity of US28 in shScr infected cells (black bar). Data are means of 4 experiments $\pm$ SD carried out in quadruplicates, *** $p < 0.001$. **B. GASP-1 levels in shGASP-1 infected cells can be restored with HA-rGASP-1.** ShScr infected cells were transfected with Flag-US28 and pcDNA3.1. ShGASP-1 infected cells were transfected with either pcDNA3.1 or HA-rGASP-1. 24 hours later, the cells were lysed, separated by SDS/PAGE and blotted with the GASP antibody. The blot shows one representative of at least three independent experiments. **C. US28 levels in shScr and shGASP-1 infected cells.** HEK293 cells were infected and transfected (see A) and ELISA was performed as described in Fig.18C.

2.1.8. Transcription factors activation by US28

US28 has been reported to activate downstream transcription factors such as NF- κ B, CREB (Casarosa et al., 2001; Waldhoer et al., 2002; Wen et al., 2009), NFAT (McLean *et al.*, 2004) and SRF (Moepps *et al.*, 2008). Following my findings that GASP-1 levels alter the ability of US28 to activate the G α q/PLC/IP pathway, I examined whether this effect would also translate to an altered downstream signal transduction profile. Therefore, I tested the impact of altered GASP-1 levels on the US28-mediated activation of the transcription factors NF- κ B and CREB. Indeed, transient expression of US28 and overexpression of GASP-1 in HEK293 cells resulted in a significant increase of both NF- κ B and CREB - luciferase activity. (Figs. 20A and B, respectively, compare black and striped bars). Next, I disrupted the interaction of US28 and endogenous GASP-1 by overexpression of cGASP-1. cGASP-1 blocked the downstream activation of both NF- κ B and CREB - luciferase activity (Figs. 20A and 20B, compare black vs. grey bars). Receptor expression levels were similar in all experiments as investigated by ELISA (Figs. 20C and D).

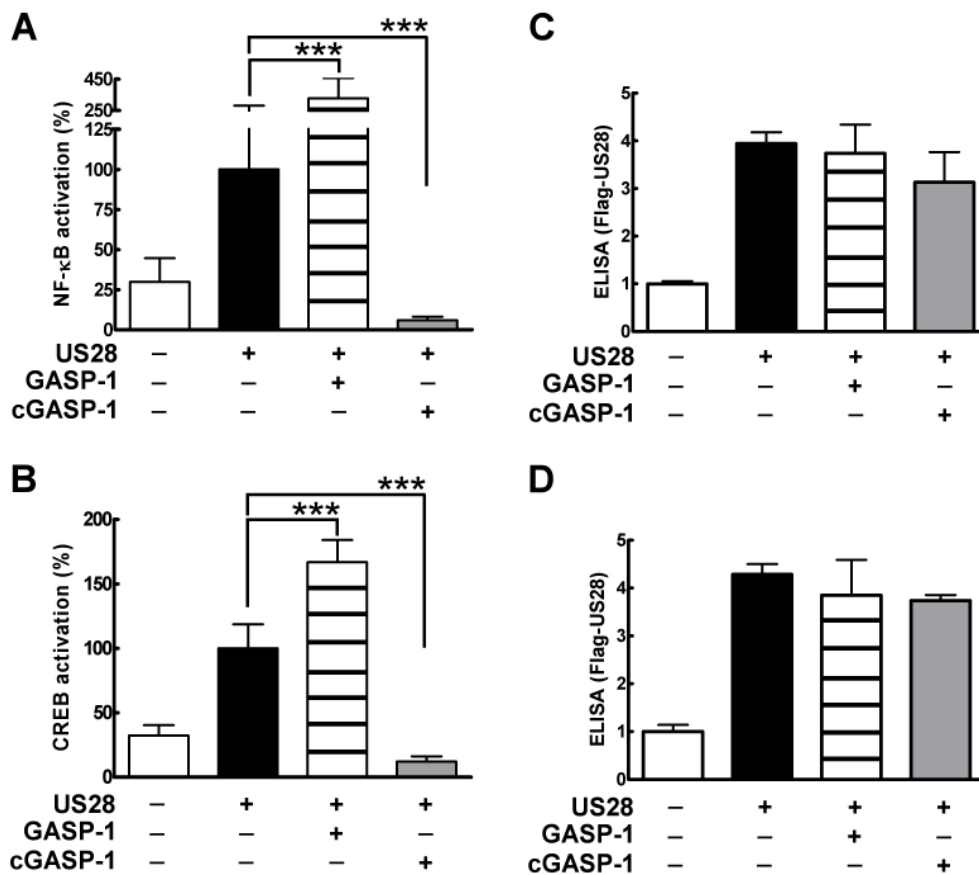
Figure 20

Figure 20. US28-mediated NF- κ B and CREB transcription is modulated by GASP-1. **A & B.** US28-mediated transcription factor activation is altered upon overexpression of GASP-1 and cGASP-1. HEK293 cells were transiently transfected with Flag-US28 (50 ng/well) in combination with 100 ng/well pcDNA3.1 (black bar), HA-GASP-1 (striped bars) or HA-cGASP-1 (grey bars) and the respective cis-reporter luciferase plasmid for either NF- κ B (50 ng/well, **A**) or CREB (200 ng/well, **B**). Controls were transfected with pcDNA3.1 (white bars). A luciferase reporter assay was conducted 24 hours post-transfection. Values were normalized to the cell number, whereby 100 % corresponds to the basal activity of US28 (black bar). Data are means of 4 experiments $\pm$ SD carried out in quadruplicates, *** p <0.001. **C & D.** US28 levels in HEK293 cells overexpressing GASP-1 and cGASP-1. HEK293 cells were transiently transfected as described in **A** and **B**. The expression levels of US28 were assayed by ELISA as described before.

To further test the hypothesis that GASP-1 is involved in the regulation of the downstream signal transduction pathways of US28, I performed the NF- κ B and CREB-luciferase assays in cells infected with the shGASP-1 and shScr virus. Indeed, the activation of transcription factors is significantly decreased in shGASP-1 infected cells in comparison to shScr infected cells (Fig. 21A and B, compare black bars vs. grey bars). To confirm that the reduced NF- κ B and CREB activation in shGASP-1 infected cells was due to the knock down of GASP-1, I re-introduced GASP-1 into shGASP-1 cells by transfection with rGASP-1. Again, rGASP-1 ‘rescued’ the activation of transcription factors via US28 in shGASP-1 infected cells (Fig. 21A and B, compare grey vs. striped bars). Receptor expression levels were similar in all experiments (Figs. 21C and D).

Figure 21

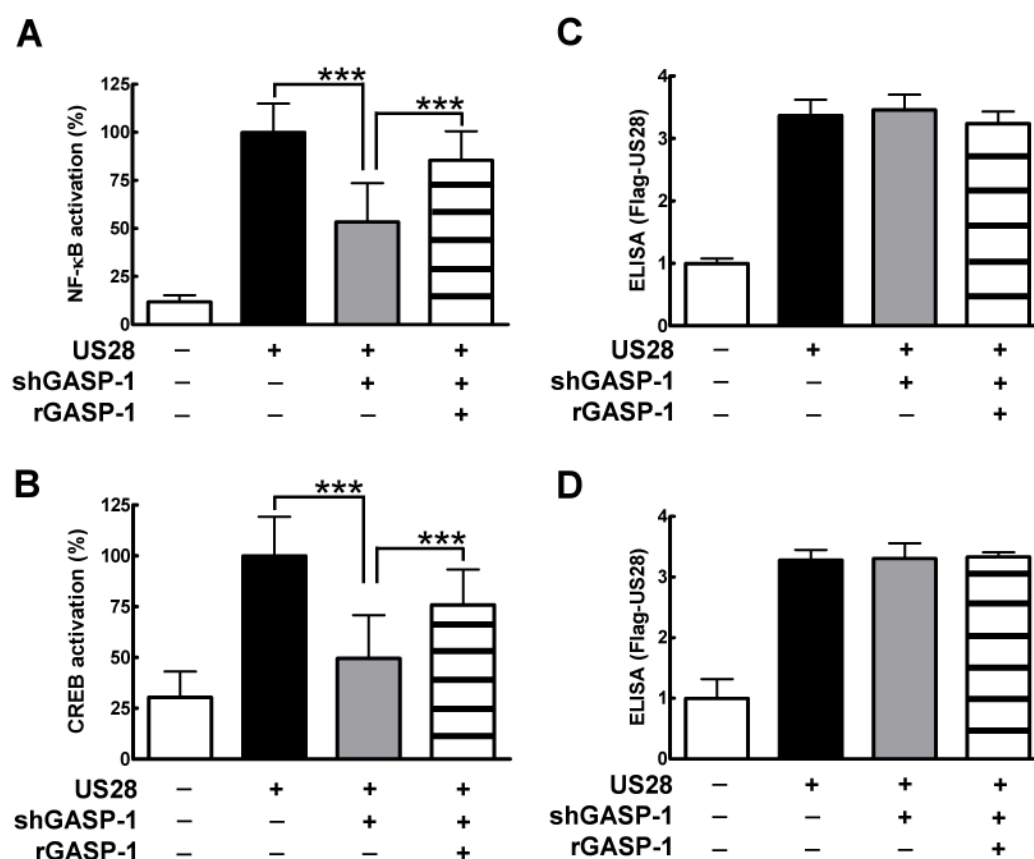


Figure 21. Reduced NF- κ B and CREB activation can be restored by overexpression of rGASP-1. A & B. US28-mediated transcription factor activation is rescued upon overexpression of rGASP-1. ShScr infected cells (black bars) and shGASP-1 infected cells (grey and striped bars) were transfected with Flag-US28 (50 ng/well) in

Results Part I

*combination with 100 ng/well of pcDNA3.1 (black bar and grey bars) or HA-rGASP-1 (striped bars) as well as the respective cis-reporter plasmids for either NF- κ B (50 ng/well, **A**) or CREB (200 ng/well, **B**). Controls were transfected with pcDNA3.1 (white bars). A luciferase reporter assay was conducted 24 hours post-transfection. Values were normalized to the cell number, whereby 100 % corresponds to the basal activity of US28 (black bar). Data are means of 4 experiments $\pm$ SD carried out in quadruplicates, *** p <0.001. **C & D. US28 levels in HEK293 cells overexpressing GASP-1 and cGASP-1.** HEK293 cells were transiently transfected as described in **A** and **B**. The expression levels of US28 were assayed by ELISA as described before.*

Due to the low transfection efficiency reached in the different mouse embryonic fibroblast cell lines, the signaling experiments could not be repeated in these cells.

2.1.9. Summary - Aim 2

In summary, the data suggest that GASP-1 not only modulates the post-endocytic sorting of US28, but also regulates the constitutive signaling capacity of US28 (visualized in Fig. 22). The results indicate that the ability of US28 to activate G α q/PLC/IP signaling is dependent on GASP-1 levels. Overexpression of the dominant negative competitor cGASP-1 or knock down of GASP-1 yielded a significantly reduced IP accumulation (Fig. 18A & 19A). The decreased signaling activity in GASP-1 knock down cells could also be rescued by restoring endogenous GASP-1 levels (Fig. 19 A, compare striped and grey bars). Furthermore, the downstream signaling events, such as the activation of transcription factors NF- κ B and CREB, were also altered by GASP-1. Disruption of the US28/GASP-1 interaction reduced the activation of NF- κ B and CREB (Fig. 20A & 20B; Fig. 21A & 21B). In contrast, overexpression of GASP-1 (Fig. 20A & B) or the restoration of endogenous GASP-1 levels (Fig. 21A & B) significantly increased the activation of transcription factors.

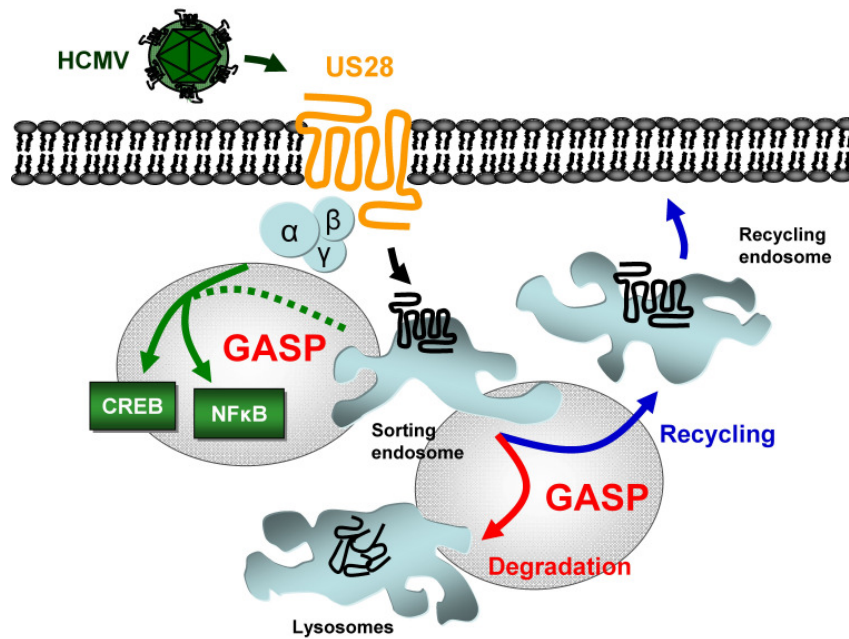
Figure 22

Figure 22. *GASP-1 regulates the post-endocytic sorting and signaling of US28. Following constitutive endocytosis, the HCMV encoded 7TM/GPCR US28 is targeted to the lysosomal/degradative pathway via direct interaction with the sorting protein GASP-1. Depletion of GASP-1 leads a redistribution of the receptor to vesicles close to the cells surface and to an enhanced recycling. Furthermore, GASP-1 is also implicated in the regulation of the constitutive signaling capacity of US28. Overexpression of GASP-1 was shown to significantly increase the activation of downstream transcription factors NF- κ B and CREB.*

2.2. Part II: Heteromerization of HCMV encoded 7TM/GPCRs

2.2.1. Background and specific aims

The activity of 7TM/GPCRs is tightly controlled by different processes, including the hetero-/oligomerization of receptors. Chemokine receptors for example were reported to form homo- and hetero-oligomeric complexes. CXCR4 was shown to form homomers that consist of a least three protomers (Hamatake *et al.*, 2009). Moreover, CXCR4 also heteromerizes with CCR5 (Guo *et al.*, 2005; Sohy *et al.*, 2009) and CCR2 (Percherancier *et al.*, 2005). Recently, the existence of a heteromeric complex consisting of CCR2, CCR5 and CXCR4 was reported. The same study showed that specific antagonists of one receptor can lead to a functional inhibition of the other heteromerization partners *in vitro* and *in vivo* (Sohy *et al.*, 2009). The interaction between CCR5 and CXCR4 has also been suggested to be involved in the modulation of T lymphocyte responses (Contento *et al.*, 2008). CCR5 and CXCR4 are known co-receptors for HIV-1 and hence their heteromerization might also alter viral entry. Indeed, it was found that dimerization of CCR5 prevents infection with HIV-1 (Vila-Coro *et al.*, 2000). It is known that CCR5 and CXCR4 mediate neuronal cell death upon infection with HIV/gp120 (Kaul *et al.*, 2007). Interestingly, it was observed that CCR5 specific ligands prevented the neurotoxicity inflicted by a CXCR4 agonist (Kaul *et al.*, 2007). These data suggest a functionally relevant interaction of CCR5 and CXCR4 and furthermore highlight the importance of receptor heteromerization in HIV-1 entry and its inflicted complications.

Based on the observation that virally encoded 7TM/GPCRs (v-GPCRs) mimic the function of endogenous chemokine receptors, I aimed to investigate a possible heteromerization of HCMV encoded receptors. Besides US28, human cytomegalovirus encodes three orphan 7TM/GPCRs, whose functions are still unknown. It was previously shown that orphan receptors are able to modulate the activity of non-orphan receptors by heteromerization (Levoye *et al.*, 2006). Thus, I hypothesized that UL78, UL33 and US27 may interact with US28 and alter its constitutive signaling capacity.

Results Part II

The 7TM/GPCRs encoded by HCMV possess high sequence homology with endogenous chemokine receptors. “Chemokine mimicry” is a potent strategy employed by the virus to evade the immune system. Considering the important functions of endogenous chemokine receptors in the immune system, heteromerization with these receptors might represent a way to modify their activity and thus benefit the virus in many ways. Hence, the ability of HCMV encoded receptors to form hetero-oligomeric complexes with cellular receptors involved in immune functions, such as the CC-chemokine receptor 5 (CCR5) and the histamine 4 receptor (H4R) was assessed.

The major aims were defined as following:

AIM 1: Determine possible co-localization of the HCMV encoded 7TM/GPCRs and evaluate the functional consequences of receptor hetero-/oligomerization

AIM 2: Assess whether HCMV encoded receptors co-localize with endogenous 7TM/GPCRs and evaluate the resulting consequences on their signaling activities

AIM 1: Determine possible co-localization of the HCMV encoded 7TM/GPCRs and assess the functional consequences

In collaboration with Ralf Jockers, Maud Kamal and Kenjiro Tagadaki (Institut Cochin, INSERM, Paris), I investigated a possible interaction/heteromerization of HCMV encoded 7TM/GPCRs. Therefore, BRET (Bioluminescence Resonance Energy Transfer) experiments were performed, where one receptor was tagged with YFP (yellow fluorescence protein; acceptor fluorophore) and the other receptor was fused to an Rluc (Renilla luciferase; donor fluorophore) sequence. In case the two respective receptors are in close proximity (1-10nm), the energy from the donor is transferred to the acceptor fluorophore and the yielding emission can be measured (illustrated in Fig.23).

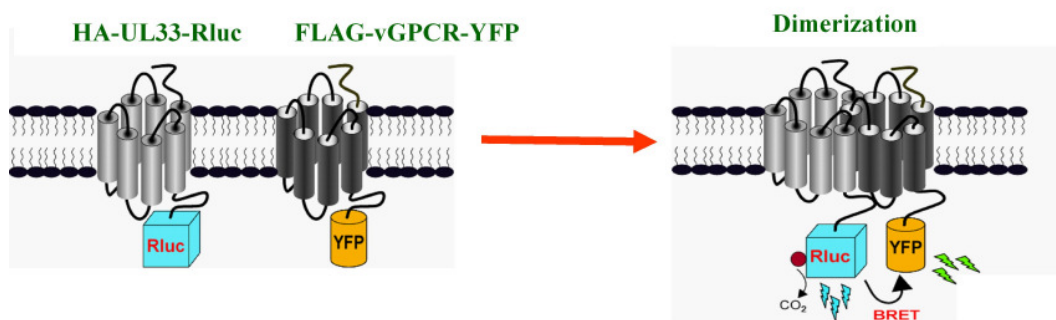
Figure 23

Figure 23. Bioluminescence resonance energy transfer (BRET). One receptor is fused to a donor fluorophore (e.g. HA-UL33-Rluc), whereas the other receptor is tagged with an acceptor fluorophore (e.g. Flag-vGPCR-YFP). Upon heteromerization/interaction, the transferred energy from the donor to the acceptor is measured.

2.2.2. Interaction of HCMV encoded 7TM/GPCRs

Preliminary BRET experiments conducted by Kenjiro Tagadaki (Institut Cochin, INSERM) indicate that HCMV encoded receptors are indeed able form homo- and heteromeric complexes. Hence, immunofluorescence studies of the identified receptor pairs were conducted in HEK293 cells to determine a possible co-localization. It was previously shown that US27 is predominantly located in LAMP positive late endosomes/lysosomes and further co-localizes with US28 (Fraile-Ramos *et al.*, 2002).

Similar to US27, UL33 was reported to be present on intracellular compartments that co-localize with the lysosomal marker LAMP (Fraile-Ramos *et al.*, 2002). A recent study indicated that UL33 co-stained with markers for early endosomes (EEA.1) and recycling endosomes (transferrin) in HCMV infected cells (Cepeda *et al.*, 2009). UL33 and US28 were shown to be predominantly localized in late endosomes/lysosomes and to co-stain with the lysosomal marker LAMP and transferrin (Waldhoer *et al.*, 2003). Indeed, antibody feeding experiments revealed that both receptors co-localize (Fig. 24A, see arrows). In addition, staining experiments after fixation were performed. Antibody feeding experiments (“intravital staining”) allow the monitoring of mature receptors in live cells that at one point traveled through the cell surface. In contrast, stainings of fixed

Results Part II

cells under permeabilizing conditions show the total amount of receptors. This approach confirmed a partial co-localization of UL33 and US28 (Fig.24B).

Figure 24

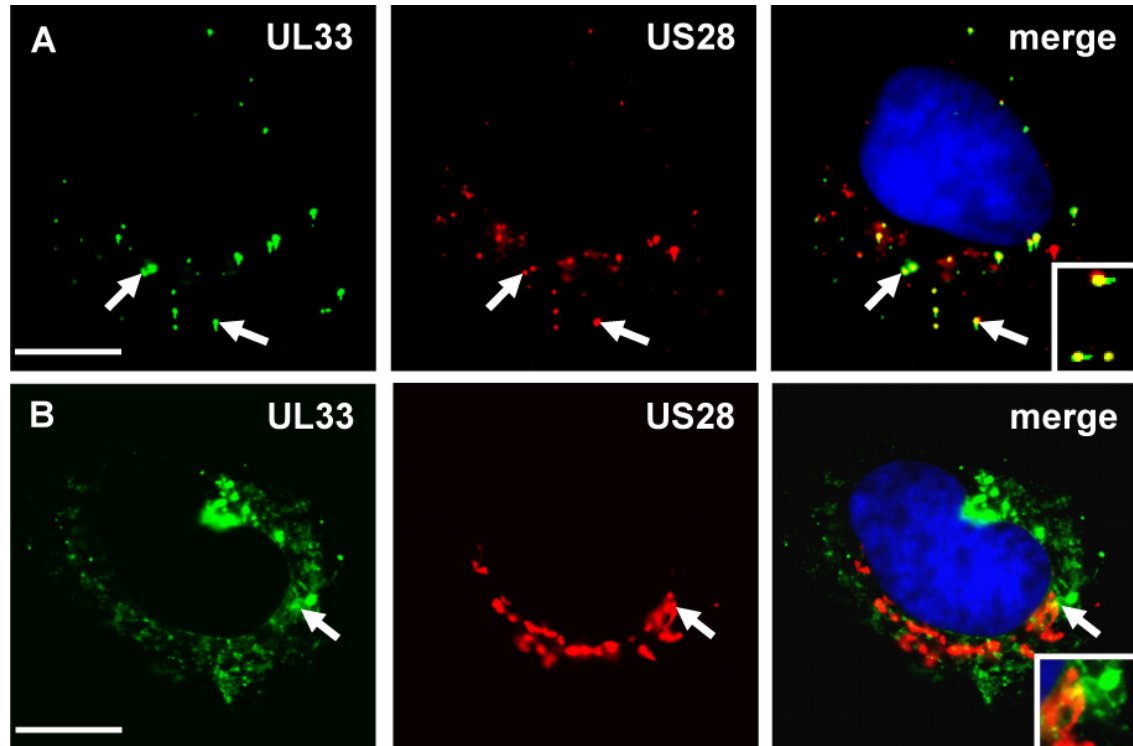


Figure 24 A. Antibody feeding of UL33 and US28. HEK293 cells transiently transfected with HA-US28 and Flag-UL33 were 'fed' antibody to the extracellular Flag-tag for 60 minutes, then fixed and permeabilized. Co-localization of the receptors (yellow) was analyzed by fluorescence microscopy. Scale bar = 10 μ m. **B Staining of UL33 and US28.** HEK293 cells were transiently transfected with HA-US28 and Flag-UL33. 24h post-transfection, cells were fixed and stained with the primary and the secondary antibodies under permeabilizing conditions. Finally, cells were mounted with vectashield (+DAPI, blue) and the co-localization of the receptors (yellow) was analyzed by fluorescence microscopy. Scale bar = 10 μ m.

Immunofluorescence studies (Fig.24 and (Fraile-Ramos *et al.*, 2002)) in combination with the BRET experiments suggest that US28 heteromerizes with US27 and UL33. Therefore, the interaction of these receptors with US28 was assessed on a function level. HEK293 cells were transiently transfected with a stable amount of US28 and increasing amounts of the respective receptor of interest. Luciferase gene reporter assays were

Results Part II

conducted to determine whether the activation of the transcription factor NF- κ B by US28 was altered in the presence of US27 or UL33. Indeed, the HCMV encoded receptors were able to differentially modulate the constitutive signaling activity of US28. US27 significantly enhanced NF- κ B activation by US28 (Fig. 25A), whereas UL33 reduced the activation of the transcription factor in a dose-dependent manner (Fig. 25B).

Figure 25

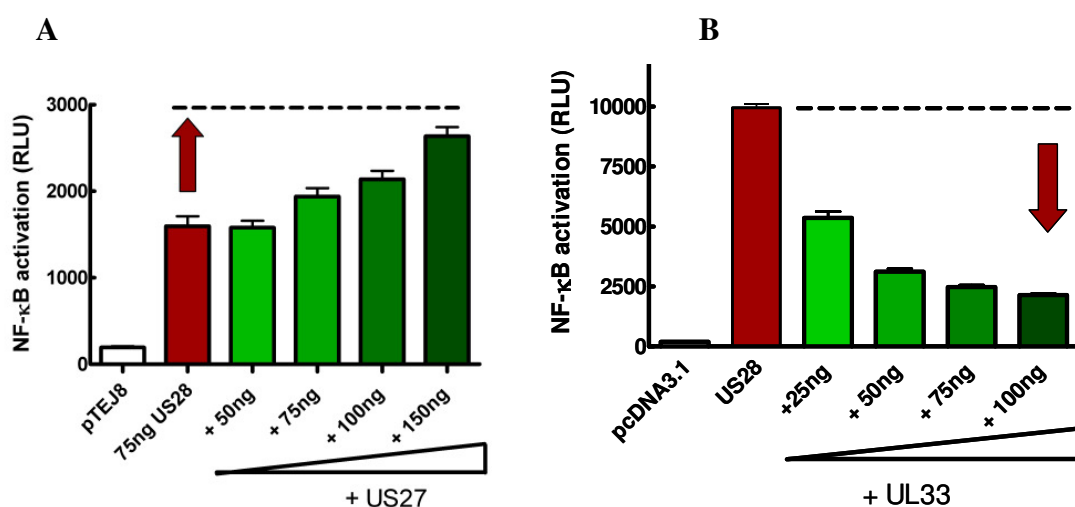


Figure 25 A. US27 enhances the activation of NF- κ B by US28. HEK293 cells were transiently transfected with Flag-US28 (75 ng/well; red bar) in combination with increasing amounts of HA-US27 and the respective cis-reporter luciferase plasmid for NF- κ B (50 ng/well). Controls were transfected with pTEJ8 vector DNA (white bars). The amount of DNA was constant for all wells. A luciferase reporter assay was conducted 24 hours post-transfection. Values were normalized to the cell number. Data are means of one representative experiment $\pm$ SEM carried out in quadruplicates. **B. UL33 decreases the activation of NF- κ B by US28.** HEK293 cells were transiently transfected with Flag-US28 (75 ng/well; red bar) in combination with increasing amounts of HA-UL33-Rluc (green bars) and the respective cis-reporter luciferase plasmid for NF- κ B (50 ng/well). Controls were transfected with pcDNA3.1 (white bars). The amount of DNA was constant for all wells. A luciferase reporter assay was conducted 24 hours post-transfection. Values were normalized to the cell number. Data are means of one representative experiment $\pm$ SEM carried out in quadruplicates.

Results Part II

So far, neither the subcellular distribution of the orphan receptor UL78, nor a possible co-localization with other viral or endogenous receptors has been investigated. To determine the localization of the receptor, co-staining experiments with the lysosomal marker LAMP 1+2 and the marker for recycling endosomes transferrin were performed. HEK293 cells transiently expressing UL78 were subjected to antibody feeding experiments, to selectively monitor the trafficking of mature receptors. These experiments showed that UL78 constitutively internalizes and is thus predominantly found in intracellular compartments. Furthermore, UL78 partially co-stains with the lysosomal marker LAMP 1+2 (see Fig. 25A) and transferrin (see Fig. 25B, merge: yellow).

Figure 26

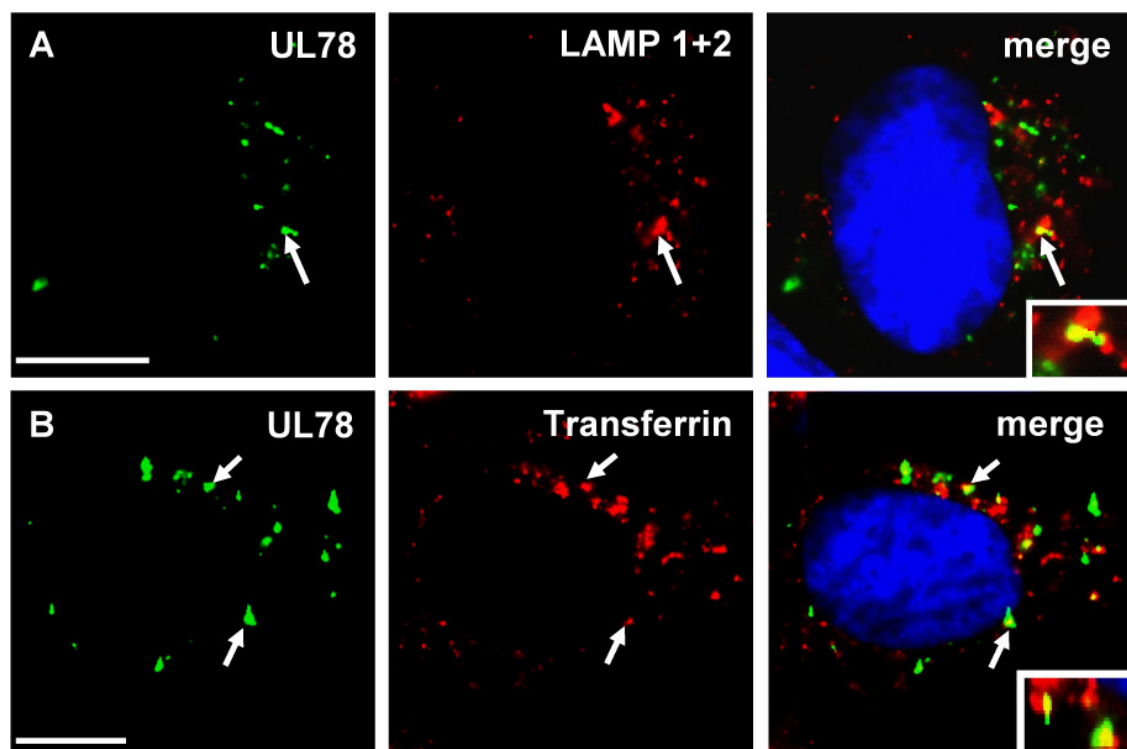


Figure 26 A. Co-localization of UL78 with LAMP 1+2. HEK293 cells transiently transfected with Flag-UL78 were ‘fed’ antibody to the extracellular Flag-tag for 60 minutes, then fixed and permeabilized. Receptors (green) were analysed for co-localization with the lysosomal markers LAMP1 and LAMP2 (red), merge: yellow. **B. Co-localization of UL78 with transferrin.** Antibody feeding experiments in HEK293 cells transiently transfected with Flag-UL78 were conducted in the presence of AlexaFluor 594-conjugated transferrin (for details see methods). Receptors (green) were

Results Part II

analysed for co-localization with the endosomal marker transferrin (red), merge: yellow. Scale bars = 10 μ m.

Next, a possible interaction and co-localization of UL78 and US28 was determined. Therefore, both receptors were transiently expressed in HEK293 cells (1:1 ratio) and antibody feeding experiments and stainings under permeabilizing conditions were performed. Indeed, as shown in Fig. 27, UL78 partially co-localizes with US28 in HEK293 cells.

Figure 27

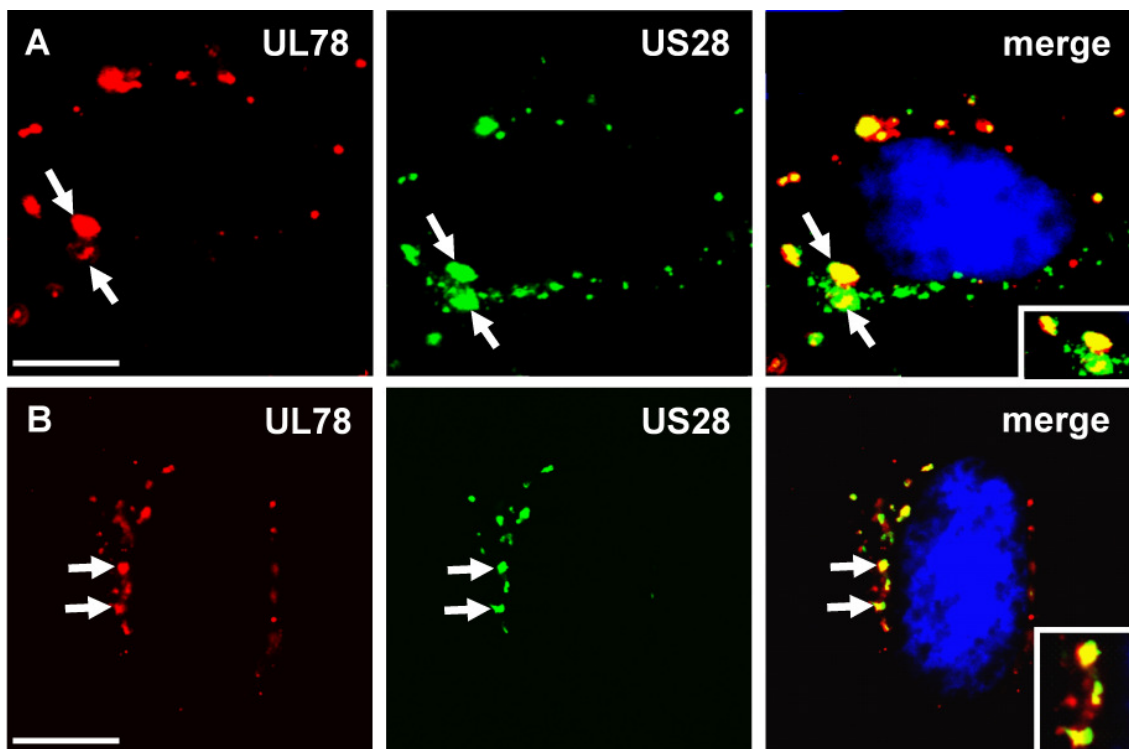


Figure 27 A. Antibody feeding of UL78 and US28. HEK293 cells transiently transfected with HA-UL78 and Flag-US28 were ‘fed’ antibody to the extracellular Flag-tag for 60 minutes, then fixed and permeabilized. Co-localization of the receptors (yellow) was analyzed by fluorescence microscopy. Scale bar = 10 μ m. **B. Staining of UL78 and US28.** HEK293 cells were transiently transfected with HA-UL78 and Flag-US28. 24h post-transfection, cells were fixed and stained with the primary and the secondary antibodies under permeabilizing conditions. Finally, cells were mounted with vectashield (+DAPI, blue) and the co-localization of the receptors (yellow) was analyzed by fluorescence microscopy. Scale bar = 10 μ m.

Results Part II

Immunofluorescence experiments in combination with the BRET data suggested that the HCMV encoded receptor US28 heteromerizes with the orphan receptor UL78. Thus, the functional consequences of this interaction on the constitutive signaling activity of US28 were assessed. HEK293 cells were transiently transfected with a stable amount of US28 and increasing amounts of UL78. 24 hours later, luciferase gene reporter assays were conducted to evaluate whether the activation of the transcription factor by US28 was altered in the presence of UL78. Indeed, the addition of increasing amounts of UL78 significantly decreased NF- κ B activation by US28 in a dose dependent manner (Fig. 28).

Figure 28

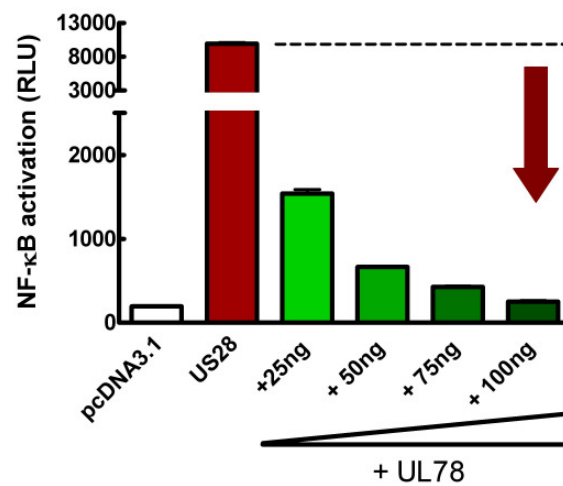


Figure 28. *UL78 decreases the activation of NF- κ B by US28. HEK293 cells were transiently transfected with Flag-US28 (75 ng/well; red bar) in combination with increasing amounts of HA-UL78 (green bars) and the respective cis-reporter luciferase plasmid for NF- κ B (50 ng/well). Controls were transfected with pcDNA3.1 (white bars). The amount of DNA was constant for all wells. A luciferase reporter assay was conducted 24 hours post-transfection. Values were normalized to the cell number. Data are means of one representative experiment $\pm$ SEM carried out in quadruplicates.*

2.2.3. Summary – Aim 1

In collaboration with the group of Ralf Jockers (INSERM, Paris), I set out to investigate interactions of HCMV encoded 7TM/GPCRs. Indeed, preliminary BRET data suggest that these vGPCRs are able to form homo- and heteromeric complexes. To verify possible interactions of HCMV encoded receptors, immunofluorescence studies were performed. As shown in Fig. 26, UL78 co-localizes with the lysosomal marker LAMP 1+2 (Fig.26A) and with transferrin, the marker for recycling endosomes (Fig. 26B). Moreover, UL78 as well as UL33 were found to co-localize with US28 in HEK293 cells (Fig. 24 and Fig. 27).

Interestingly, HCMV receptor heteromerization was found to have functional consequences on the signaling capacity of US28. Co-expression of UL33 (Fig. 25B) and UL78 (Fig. 28) decreased the activation of NF- κ B by US28, whereas US27 (Fig. 25A) potentiated the signaling capacity of US28. These findings indicate that the constitutive signaling of US28 is differentially modulated by heteromerization with HCMV encoded 7TM/GPCRs (summarized in Table 1).

Table 1

US28	+ US27	+ UL33	+ UL78
BRET	n.d.	yes	yes
Co-localization	yes (Fraile-Ramos <i>et al.</i> , 2002)	yes	yes
NF-κB activation	increased	decreased	decreased

Table 1. Interaction of US28 with other HCMV encoded 7TM/GPCRs. US28 co-localizes with US27 (Fraile-Ramos *et al.*, 2002), UL78 (Fig.27) and UL33 (Fig. 24). US28 was suggested to heteromerize with UL33 and UL78 as determined by BRET (bioluminescence resonance energy transfer) assays (performed by Tagadaki K, INSERM). Co-expression of US27 led to an enhanced activation of NF- κ B, whereas co-expression of UL33 and UL78 reduced the activation of the transcription factor by US28. n.d.: not determined.

AIM 2: Assess whether HCMV encoded receptors co-localize with endogenous 7TM/GPCRs and evaluate the functional consequences

The finding that virally encoded 7TM/GPCRs are able to heteromerize provoked the question whether they might also be able to form hetero-/oligomeric complexes with endogenous receptors. A previous study hinted that US28 might heteromerize/interact with the endogenous G α i/0 coupled chemokine receptor 1 (CCR1). Co-expression of CCR1 and US28 yielded a significantly increased activation of NF- κ B upon stimulation with CCL5 (Bakker *et al.*, 2003). Moreover, this increased activation of the transcription factor was sensitive to pertussis toxin, implying that G α i/0 proteins were involved in this response (Bakker *et al.*, 2003). BRET assays conducted by Kenjiro Tagadaki (Institut Cochin, INSERM) suggested that US28, UL33 and UL78 heteromerize with the endogenous CC-chemokine receptor 5 (CCR5).

2.2.4. Co-localization and interaction of CCR5 and US28

To verify a possible interaction between CCR5 and HCMV encoded receptors, antibody feeding and immunofluorescence studies were conducted in HEK293 cells. To simplify the experimental set-up, stable HA-CCR5 HEK293 cell lines were generated and the receptors of interest were transiently co-expressed in these cells. In agreement with previous studies (Dong *et al.*, 2005), antibody feeding experiments showed that HA-CCR5 was predominantly expressed on the cell surface in stable transfected cells (Fig. 29A). Next, a possible co-localization of the endogenous chemokine receptor CCR5 and US28 was investigated by conducting antibody feeding experiments. It was previously reported that US28 is able to downregulate the expression of CCR5 on the plasma membrane (Pleskoff *et al.*, 1998). However, the mechanism by which US28 influences CCR5 surface expression was not investigated. Indeed, co-expression of US28 reduced the expression of CCR5 on the plasma membrane in comparison to cells only expressing CCR5 (compare Fig.29A and 29B)

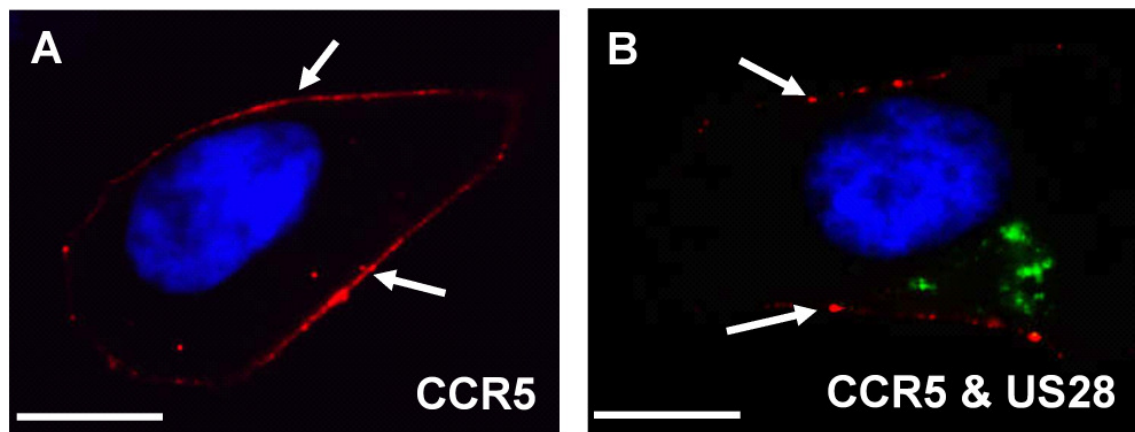
Figure 29

Figure 29 A. Surface expression of CCR5 in HEK293 cells. HEK293 stably expressing HA-CCR5 (red) were ‘fed’ antibody to the extracellular HA-tag for 60 minutes, then fixed and stained under permeabilizing conditions. Finally, cells were mounted with vectashield (+DAPI, blue) and analyzed by fluorescence microscopy. **B. Co-expression of CCR5 and US28.** HEK293 stably expressing HA-CCR5 (red) and transiently transfected with Flag-US28 (green) were ‘fed’ antibodies to the extracellular Flag- and HA-tag for 60 minutes. Next, cells were fixed and stained under permeabilizing. Finally, cells were mounted with vectashield (+DAPI, blue) and analyzed by fluorescence microscopy. Scale bars = 10 μ m.

These findings suggest that co-expression of US28 “prevented” the expression of CCR5 on the cell surface. To further investigate whether the trafficking of CCR5 is disrupted in the presence of US28, stainings of the total receptor pool were performed. Stable CCR5 cells transiently expressing US28 were fixed and stained with the primary and secondary antibodies under permeabilizing conditions. Interestingly, this experimental set-up revealed a prominent co-localization of CCR5 and US28 (Fig. 30; co-localization: yellow). The pattern of the co-localization furthermore closely resembled the staining of the endoplasmatic reticulum (ER). Hence, it might be possible that the co-expression of US28 leads to an altered export of CCR5 to the cell surface and its retention in the ER.

Figure 30



Figure 30. Staining of CCR5 and US28. HEK293 cells stably expressing HA-CCR5 were transiently transfected with Flag-US28. 24h post-transfection, cells were fixed and stained with the primary and the secondary antibodies under permeabilizing conditions. Finally, cells were mounted with vectashield (+DAPI, blue) and the co-localization of the receptors (yellow) was analyzed by fluorescence microscopy. Scale bar = 10 μ m.

Next, the functional consequences of the CCR5/US28 heteromerization on the constitutive signaling activity of US28 were assessed. HEK293 cells were transiently transfected with a stable amount of Flag-US28 and increasing amounts of HA-CCR5. 24 hours post transfection, luciferase gene reporter assays were conducted to determine the activation of the transcription factor NF- κ B by US28. Indeed, the addition of increasing amounts of CCR5 significantly reduced the signaling capacity of US28 in a dose dependent manner (Fig. 31).

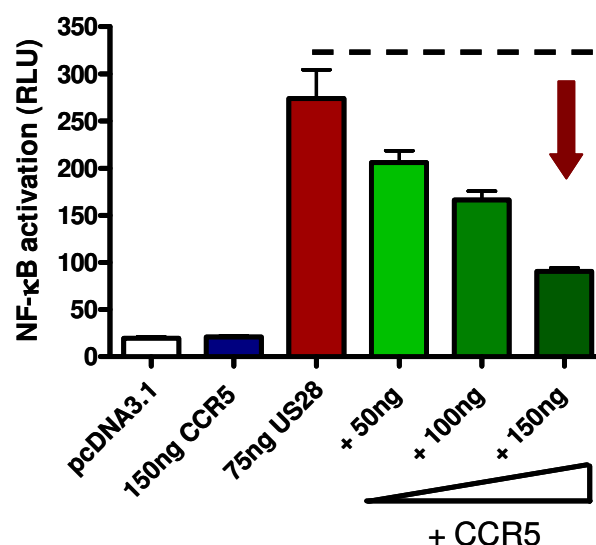
Figure 31

Figure 31. *CCR5 decreases the activation of NF-κB by US28.* HEK293 cells were transiently transfected with Flag-US28 (75 ng/well; red bar) in combination with increasing amounts of HA-CCR5 (green bars) and the cis-reporter plasmid for NF-κB (50 ng/well). Controls were transfected with only pcDNA3.1 (white bars) or HA-CCR5 (150ng/well; blue bar). The amount of DNA was constant for all wells. A luciferase reporter assay was conducted 24 hours post-transfection. Values were normalized to the cell number. Data are means of one representative experiment $\pm$ SEM carried out in quadruplicates.

The finding that co-expression of CCR5 and US28 (i) reduces the surface expression of CCR5 and (ii) decreases the constitutive signaling activity of US28 suggests that these receptors indeed interact *in vitro*. Since the endogenous chemokine receptor CCR5 is implicated in immune functions, the heteromerization with US28 might represent another strategy of HCMV to evade the host immune system. However, further studies need to be conducted to investigate whether CCR5 mediated signaling/functions are altered upon co-expression of US28.

2.2.5. Co-localization of UL78, UL33 and CCR5

BRET studies performed by Kenjiro Tagadaki (Institut Cochin, INSERM) indicated that the orphan HCMV encoded receptors UL78 and UL33 heteromerize with CCR5. Hence,

Results Part II

immunofluorescence studies were conducted to assess a possible co-localization in HEK293 cells. Intriguingly, antibody feeding experiments revealed that the co-expression of UL33 leads to a re-distribution of CCR5 – from the cell surface to the cytosol. Moreover, both receptors were found to co-localize intracellularly (Fig. 32A). To further verify the co-localization of UL33 and CCR5, stainings of fixed cells under permeabilizing conditions were performed. Indeed, this approach further confirmed that UL33 and the endogenous receptor CCR5 co-localize (Fig. 32B).

Figure 32

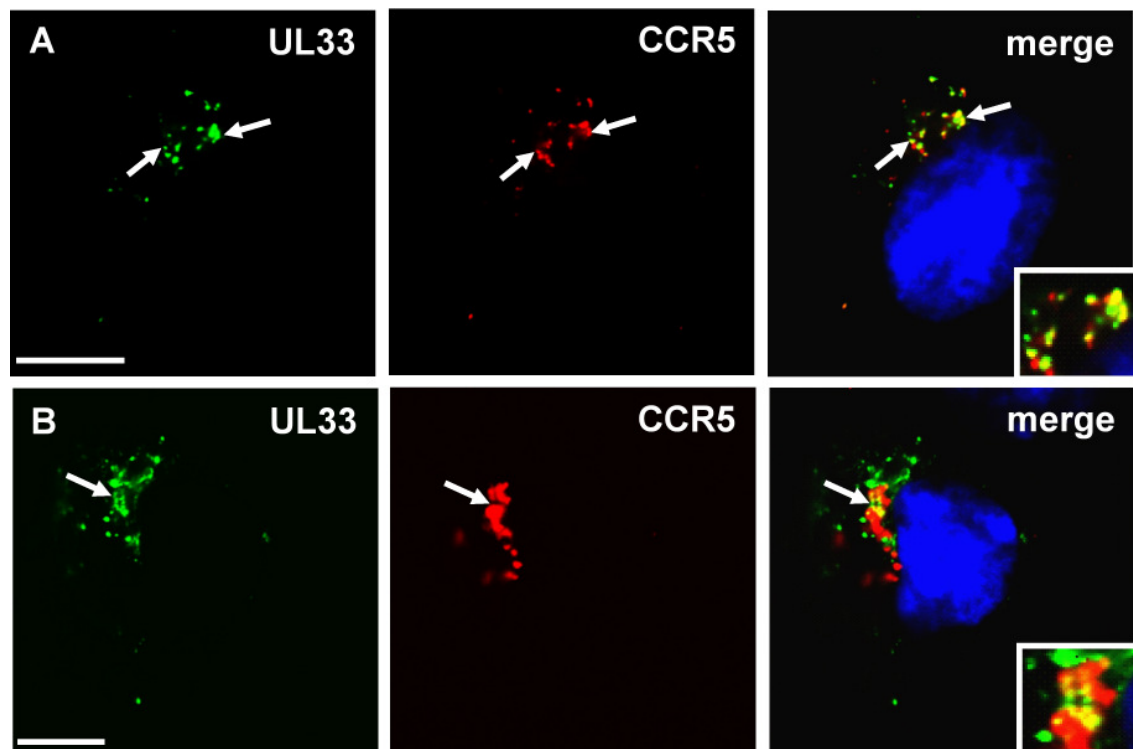


Figure 32. Antibody feeding of CCR5 and UL78. HEK293 stably expressing HA-CCR5 were transiently transfected with Flag-UL78. 24h post-transfection, cells were fixed and stained under permeabilizing conditions. Finally, cells were mounted with vectashield (+DAPI, blue) and analyzed by fluorescence microscopy. Scale bar = 10 μ m. **B. Staining of UL33 and CCR5.** HEK293 cells were transiently transfected with HA-CCR5 and Flag-UL33. 24h post-transfection, cells were fixed and stained with the primary and the secondary antibodies under permeabilizing conditions. Finally, cells were mounted with vectashield (+DAPI, blue) and the co-localization of the receptors (yellow) was analyzed by fluorescence microscopy. Scale bar = 10 μ m.

Results Part II

BRET data indicated a possible heteromerization between CCR5 and UL78, however, antibody feeding experiments did not show any co-localization of these two receptors. UL78 was localized intracellularly, whereas CCR5 was predominantly located on the cells surface (Fig. 33A). On the other hand, stainings of the whole receptor pool revealed that UL78 and CCR5 indeed co-localize (Fig.33B).

Figure 33

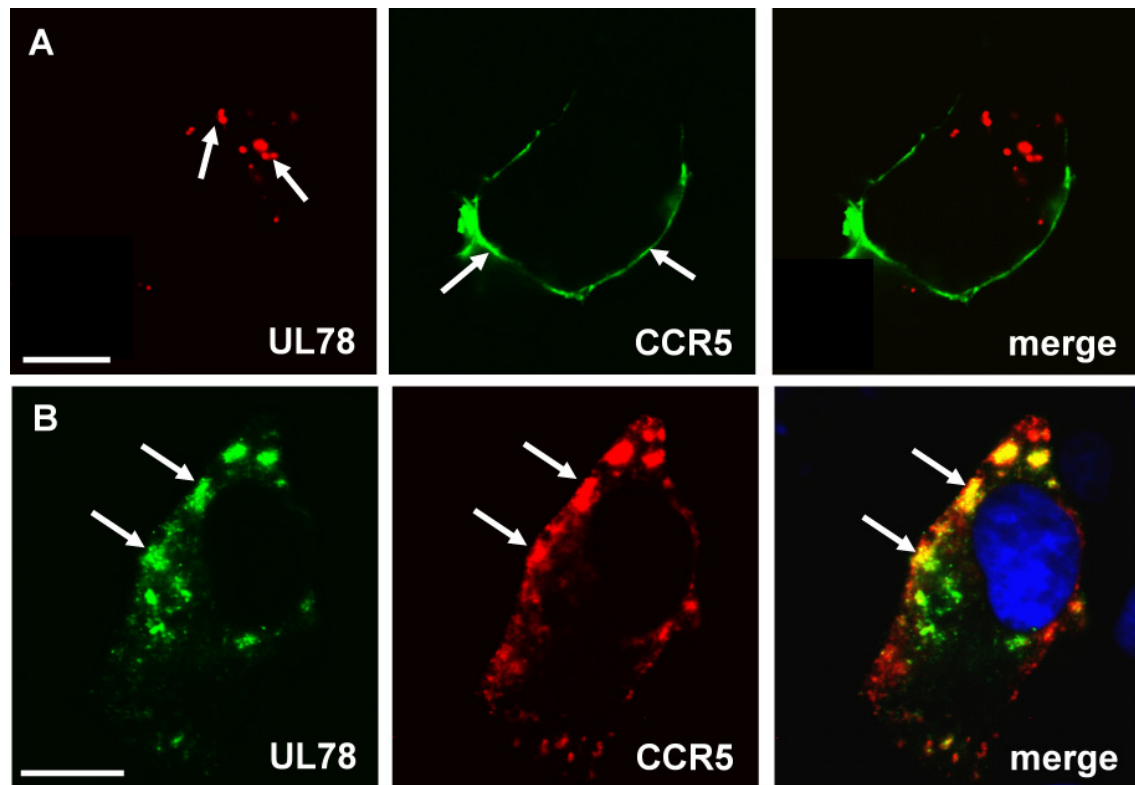


Figure 33. Antibody feeding of CCR5 and UL78. HEK293 stably expressing HA-CCR5 were transiently transfected with Flag-UL78. 24h post-transfection, cells were fixed and stained under permeabilizing conditions. Finally, cells were mounted with vectashield (+DAPI, blue) and analyzed by fluorescence microscopy. Scale bar = 10 μ m. **B. Staining of UL78 and CCR5.** HEK293 cells were transiently transfected with HA-CCR5 and Flag-UL78. 24h post-transfection, cells were fixed and stained with the primary and the secondary antibodies under permeabilizing conditions. Finally, cells were mounted with vectashield (+DAPI, blue) and the co-localization of the receptors (yellow) was analyzed by fluorescence microscopy. Scale bar = 10 μ m.

2.2.6. Co-localization of US28 and H4R

In collaboration with Martine Smit, Henry Vischer and Richard van Rijn (Vrije University Amsterdam), I set out to investigate a possible interaction between US28 and the histamine receptor 4. The histamine 4 receptor (H4R) belongs to the superfamily of G α i/0 coupled receptors, thus inhibiting AC and decreasing the intracellular accumulation of cAMP. Furthermore, H4R was also reported to induce Ca²⁺ release via activation of PLC (Hofstra *et al.*, 2003). Due to its expression mainly on hematopoietic cells, H4R was suggested to play a role in immune functions, especially in inflammation (De Esch *et al.*, 2005). Intriguingly, H4R-induced enhancement of COX-2 expression was also reported to contribute to colorectal cancer (Cianchi *et al.*, 2005).

Similar to endogenous chemokine receptors and US28, H4R is expressed on cells of the hematopoietic lineage, such as leukocytes (Oda *et al.*, 2000). Moreover, H4R also functions as a chemotactic receptor that is involved in the release of cytokines and chemokines (Gutzmer *et al.*, 2005; Dunford *et al.*, 2006; Dijkstra *et al.*, 2007). Co-expression of US28 and CCR1, for example was shown to possess synergistic effects that might be caused by heteromerization (Bakker *et al.*, 2003). Moreover, our data indicate that US28 forms heteromeric complexes with CCR5. Hence, it might be possible that US28 and H4R also interact/heteromerize thus influencing the function/signaling of the other receptor.

In support of this hypothesis, preliminary studies conducted by Richard van Rijn indicate that H4R and US28 are indeed able to form hetero-/oligomeric complexes. Therefore, the localization of US28 and H4R in HEK293 cells was investigated. Transient expression of H4R in HEK293 cells showed that the receptor is predominantly expressed on the cell surface (Fig.33A). To assess a possible co-localization of US28 and H4R, both receptors were transiently expressed in HEK293 cells (1:1 ratio) and antibody feeding experiments were conducted. Interestingly, this approach revealed that the cells either expressed one or the other receptor. No cells co-expressing both constructs could be identified (see Fig.33B; two adjoining cells, one expressing H4R and the other US28). Furthermore, it was observed that the overall expression level of US28 was much higher in comparison to H4R. These findings suggest that US28 possibly suppresses the expression or alters the trafficking of H4R. To determine whether the low expression level of H4R could be

Results Part II

elevated, a five fold excess of H4R was used. Indeed, antibody feeding experiments showed that this approach slightly increased the overall H4R expression levels and further revealed the co-expression of US28 and H4R in a few cells (see Fig.33 C).

Figure 33

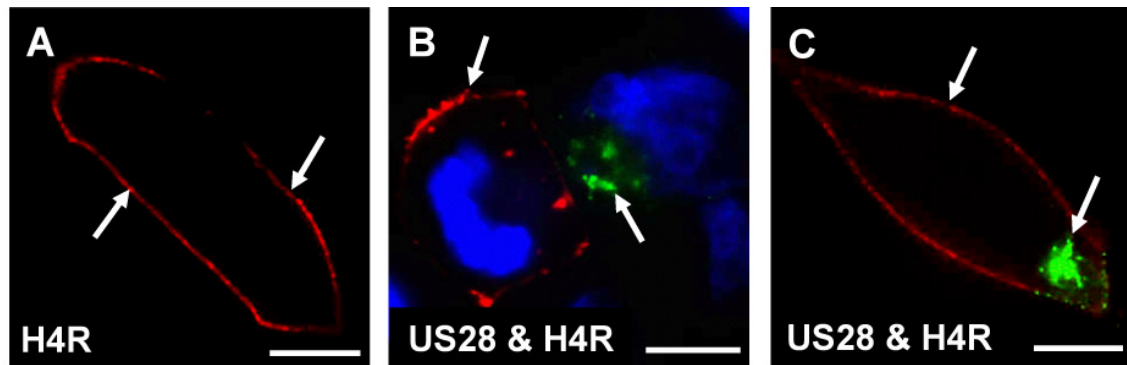


Figure 33. A. Surface expression of H4R. HEK293 cells transiently transfected with HA-H4R were ‘fed’ antibody to the extracellular HA-tag for 60 minutes. Then, cells were fixed and stained under permeabilizing conditions. **B. Co-transfection of H4R and US28 (1:1).** HEK293 cells transiently transfected with Flag-US28 and HA-H4R (1:1 ratio) were ‘fed’ antibody to the extracellular Flag-tag for 60 minutes. Then, cells were fixed and stained under permeabilizing conditions. Red: H4R; green: US28; blue: DAPI. **C. Co-expression of US28 and H4R (1:5).** HEK293 cells transiently transfected with Flag-US28 and HA-H4R (1:5 ratio) were ‘fed’ antibody to the extracellular Flag-tag for 60 minutes. Then, cells were fixed and stained under permeabilizing conditions. Red: H4R; green: US28. Scale bars = 10 μ m.

To determine whether the expression or export of H4R might be impaired in the presence of US28, stainings of fixed cells under permeabilizing conditions were performed. HEK293 cells transiently expressing Flag-US28 and HA-H4R were first fixed and then stained with the primary and secondary antibodies under permeabilizing conditions. This approach showed that US28 was localized in vesicles in the cells. However, H4R was not found on the cell surface (as shown in Fig. 33A), but localized intracellularly in a pattern that closely resembled the staining of the endoplasmatic reticulum (ER) (see Fig. 34). Moreover, this experimental set-up revealed that both receptors co-localized, possibly in the ER of transfected cells (Fig. 34, merge). These data suggest that the co-expression of US28 might disrupt the trafficking/export of H4R from the ER to the cell surface.

Figure 34

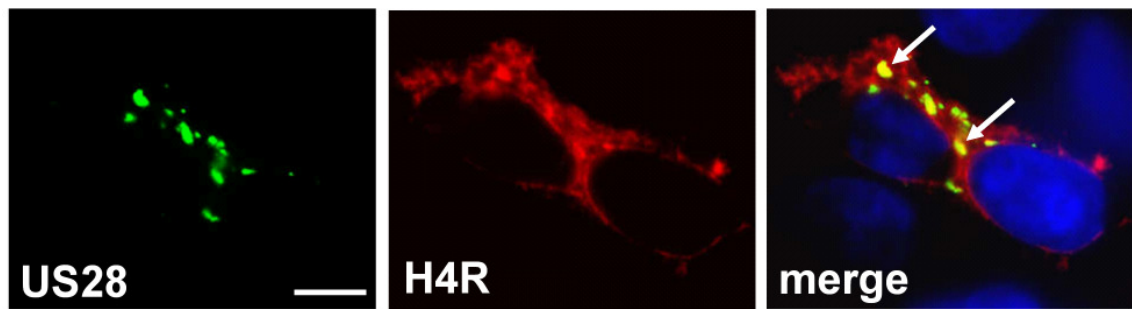


Figure 34. Staining of US28 and H4R in HEK293 cells. HEK293 cells transiently expressing Flag-US28 and HA-H4R (1:1 ratio) were fixed and stained under permeabilizing conditions. The localization of the respective receptors was analyzed by fluorescence microscopy. The HCMV-encoded receptor US28 (green) was found intracellularly, whereas H4R showed an atypical distribution mainly in the ER (red). DAPI (blue); merge (yellow). Scale bar = 10 μ m.

Next, I investigated whether the altered expression of H4R was connected with the constitutive internalization and trafficking of US28. Therefore, a c-terminally truncated, internalization deficient US28 mutant (US28 Δ 300) was used for the co-staining experiments (see Fig. 33A. and (Waldhoer *et al.*, 2003)). Indeed, in transiently transfected HEK293 cells (1:1 ratio), both, H4R and US28 Δ 300 (1:1 ratio) were found to co-localize on the cell surface (Fig. 35A, merge). On the other hand, staining of fixed cells under permeabilizing conditions did not reveal a prominent co-localization of H4R and US28 Δ 300 in the ER as it was seen with wild type US28 (compare Figs.34 and 35B).

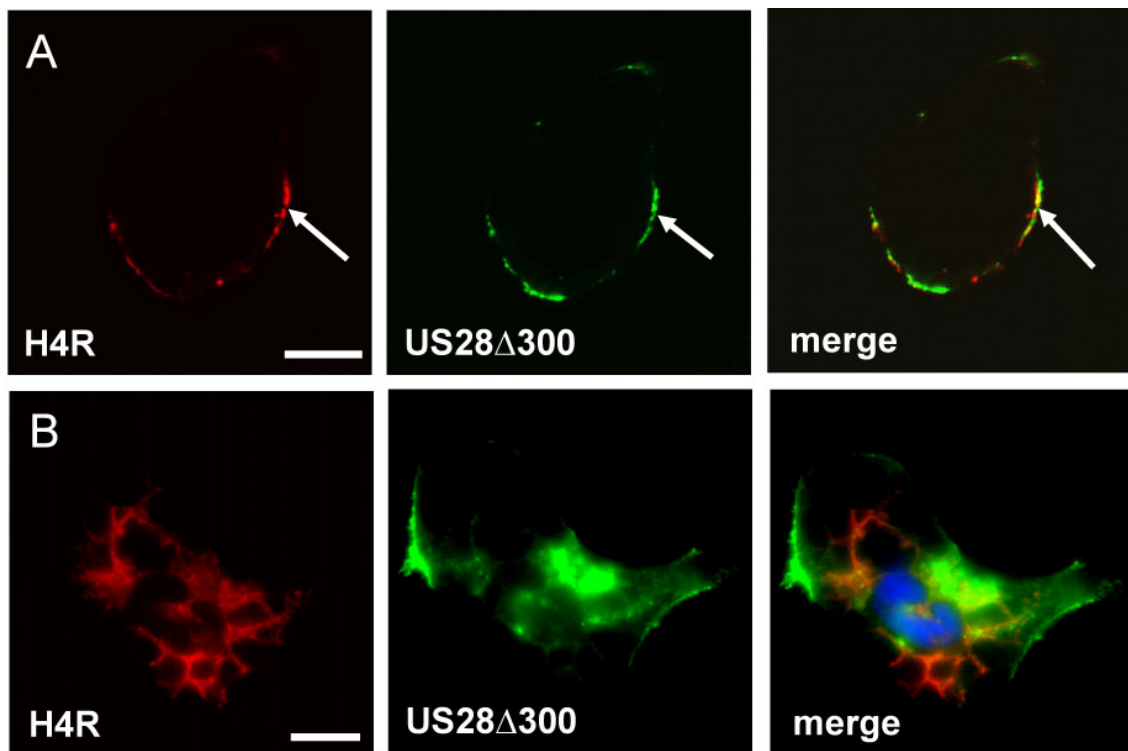
Figure 35

Figure 35. A. Antibody feeding of H4R and US28Δ300. HEK293 cells transiently transfected with Flag-US28 and HA-H4R (1:1 ratio) were 'fed' antibody to the extracellular Flag-tag for 60 minutes. Then, cells were fixed and stained under permeabilizing conditions. Red: H4R; green: US28Δ300; blue: DAPI. Scale bar = 10 μm. **B. Dead staining of H4R and US28Δ300.** HEK293 cells transiently expressing Flag-US28 and HA-H4R (1:1 ratio) were first fixed and then stained under permeabilizing conditions. The localization of the respective receptors was analyzed by fluorescence microscopy. Red: H4R; green: US28Δ300; blue: DAPI. Scale bar = 10 μm.

2.2.7. Summary- Aim 2

I provide evidence that the 7TM/GPCR US28 is not only able to form hetero-oligomers with the other HCMV encoded receptors, but also with endogenous receptors, such as CCR5. Preliminary BRET experiments indicated that US28 heteromerizes with CCR5. Interestingly, antibody feeding experiments revealed that the surface expression of CCR5 was significantly reduced upon co-expression of US28 (Fig. 29B). Intriguingly, stainings under permeabilizing conditions showed a prominent co-localization of CCR5 and US28 – probably in the ER of the cells (Fig. 30). Hence, it might be possible that US28

Results Part II

prevents the export of CCR5 from the ER to the cell surface. The heteromerization of US28 and CCR5 was further found to have functional implications. Co-expression of CCR5 reduced the constitutive activation of the transcription factor NF- κ B by US28 in a dose dependent manner (Fig. 31).

Moreover, the HCMV encoded receptor UL33 was found to heteromerize with CCR5. Antibody feeding experiments revealed that co-expression of UL33 leads to a dramatic re-distribution of CCR5 from the cell surface to the cytosol. Furthermore, both receptors were found to co-localize in HEK293 cells (Fig. 32). The orphan receptor UL78 was also suggested to heteromerization with CCR5. Although antibody feeding experiments did not show any co-localization (Fig. 33A), stainings of the whole receptor pool revealed that UL78 indeed co-localizes with CCR5 in HEK293 cells (Fig.33B).

Additionally, US28 was found to interact with yet another endogenous receptor implicated in immune functions, the histamine 4 receptor (H4R). Antibody feeding experiments showed that the overall expression level of H4R was reduced by the presence of US28 (1:1 ratio). This decreased expression of H4R could be partially restored by a five fold excess of H4R (Fig. 33C). Interestingly, US28 was found to co-localize with H4R in the ER of transiently transfected cells (Fig. 34). The importance of the trafficking of US28 for the altered export of H4R was evaluated by conducting immunofluorescence studies with the internalization deficient US28 Δ 300. Indeed, antibody feeding experiments showed that H4R and US28 Δ 300 co-localized on the cell surface (Fig. 35A). Further studies will be necessary to assess the interaction between US28 and H4R and their functional implications.

3. Discussion

The post-endocytic sorting of US28 is regulated by GASP-1

Human cytomegalovirus (HCMV) encodes four 7TM/GPCRs - US28, US27, UL33 and UL78 – with high homology to endogenous chemokine receptors. US28, the by far best characterized HCMV receptor, was shown to constitutively internalize and is further predominantly located in late endosomes/lysosomes (see (Waldhoer *et al.*, 2003) and Fig. 9A). The G protein coupled receptor associated sorting protein (GASP-1) targets several 7TM/GPCRs to the lysosomal pathway for degradation (for review see (Moser *et al.*, 2010)). In my thesis, I verified the hypothesis that GASP-1 is an important cellular determinant that regulates the post-endocytic trafficking of the viral receptor US28. My data suggest that US28 is targeted for degradation via a direct protein-protein interaction with the sorting protein GASP-1 (see (Tschische *et al.*, 2010), Fig.1A and 1C). Disruption of this process results in a redistribution of US28 into vesicles close to the plasma membrane (Figs. 9B and 10C) that co-localize with transferrin (Figs. 12B and 12D), but not with the early endosomal marker EEA.1 (Figs. 11B and 11D). The importance of GASP-1 was further confirmed in GASP-1 knock-out (KO) mouse embryonic fibroblasts (MEFs), which were generated from conditional GASP-1 KO mice. In these cells, US28 showed a similar redistribution to the cell surface (Fig.16B) as it was seen in HEK293 cells overexpressing cGASP-1 (Fig.9B) or infected with shGASP-1 virus (Fig.10C). Importantly, the constitutive internalization kinetics of US28 are not altered, however the degradation of US28 is significantly delayed in cells disrupted for the US28/GASP-1 interaction (see (Tschische *et al.*, 2010), Fig. 4). In addition, in GASP-1 knock down cells and in cells overexpressing cGASP-1, recycling of US28 is enhanced (Fig. 17B and 17D) and results in a higher surface expression of US28.

GASP-2 is the closest homologue to GASP-1 and although the c-terminus of US28 binds GASP-2 *in vitro* (see (Tschische *et al.*, 2010), Fig. 1B), no regulatory and/or compensatory effects of GASP-2 in any of our functional assays were observed. GASP-2 levels remain unchanged in cells either infected with shGASP-1 lentiviruses or overexpressing cGASP-1 (see Figs. 10A and 18B). Consistent with previous findings, I

Discussion

verified that GASP-2 is not involved in the lysosomal sorting of US28. In GASP-2 knock out MEF cells, US28 was found to localize predominantly in vesicles in the cells, in pattern that closely resembled the wild type MEFs (compare Fig. 16 A and C).

GASP-1 controls the constitutive signaling activity of US28

The sorting of individual receptors to either the recycling or degradation pathways is a tightly regulated process that is of fundamental importance for the regulation of 7TM/GPCR signaling. It is well-established that 7TM/GPCRs mainly transmit their signals through G proteins from the cell surface. Following their activation, 7TM/GPCRs are often desensitized and then endocytosed. After endocytosis receptors can be either recycled to the plasma membrane or targeted to lysosomes for degradation. However, there is accumulating evidence that the relationship between receptor trafficking and signaling is more complex than this. In particular, endosomes have been implicated as important sites for receptor-initiated signaling, as they contain a wide range of signaling molecules in their membranes and are able to recruit scaffolding proteins and signaling mediators. Furthermore, recent findings insinuate that, in some cases, endocytosis might even be required as it can enhance receptor-mediated signaling for some pathways (for reviews see (Sadowski *et al.*, 2008; Sorkin & von, 2002; von & Sorkin, 2007; Calebiro *et al.*, 2010)).

I substantiated the hypothesis that internalized receptors are able to signal from intracellular compartments. The 7TM/GPCR US28 has previously been reported to constitutively activate the $G\alpha_q$ /PLC/IP pathway and the transcription factors NF- κ B and CREB. I found that GASP-1 is crucial for US28-mediated IP production, i.e. overexpression of GASP-1 enhances IP turnover, whereas competition with cGASP-1 (Fig. 18A) or knock-down of GASP-1 expression (Fig. 19D) decreases IP accumulation. In addition, the activation of the transcription factors NF- κ B and CREB via US28 is also dependent on GASP-1 levels (see Figs. 20 and 21).

My data suggest that the sorting protein GASP-1 is crucial for the constitutive signaling activity of US28. Hence, GASP-1 may be one such 'signal mediator' for US28 once it is present in early sorting endosomes. I can only speculate on the reasons for the decreased ability of US28 to activate $G\alpha_q$ /PLC mediated signaling in cells where the US28-GASP-

Discussion

1 interaction is disrupted (see Fig. 19). One possible explanation would be the changed localization of the receptor in the absence of GASP-1 (see Fig. 10). If US28 transmits its signals from the cell surface, this process might be disturbed when the receptor is localized in vesicles close to the cell membrane, such as observed in cells where GASP-1 expression has been knocked down or in cells overexpressing the dominant negative cGASP-1. For instance, US28 cannot be found in EEA1 positive early endosomes in cells devoid of GASP-1 or in the presence of dominant negative cGASP-1 (Fig. 11), hence one might speculate that these vesicles represent the 'signalosome' for US28. On the other hand, overexpression of GASP-1 appears to upregulate signaling from US28, suggesting that GASP-1 plays a direct role in signal transduction. Thus, one could speculate that GASP-1 enhances signaling due to a conformational change of the receptor to a more active state upon interaction with GASP-1. Alternatively, it could be possible that GASP-1 is necessary for targeting the receptor to a particular compartment for signal transduction, especially since only a minor fraction of this receptor is typically found on the cell surface (Waldhoer *et al.*, 2003; Fraile-Ramos *et al.*, 2001).

US28 is regulated via heteromerization with orphan HCMV encoded receptors

In addition to the regulation of 7TM/GPCRs through sorting processes, their activity can also be controlled via hetero-/oligomerization. Receptor heteromerization has been demonstrated to significantly alter the trafficking and the signaling properties of the involved proteins (Neote *et al.*, 1993; Milligan, 2004; Ferre *et al.*, 2009). Intriguingly, also orphan receptors were shown to be capable of influencing the activity of non-orphan receptors through heteromerization. The orphan receptor GPR50, for example, was reported to antagonize the functions of the melatonin 1 (MT1) receptor. Heteromerization of GPR50 with the MT1 receptor prevented the agonist binding and G protein coupling (Levoye *et al.*, 2006). Moreover, it was found that the c-terminus of GPR50 is involved in these inhibitory effects. As opposed to the wild type receptor, heteromerization with a c-terminally deleted GPR50 did not alter MT1 related functions (Levoye *et al.*, 2006).

HCMV encodes three orphan 7TM/GPCRs -UL33, US27 and UL78 – whose functions have not been identified so far. In my thesis, I determined whether these orphan receptors regulate the activity of US28 via the formation of hetero-/oligomeric complexes. Based on preliminary BRET data that suggested the ability of these vGPCRs to heteromerize, a

Discussion

possible co-localization was assessed. Similar to other HCMV encoded receptors, UL78 was found to be predominantly localized intracellularly and co-stained with the lysosomal marker LAMP 1+2 (Fig.27A) and transferrin, the marker for recycling endosomes (Fig. 27B). US28 partially co-localizes with UL78 in HEK293 cells (Fig. 27C) and with US27 in HeLa cells (Fraile-Ramos *et al.*, 2003). In addition, antibody feeding experiments revealed a co-localization of US28 and UL33 in HEK293 cells (Fig.32).

To evaluate the functional consequences of viral GPCR heteromerization, gene reporter assays were performed. Indeed, I found direct evidence that the signaling activity of US28 is differentially modulated by HCMV encoded 7TM/GPCRs. The co-expression of US28 with either UL33 (Fig.26B) or UL78 (Fig.28) significantly decreases the activation of NF- κ B by US28. In contrast, co-transfection of US27 potentiates the constitutive signaling capacity of US28 in a dose-dependent manner (Fig.26A). Consistent with previous studies I demonstrate that orphan receptors are able to alter the functions of non-orphan receptors via heteromerization.

The 7TM/GPCRs encoded by HCMV are highly conserved. Moreover, their rat and mouse homologues were shown to be relevant for CMV infection and pathogenesis, since deleted viruses yielded reduced mortality rates (Beisser *et al.*, 1998; Beisser *et al.*, 1999). However, the functions of the HCMV receptors US27, UL33 and UL78 are still the cause of speculations. US28 was reported to be expressed at early and late time point during HCMV infection and is thought to be involved in different processes, including immune evasion, viral dissemination or early reprogramming of host cells (see (Vischer *et al.*, 2006)). UL33 and US27, both expressed during the early phases of HCMV infection, could thus control the signaling of US28 in opposing ways depending on the cellular environment. UL78, on the other hand, is co-expressed with US28 during late stages of HCMV infection. After the initial re-programming of newly infected cells, the downregulation of US28 via heteromerization with UL78 may thus be useful for the establishment of viral latency.

Heteromerization with endogenous 7TM/GPCRs

Discussion

Virally encoded chemokine receptors are thought to mimic the functions of endogenous chemokine receptors for the benefit of the virus in immune evasion. Hence, I assessed whether HCMV encoded receptors are able to interact/heteromerize with cellular receptors, such as the CC-chemokine receptor 5 (CCR5) or the histamine 4 receptor (H4R). Indeed, preliminary BRET experiments suggested that US28 heteromerizes with the chemokine receptor CCR5. Interestingly, antibody feeding experiments in cells transiently expressing both receptors did not reveal any co-localization, however showed a reduced surface expression of CCR5 in the presence of US28 (compare Fig. 29A and B). Stainings of the whole receptor pool showed a prominent co-localization of both receptors, possibly in the endoplasmatic reticulum (ER) (Fig. 30). These data indicate that US28 might heteromerize with CCR5 in the ER thus preventing its export to the plasma membrane and leading to ER retention. Interestingly, I further established that the interaction between US28 and CCR5 alters US28 induced signaling pathways. Transient expression of CCR5 inhibits the activation of the transcription factor NF- κ B by US28 in a dose-dependent manner (Fig. 31).

In addition, the HCMV encoded 7TM/GPCRs UL33 and UL78 were found to co-localize and possibly heteromerize with CCR5. Intriguingly, co-expression of UL33 caused a re-distribution of CCR5 from the cell surface to the cytosol (Fig. 32). This finding might represent yet another mechanism to reduce the expression of CCR5 on the plasma membrane. The chemokine receptor CCR5 is implicated in important processes of the immune system, such as the regulation of the trafficking and function of T-lymphocytes, macrophages and immature dendritic cells (for review see (Oppermann, 2004)). Hence, the heteromerization of viral receptors with CCR5 might represent a strategy employed by HCMV to control the activity of this host receptor. Whether the reduced ER export of CCR5, or its re-distribution alter its functions associated with its surface expression – e.g. cell migration – needs to be further investigated.

Another endogenous receptor that might be “hijacked” by HCMV is the histamine 4 receptor (H4R). Similar to endogenous chemokine receptors and US28, H4R is expressed on leukocytes (Oda *et al.*, 2000). Moreover, H4R also functions as a chemotactic receptor that is involved in the release of cytokines and chemokines (Dijkstra *et al.*, 2007). Indeed, preliminary studies suggest that US28 heteromerizes with the histamine 4 receptor. Co-transfection of US28 and H4R (1:1 ratio) showed a reduced expression level

Discussion

of H4R, which was partially rescued by a 5-fold excess of H4R (Fig. 33C). Similar to CCR5, a prominent co-localization of US28 and H4R in the ER was observed (Fig. 34). Moreover, the constitutive internalization property of US28 seems to be important for its heteromerization with H4R its retention in the ER. As opposed to the wild type receptor, the internalization deficient US28 Δ 300 was found to be co-expressed with H4R on the cell surface (Fig. 35A).

ER retention, molecular chaperones and heteromerization

Multiple mechanisms and proteins facilitate the ER export and transport of receptors to the cell surface. The GABA_BR1, for example, contains a specific sequence in its cytoplasmic tail, which results in its retention in the ER. Heteromerization with the GABA_BR2 was shown to mask this signal thus leading to the delivery of the functional heteromer to the cell surface (Margeta-Mitrovic *et al.*, 2000; Pagano *et al.*, 2001). Another way to guide the trafficking of a receptor from the ER to the plasma membrane is given by the interaction with molecular chaperones. The transport of the calcitonin receptor like receptor (CRLR) to the cell surface was shown to be dependent on the interaction with the chaperone RAMP1 (receptor activity modifying protein 1). Moreover, calnexin was reported to be essential for the trafficking and surface expression of the dopamine D1 and D2 receptors (Free *et al.*, 2007).

The endogenous 7TM/GPCRs CCR5 and H4R are both predominantly expressed on the cell surface in HEK293 cells. Interestingly, the presence of US28 yields the retention of these receptors in the ER, where they are found to co-localize with US28. Moreover, BRET experiments indicate that US28 heteromerizes with CCR5 and H4R. Thus, the interaction with US28 might (i) reveal an ER-retention sequence in the c-tails of the involved receptors or (ii) prevent the binding of a molecular chaperone that guides the export to the cell surface. Further studies will be needed to determine this.

Clinical implications

Human cytomegalovirus is a widespread pathogen that has been shown to be present in various malignancies, including breast cancer (Soderberg-Naucler, 2008), colon cancer (Harkins *et al.*, 2002) or malignant glioblastoma (Sabatier *et al.*, 2005). US28 was

Discussion

recently shown to induce tumours in an NIH3T3 nude mouse model (Maussang *et al.*, 2006). US28 is also able to up-regulate several proteins involved in oncogenesis, especially cyclooxygenase-2 (COX-2), a transcriptional target of NF- κ B suggesting a possible role for US28 in tumorigenesis (Maussang *et al.*, 2009). Moreover, US28 was reported to possess co-receptor functions for HIV-1 (Pleskoff *et al.*, 1997) and is further thought to link HCMV infection to vascular diseases (Streblow *et al.*, 2003).

For the first time, I provide evidence that the constitutive activity of US28 is regulated by (i) a direct interaction with GASP-1, (ii) the heteromerization with orphan HCMV encoded receptors and (iii) the interaction with endogenous 7TM/GPCRs. Identifying the complex mechanisms that control the trafficking and signaling of US28 might bring us one step closer to gaining a better understanding of this viral receptor and its significance in the pathogenesis implicated by HCMV. Most importantly, this knowledge might also guide new therapeutic applications.

The modulation of endogenous GASP-1 levels – e.g. by the use of blocking antibodies – might represent a way to prevent US28-mediated signaling and its insinuated complications *in vivo*. Blocking the US28/GASP-1 interaction is sufficient to significantly reduce the activation of NF- κ B. Hence, it is tempting to speculate that US28-induced tumorigenesis via up-regulation of COX-2 might be reduced by this approach. Furthermore, the finding that US28 heteromerizes with HCMV-encoded and endogenous receptors might guide the development of heteromer-specific ligands. Both, US28 and CCR5 are known co-receptors for HIV-1 entry. Moreover, it was reported that HCMV serum positivity often correlates with HIV infection and enhances associated complications, such as retinitis (Kuppermann *et al.*, 1993) or immune reconstitution inflammatory syndrome (IRIS) (Anderson *et al.*, 2010). The synthetic inverse agonist VUF2274 was shown to prevent the activation of PLC by US28 and importantly, also blocks its co-receptor function for HIV-1 (Casarosa *et al.*, 2003). Hence, selective ligands that specifically target the US28/CCR5 heteromer might be useful tools for antiviral therapy.

4. Materials

4.1. Reagents

Mouse M1 and M2 monoclonal antibody, ANTI-Flag M2 Affinity Gel, Albumin from bovine serum, gelatine from bovine skin Type B, Triton X-100, Kodak BioMax light films, 3, 3', 5, 5'-Tetramethylbenzidine Liquid substrate system, trypsin-EDTA solution, Pen/Strep solution (100x) and Tween 20 were purchased from Sigma Aldrich (Austria). 4-20% Tris-Glycine gradient gels, cell culture media (DMEM), foetal bovine serum (FBS), Dulbeccos phosphate buffered saline (DPBS), ViraPower™ Lentiviral Expression Systems and Lipofectamine 2000, Alexa Fluor 647 nm conjugated IgG2b, 594 nm conjugated IgG1a, 594 nm conjugated IgG2b, 594 nm conjugated donkey anti rabbit IgG, 594 nm conjugated transferrin were purchased from Invitrogen (Austria). QuickTiter™ Lentivirus Quantification Kit (HIV p24 ELISA) was purchased from Cell Biolabs (THP, Austria). HRP-conjugated anti-rabbit antibody and HRP-conjugated anti-mouse antibody were from Jackson Immuno Research (Dianova, Germany). Generation of anti-GASP1 antibodies has been described previously (Whistler *et al.*, 2002). Mouse monoclonal LAMP1 and LAMP2 antibodies were from the Developmental Studies Hybridoma Bank developed under the auspices of the NICHD and maintained by the University of Iowa, Department of Biological Sciences, Iowa City, IA 52242. Complete Protease inhibitor cocktail tablets and CBM cyclin were from Roche (Austria). Immobilon-P Transfer Membrane was purchased from Millipore (Austria). ECL Western Blotting Substrate was from Pierce (THP, Austria). Vectashield mounting medium and Vectastain ABC Kit were purchased from Vector Laboratories (Szabo-Scandic, Austria). The white 96-well culture plates and the Steadylite Plus Assay Kit, high sensitivity luminescence reporter gene assay system, [³H]myo-inositol and EasyTag ³⁵S-methionine were purchased from PerkinElmer (Austria). Yttrium silicate (YSi) SPA beads were from GE Healthcare (Austria). NaOH, KCl and CaCl₂*2H₂O were obtained from Merck (Austria), NaCl, Tris and Formaldehyde were from Roth (Lactan, Austria). PAGEBlue was purchased from Fermentas (Austria). Polyclonal rabbit anti early endosomal antigen 1 (EEA.1) was purchased from Calbiochem (Merck Austria). G418 sulphate solution was purchased from PAA Laboratories (Austria). CalPhos Mammalian Transfection Kit was purchased from Clontech (France).

4.2.DNA constructs

A Flag-tagged version of US28 (Flag-US28) was cloned by using the BamHI and XbaI restriction sites in the SS-Flag pcDNA3.1 zeo (+) vector. The cloning of HA-tagged GASP-1 (HA-GASP-1), cGASP-1 (HA-cGASP-1), GFP-tagged cGASP-1 (GFP-cGASP-1), were described previously (Whistler *et al.*, 2002;Thompson *et al.*, 2007). Oligonucleotides for shRNA were constructed using AAG(N18)TT criteria to select an appropriate sequence. The oligonucleotides were annealed and cloned into the lentilox vector pLL3.7 (ATCC, USA) using the XhoI and HpaI restriction sites. The target sequence for GASP-1 (GTACCTGTCTGAGG ATAGA) and the scrambled shRNA (GTTAAAGGTTTCGGCACGAA) were synthesised by Operon (USA). Lentiviruses encoding for either shRNA against GASP-1 (shGASP-1) or scrambled shRNA (shScr) were produced in HEK293FT cells. For rescue experiments we designed a rescue full length GASP-1 (rGASP-1). To inactivate the shRNA recognition site we performed targeted mutagenesis (QuikChange Site-Directed Mutagenesis Kit, Stratagene) according to the instruction manual. Three point mutations were introduced without altering the protein sequence by the use of oligonucleotide primers containing three point mutations (underlined and blot) (see table 2). The construction of the HA-tagged H4R was described previously (van Rijn *et al.*, 2006). CCR5 and UL78 were cloned in the SS-Flag pcDNA3.1 zeo (+) vector and SS-HA pcDNA3.1 zeo (+) vector using BamHI and EcoRV restriction sites. UL33 (exon 1+2) was cloned in the SS-Flag pcDNA3.1 zeo (+) vector using BamHI and XhoI restriction sites. All DNA constructs were verified by sequencing.

Table 2 .Primers

Construct	Forward primer	Reverse primer
SS-Flag-US28 pcDNA3.1 zeo (+)	5'-GCCGCGGATCCA ACGCCGACGACGA-3'	5'-GGCCCTCTAGATTAC GGTATAATTTGTGA-3'
SS-Flag-US28 (AD169) pcDNA3.1 zeo (+)	5'-GCCGCGGATCCA ACGCCGACGACGA-3'	5'-GGCCCTCTAGATTAC GGTATAATTTGTGA-3'

Materials

SS-Flag-CCR5 pcDNA3.1 zeo (+)	5'-CGGCCGCGGATCCA GATTATCAAGTGTCA-3'	5'-CTGTGCTGGATATCT CACAAGCCCACAGA-3'
SS-HA-CCR5 pcDNA3.1 zeo (+)	5'-CGGCCGCGGATCCA GATTATCAAGTGTCA-3'	5'-CTGTGCTGGATATCT CACAAGCCCACAGA-3'
SS-Flag-UL78 pcDNA3.1 zeo (+)	5'-GGCCGCGGATCCAC CTTCTGTGGAGGAGA-3'	5'-CTGTGCTGGATATC TCATAATGCCGTCAC-3'
SS-HA-UL78 pcDNA3.1 zeo (+)	5'-GGCCGCGGATCCAC CTTCTGTGGAGGAGA-3'	5'-CTGTGCTGGATATC TCATAATGCCGTCAC-3'
Rescue GASP-1 (rGASP-1)	5'-CTGTCTTGTTGCTA AAACAAAGTA <u>T</u> CTGTC <u>C</u> GAGGA <u>C</u> AGAGAACTG GTTAATAC-3'	5'-GTATTAACCAGTTCTC <u>T</u> <u>G</u> TCCTC <u>G</u> GACAG <u>A</u> TAC TTTGTTTTAGCAACCAA GACAG-3'
SS-Flag-UL33 pcDNA3.1 zeo (+)	5'-GTACCAGGATCCAGA CACCATCATCCAC-3'	5'-CTCAGCGGGGTATGA GCTCGAGTAGCAC-3'

4.3. Equipment and Instrumentation

Flex Station II (Molecular Devices)

TopCount NXT (PerkinElmer)

BioRad xMark Microplate Spectrophotometer

Zeiss LSM 510 META confocal microscope

Olympus IX70 fluorescence microscope

5. Methods

5.1. Cell culture

HEK293 cells were maintained at 5 % CO₂ at 37 °C in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10 % fetal bovine serum (FBS). HEK293 cells stably expressing HA-CCR5 were maintained as described above plus 0.2 mg/ml G418. Mouse embryonic fibroblast cells were cultured in DMEM containing 10% FBS, 2mM glutamine and 1% Pen/Strep. For the first 3-4 passages 0.5µg/ml CBM cyclin was added to the growth medium to remove mycoplasma.

5.2. Lentivirus production and knock-down of endogenous GASP-1

The Lentiviruses with scrambled or GASP-1 shRNA were produced in HEK293FT cells. 2 x 10⁷ HEK293FT cells were grown in DMEM supplemented with 10 % FBS and transfected with 7 µg of scrambled shRNA LentiLox or GASP-1 shRNA LentiLox vectors and 20 µg of Virapower packaging mix. 48 hours post-transfection viruses were harvested and purified by ultracentrifugation (26000 rpm, 90 min, 4°C). The virus pellets were resuspended in 25 µl salt-free PBS, frozen in liquid nitrogen and stored at -80 °C. Virus titer was estimated by ELISA using the QuickTiter™ Lentivirus Quantification Kit. For the knock down of endogenous GASP-1, HEK293 cells were infected with 10 MOI (multiplicity of infection) of Lenti-shGASP-1 (shGASP-1) or Lenti-shScrambled (shScr) virus and incubated at 37 °C and 5 % CO₂ for 48 - 72 hours. Successful Lenti-shRNA infection was monitored by immunofluorescence of EGFP, which is under the control of a CMV promoter following the LoxP site of the LentiLox 3.7 vector. GASP-1 knock down was verified by western blot analysis of GASP-1 protein levels.

5.3. Immunocytochemistry

Antibody feeding experiments: Wild type HEK293 cells or HEK293 cells stably expressing GFP-cGASP-1 were grown on gelatine coated cover slips and transfected with Flag-US28 (4 µg/coverslip). 24 hours post-transfection, antibody feeding experiments were conducted essentially as described before (Martini *et al.*, 2007). In

Methods

brief, living cells were incubated with 1:500 anti-Flag M1 antibody for 45 minutes, fixed with 3.7 % formaldehyde in PBS and permeabilized in blotto (50 mM Tris-HCl, pH 7.5, 1 mM CaCl₂, 0.1 % Triton X-100 and 3 % milk). After co-localization staining (see below), all cells were mounted with Vecta shield mounting medium.

Co-localization experiments with LAMP1 and LAMP2. Following the feeding experiments described above, cells were incubated with a monoclonal antibody directed against LAMP 1/2 (IgG1 1:500) for 60 minutes. Subsequently, the cells were stained with the subtype-selective antibodies AlexaFluor 647-conjugated IgG2b against the Flag-tag (1:1000, 20 minutes) and AlexaFluor 594-conjugated IgG1a against LAMP 1/2 (1:500, 20 minutes).

Co-localization experiments with EEA.1. Following the feeding experiments described above, cells were incubated with a polyclonal antibody directed against EEA.1 (IgG, 1:500) for 60 minutes. Cells were stained with the subtype-selective antibodies AlexaFluor 647-conjugated IgG2b against the Flag-tag (1:1000) and the AlexaFluor 594-conjugated goat-anti rabbit IgG (1:1000) for 20 minutes.

Co-localization experiments with transferrin. For co-localization studies with transferrin, cells were serum-starved for 60 minutes prior to incubation with M1 antibody directed against the FLAG epitope (1:1000, 45 min). Then cells were labeled with AlexaFluor 594 nm - conjugated transferrin (1:500) for the last 30 minutes. After fixation and permeabilization, the cells were stained with the subtype-selective antibody AlexaFluor 647 nm conjugated IgG2b against the Flag-tag (1:1000, 20 minutes).

Antibody feeding experiments in shScr and shGASP-1 infected cells. Cells infected with either shScr or shGASP-1 viruses for 48 hours were transiently transfected with 4 µg/coverslip Flag-US28. 24 hours post-transfection antibody feeding and co-localization experiments were performed as described above.

Co-localization experiments of Flag- and Ha-tagged receptors. Following the feeding experiments as described above, cells were incubated with the subtype-selective antibodies AlexaFluor 488-conjugated IgG2b against the Flag-tag (1:1000, 20 minutes) and AlexaFluor 594-conjugated IgG1a against the HA-tag (1:1000, 20 minutes).

Staining of fixed cells. 24 hours post-transfection, cells were fixed with 3.7 % formaldehyde in PBS and permeabilized in blotto (50 mM Tris-HCl, pH 7.5, 1 mM CaCl₂, 0.1 % Triton X-100 and 3 % milk). Then, cells were incubated with anti-Flag M1 antibody (1:500) and anti-HA antibody (HA11, 1:500) for 60 minutes. Subsequently, cells were washed and stained with the subtype-selective antibodies

Methods

AlexaFluor 488-conjugated IgG2b against the Flag-tag (1:1000, 20 minutes) and AlexaFluor 594-conjugated IgG1a against the HA-tag (1:1000, 20 minutes). After the staining, all cells were mounted with Vecta shield mounting medium (+DAPI).

Microscopy. Cells were analysed with a Zeiss LSM 510 META confocal microscope or an Olympus IX70 fluorescence microscope. To determine the exact localization of the receptor, Z-stacks of representative cells were taken and the pictures of the middle of the cells are shown (confocal microscope).

5.4. Recycling experiments

Wild type HEK293 cells, cells stably expressing GFP-cGASP-1 or cells infected with either shScr or shGASP-1 were grown on gelatine coated coverslips. Subsequently, the cells were transiently transfected with Flag-US28 (4 µg/coverslip). 24 hours post-transfection, recycling experiments were performed essentially as described in (Martini *et al.*, 2007). In brief, living cells were fed with the Ca²⁺-sensitive M1 antibody directed against the Flag-epitope (1:500, 45 minutes). Then residual surface receptors were stripped of antibody by washing twice with cold 0.04 mM PBS-EDTA solution. To determine the constitutive recycling of the receptor, warm medium was re-added to the cells. After 15 or 30 minutes the medium was aspirated and the cells were fixed with 3.7 % formaldehyde in PBS. Cells were stained with the subtype-selective antibody AlexaFluor 594-conjugated IgG2b against the Flag-tag (1:1000, 20 minutes) under non-permeabilizing (minus Triton-X) conditions. Finally, the cells were mounted with Vecta shield mounting medium.

5.5. Reporter gene assays

Transcription factor luciferase assays were performed essentially as described in (Waldhoer *et al.*, 2002). In brief, HEK293 cells (40000 cells/well) were seeded on gelatine coated 96-well plates. For GASP-1 knock down experiments, HEK293 cells were infected with either shScr or shGASP-1 virus 48 hours previous to transfection. Cells were transiently transfected with 50 ng/well Flag-US28 and the cis-reporter plasmid for CREB (200 ng/well) or NF-κB (50 ng/well). Depending on the experiment (indicated at the figure legends) US28 was co-transfected with 100 ng/well pcDNA3.1,

Methods

HA-GASP-1, HA-cGASP-1 or HA-rGASP-1. The amount of DNA was kept constant for all samples. In parallel to each assay, an additional plate was prepared for ELISA (see below) to control for receptor expression levels. The luciferase reporter gene assay was conducted 24 hours post-transfection according to the manufacturer's guidelines. In brief, cells were washed twice with PBS and the cell number was determined by optical density in a FlexStation II Device (Molecular Probes, Endpoint Measurement). Then 100 μ l/well Steadylight luciferase assay reagent were added to 100 μ l/ well PBS. Following a 10 min incubation period, luminescence was measured using a TopCounter Device (TopCount NXT, PerkinElmer).

5.6.ELISA (enzyme linked immunosorbent assay)

On the same day as the respective signaling assay (see above), cells were fixed with 3.7 % formaldehyde in PBS and permeabilized in blotto (50 mM Tris-HCl, pH7.5, 1 mM CaCl_2 , 0.1 % Triton X-100 and 3 % milk) for one hour. Next, the cells were incubated with 1:500 anti-Flag M1 antibody overnight at 4 °C. On the next day, the cells were washed with TBS (25 mM Tris base, 135 mM NaCl, 2.5 mM KCl, 1 mM CaCl_2 , pH 7.4) and incubated with HRP-conjugated anti-mouse antibody (1:2500) in blotto (50 mM Tris-HCl, pH7.5, 1 mM CaCl_2 and 1.5 % milk) for two hours at room temperature. After three washes with TBS, the cell number was determined by optical density in a FlexStation II Device. Then 100 μ l/well TMB (3, 3', 5, 5'-Tetramethylbenzidine) substrate was added and the colouring reaction was stopped by the addition of 50 μ l/well 0.5 M sulfuric acid after 2 minutes at room temperature. Receptor levels were measured at the optical density of 450 nm in a BioRad xMark Microplate Spectrophotometer.

5.7.Inositol Phosphate (IP) accumulation assay

HEK293 cells were seeded on gelatine coated 96-well plates (40 000 cells/well). For GASP-1 knock down experiments, HEK293 cells were infected with either shScr or shGASP-1 virus 48 hours previous to transfection. Cells were transfected with 75 ng/well Flag-US28 and depending on the experimental setting indicated in the figure legends, co-transfected with 100 ng/well of either pcDNA3.1, HA-GASP-1, HA-cGASP-1 or HA-rGASP-1 (rescue experiments). Controls were transfected with 175

Methods

ng/well pcDNA3.1 in the absence of US28. 24 hours post-transfection the cells were loaded with 1 $\mu\text{Ci/well}$ [^3H]myo-inositol in 100 $\mu\text{l/well}$ Optimem and incubated overnight at 37 °C and 5 % CO_2 . The next day, the labelling medium was aspirated, 100 $\mu\text{l/well}$ HBSS buffer (including CaCl_2 and MgCl_2) containing 10 mM LiCl were added and the cells were incubated for 45 minutes at 37 °C and 5 % CO_2 . The reaction was terminated by aspiration and the cell number was determined by optical density in a FlexStation II Device. Subsequently, cells were lysed with 50 $\mu\text{l/well}$ 10 mM formic acid for 90 minutes on ice. 40 μl of the resulting cell extract were transferred to 160 μl of YSi-SPA beads (12.5 mg/ml) and shaken for 90 minutes at 4°C. The plates were then stored at 4°C and the accumulation of inositol phosphate was counted in a TopCount microplate scintillation counter (TopCount NXT, PerkinElmer) 24 hours later.

5.8. Isolation and culturing of Mouse Embryos Fibroblasts (MEFs)

Between day 13-17pc pregnant GASP-1 floxed and GASP-2 KO mice were anesthetized with isoflourane and subsequently sacrificed by cervical dislocation. The uterine horns were dissected and placed into pre-warmed PBS. Next, the individual embryos were separated from the placenta and the surrounding membranes.

Each embryo was washed with clean and pre-warmed PBS. The head and organs of the embryos were removed and kept for genotyping. Then, PBS was aspirated and the embryos were finely minced using forceps. After adding about 1ml of 0.25% trypsin to each embryo, they were forced through a 21 gauge needle 5-6 times and then incubated at 37 degrees Celsius for 20–30 minutes. Next, the cells were resuspended in about 3-4ml of pre-warmed growth medium and again forced through a 21 gauge needle until there a no big chunks of tissue left. Finally, cells were plated and cultivated according to the 3T3 protocol of Todaro and Green (Todaro & Green, 1963). For the spontaneous immortalization of the MEFs, cells were plated at the same inoculation density (e.g. 3×10^5 cells/dish with 50 mm diameter) each time and kept on a three day transfer schedule for about 25 passages. MEF cells were maintained in DMEM containing 10% FBS, 2mM glutamine and 1% Pen/Strep. For the first 3-4 passages CBM cyclin was added to the growth medium to remove mycoplasma. For passaging the cells 0.01% trypsin solution was used.

5.9. Stable transfection of HEK293 cells

HEK293 cells maintained in DMEM containing 10% FBS were grown to 70-80% confluence on a 10cm dish. Then, cells were transfected using the CalPhos mammalian Transfection Kit. The growth medium was replaced with 10ml fresh DMEM about 3h before the transfection. The transfection was performed according to the manufacturer's protocol. In brief, two separate solutions were prepared. Solution A contained 700µl of the provided 2x HBS buffer. Solution B consisted of a mixture of 10µg HA-CCR5 plasmid DNA, 86.8µl 2M CaCl₂ solution and complemented with water to a final volume of 700µl. Then, solution A was vortexed while solution B was added dropwise. After 30min incubation at room temperature, the mixture was again vortexed and then slowly added to the cells. The cells were incubated with the transfection mixture for about 5-6h and finally, the growth medium was replaced with fresh DMEM (+10%FBS).

The HA-CCR5 construct used for the transfection contained a neomycin resistance cassette. Therefore, selection of clones stably expressing HA-CCR5 was performed by culturing the cells in growth medium containing 0.3mg/ml neomycin (G418). The cells stably expressing HA-CCR5 were then maintained in DMEM containing 10% FBS and 0.2 mg/ml neomycin.

5.10. Statistical analysis

Statistical analyses were performed using ANOVA analysis of variance for comparisons between multiple groups, followed by a Bonferroni's post-hoc analysis using GraphPad Prism software.

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Appendix

6. Appendix

Appendix I

The G-protein Coupled Receptor Associated Sorting Protein GASP-1 Regulates the Signalling and Trafficking of the Viral Chemokine Receptor US28

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Human cytomegalovirus (HCMV) encodes the seven transmembrane (7TM)/G-protein coupled receptor (GPCR) US28, which signals and endocytoses in a constitutive, ligand-independent manner. Here we show that, following endocytosis, US28 is targeted to the lysosomes for degradation as a consequence of its interaction with the GPCR-associated sorting protein-1 (GASP-1). We find that GASP-1 binds to US28 *in vitro* and that disruption of the GASP-1/US28 interaction by either (i) overexpression of dominant negative cGASP-1 or by (ii) shRNA knock-down of endogenous GASP-1 is sufficient to inhibit the lysosomal targeting of US28 and slow its post-endocytic degradation. Furthermore, we found that GASP-1 affects US28-mediated signalling. The knock-down of endogenous GASP-1 impairs the US28-mediated $G_{\alpha q}$ /PLC/inositol phosphate (IP) accumulation as well as the activation of the transcription factors Nuclear Factor- κ B (NF- κ B) and cyclic AMP responsive element binding protein (CREB). Overexpression of GASP-1 enhances both IP accumulation and transcription factor activity. Thus, GASP-1 is an important cellular determinant that not only regulates the post-endocytic trafficking of US28, but also regulates the signalling capacities of US28.

Key words: chemokine, cytomegalovirus, GASP-1, G-protein coupled receptor, post-endocytic sorting, signalling, US28

Received 10 July 2009, Revised and accepted for publication 20 January 2010, published online 22 February 2010

Cytomegaloviruses are widespread opportunistic pathogens that cause acute, latent and chronic infections. Although primary infection is asymptomatic in immunocompetent individuals, these viruses are implicated in a great variety of diseases in immunocompromised hosts (1). Human cytomegalovirus (HCMV) encodes four putative seven transmembrane (7TM) spanning/G-protein coupled receptors (GPCRs), namely US27, US28, UL33 and UL78 (2), all of which have structural homology to chemokine receptors. US28 – by far the best characterized of these four receptors – has been shown to bind a wide range of endogenous chemokines. In addition, US28 is able to endocytose and signal in a constitutive, i.e. ligand-independent, manner (3–5). Although US28 may utilize arrestin for internalization (6), this receptor can be targeted to intracellular compartments in arrestin-deficient cells (7) and moreover is able to use both clathrin-dependent and independent endocytic pathways (8).

In most cell types studied so far, US28 is typically located in the membranes of intracellular organelles, especially late endosomes/lysosomes and multi-vesicular bodies (MVBs) (9), whereas only a minor fraction is found on the cell surface (5,9). The majority of receptors, however, reaches these intracellular compartments after first travelling to the cell surface (5). It has been suggested that the virions of HCMV may be assembled in these MVBs, whereby the viral receptors are incorporated into the viral membranes during the final stages of virus assembly (10,11). US28 has further been implicated to be important for the viral life cycle as it might enhance cell–cell fusion, thus promoting viral spread (12,13).

Several proteins have been identified that specifically target 7TM/GPCRs to either recycling or lysosomal pathways. One such protein is the GPCR-associated sorting protein-1 (GASP-1) (14,15). For a variety of 7TM/GPCRs it has been shown that the interaction with GASP-1 favours their targeting to the lysosomal pathway, whereas receptors that do not interact with GASP-1 recycle to the cell surface both *in vitro* (14,16–18) and *in vivo* (19,20). Moreover, GASP-1 regulates dopamine and cannabinoid (16,19,20) receptor signalling and it was recently reported that acute and sensitized cocaine locomotor effects were attenuated in GASP-1 knock-out mice (21). One study suggested the existence of nine additional GASP paralogues (15). However, except for

GASP-2 (~150 kDa protein, 50% identity with the amino-terminal- and 68% identity with the carboxy-terminal sequence of GASP-1), none of the other eight family members has a more than 20% homology to GASP-1, and whether any of these paralogues are involved in 7TM/GPCR sorting has not been determined.

The post-endocytic sorting of 7TM/GPCRs is a fundamental process that can also control the signalling capacity of these receptors. US28 has been reported to activate downstream transcription factors such as NF- κ B, cyclic AMP responsive element binding (CREB) (3,4), the nuclear factor of activated T-cells (NFAT) (22) and the serum response factor (SRF) (23). This 'pirating' of signalling networks and modulation of cellular functions may be an effective strategy of HCMV, and viruses in general, to evade the host immune system. Here, we show that the sorting protein GASP-1 is not only a key regulator in the post-endocytic sorting of US28, but is also crucially involved in the signalling capacities of this viral receptor.

Results

Protein–protein interaction of GASP and US28

GASP-1 interacts with the cytoplasmatic portions of many 7TM/GPCRs (24). For members of the opioid (14), dopamine (18,20), bradykinin (17) and cannabinoid (16,19) receptor families, GASP-1 has been shown to not only bind but also regulate the trafficking of these receptors, suggesting that interaction with GASP-1 could facilitate degradation of diverse 7TM/GPCRs. The GASP-2 homologue displays similar binding patterns for receptors as GASP-1 (18); however, to date no functional consequences of this interaction have been reported.

As US28 has been reported to constitutively endocytose and sort to vesicles of the classical degradation pathway, i.e. late endosomes and lysosomes (5,9), we examined whether degradation of the virally encoded receptor US28 was modulated by GASPs.

First, we examined whether GASP-1 and GASP-2 interacted with a glutathione S-transferase (GST)-fusion protein containing the 59 amino acid carboxyterminus (C-terminus) of US28. *In vitro*, both GASP-1 (Figure 1A) and its homologue GASP-2 (Figure 1B) bind to the cytoplasmic tail of US28 as did the 'degrading' delta opioid receptor (DOP) (14), which served as positive control. In contrast, a 'recycling' receptor, the mu opioid receptor (MOP), did not bind to either GASP-1 or GASP-2 (14). Consistent with its ability to interact with GASP-1 and GASP-2 *in vitro*, US28 co-immunoprecipitated endogenous GASP-1 and GASP-2 in HEK293 cells (Figure 1C, upper panel, 1D for quantification). Indeed, US28 bound more GASP-1 and GASP-2 than DOP, which served as positive control (Figure 1C,D). Neither GASP-1 nor GASP-2 co-immunoprecipitated with the recycling β 2-adrenergic receptor (β 2AR) (18) (for graphic representation see

Figure 1D), which together with HEK293 cells transfected with empty vector (pcDNA) served as the negative controls.

Summarized, these data suggest that US28 directly interacts with GASP-1 and GASP-2 via its C-terminus.

GASP-1 mediates lysosomal targeting of US28

We next sought to elucidate the role of GASP-1 in the post-endocytic trafficking of US28. We have previously shown that US28 is constitutively internalized, and that the majority of the receptor accumulates in late endosomes and lysosomes (5,9). As GASP-1 has been reported to be a sorting protein that targets various 7TM/GPCRs to the lysosomal pathway, we tested whether preventing the interaction of US28 and GASP-1 could alter the post-endocytic localization of US28. We approached this question using two independent techniques. We examined the effects of (i) dominant negative cGASP-1 and of (ii) shRNA knock-down of GASP-1 on the localization of US28.

In the previous studies (14,16), the C-terminal fragment of GASP-1, corresponding to the COOH-terminal 497 residues of GASP-1 (cGASP-1), has been used as a dominant negative to compete with GASP-1 for receptor binding, thus changing the sorting of receptors targeted for degradation by GASP-1. We found that dominant negative cGASP-1 binds to the c-tail of US28 (Figure 2A) and competes for binding to the US28 c-tail with full-length GASP-1 (Figure 2B). To examine whether overexpression of cGASP-1 altered the trafficking of US28, we generated a cell line that stably expressed GFP-cGASP at ~100-fold over endogenous GASP-1 (Figures S1 and 4A). Cells overexpressing GFP-cGASP-1 and untransfected HEK293 cells were transiently transfected with Flag-US28 and 'fed' antibody to the extracellular Flag-tag of US28 for 45 min to monitor the constitutive endocytosis and trafficking of US28 in live cells (14,16). As has been shown previously (5), and in Figure 2C, US28 is predominantly found in lysosomes after 45 min in HEK293 cells, as evidenced by its colocalization with the lysosomal markers LAMP1 and LAMP2. In contrast, in the presence of GFP-cGASP-1, US28 undergoes a dramatic redistribution to vesicles close to the cell membrane that do not colocalize with the lysosomal markers (Figure 2D, see arrows).

To further address the role of GASP-1 in the lysosomal targeting of US28, we developed shRNAs that knock down the expression of GASP-1. Lentiviral infection of HEK293 cells with shRNA targeting GASP-1 (Figure 2E, shGASP-1) for 48 h showed an efficient knock-down of GASP-1 protein levels, while scrambled shRNA virus had no effect on GASP-1 levels (Figure 2E, shScr). In both, shScr and shGASP-1 infected cells, GASP-2 levels remained unchanged (Figure 2E, see 130-kDa band). US28 colocalized with LAMP positive endosomes in cells infected with shScr virus (Figure 2G). In contrast, in cells infected with the shGASP-1 virus, US28 showed

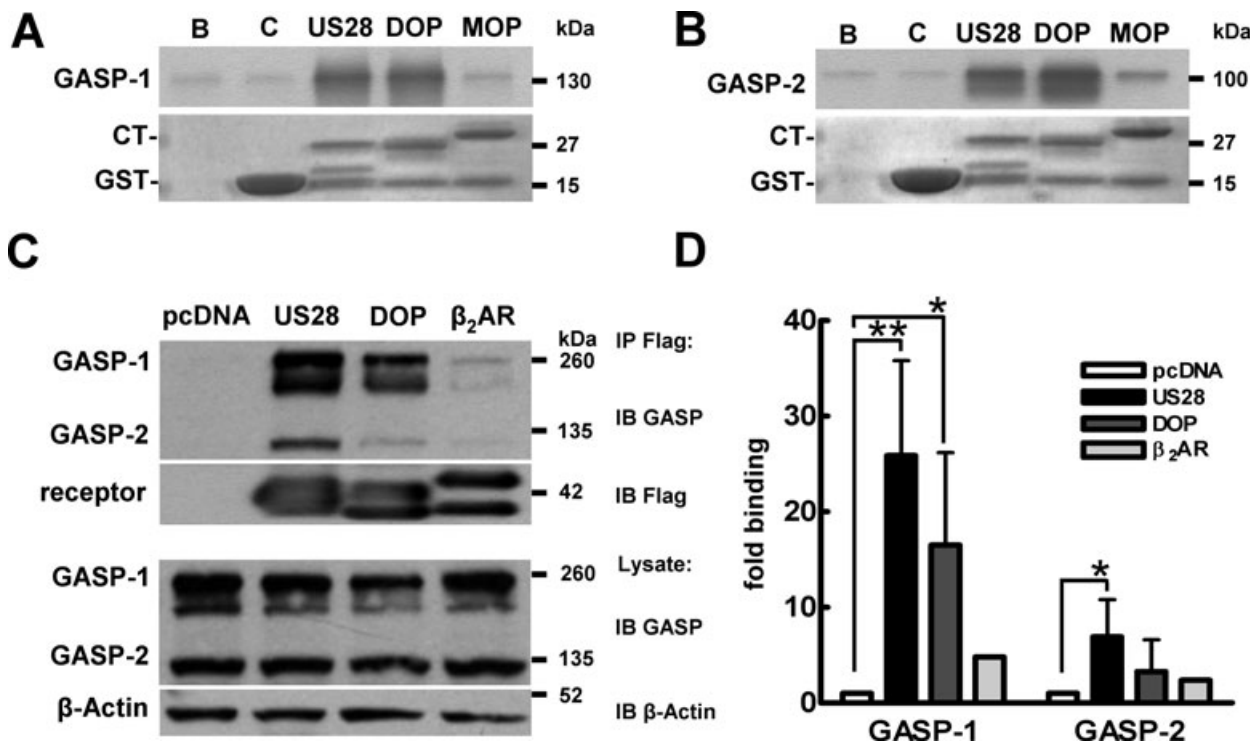


Figure 1: US28 interacts with GASP-1 and GASP-2 *in vitro*. A,B) *In vitro* translation assay of GASP binding. ^{35} S-methionine-labelled recombinant GASP-1 (A) and GASP-2 (B) bind to GST-fusion proteins containing the CT of US28 and the DOP, but not the GST-protein (C), beads (B) alone or the MOP. The corresponding input protein levels are shown below the radiographs. Blots are representative of at least three independent experiments. C) Co-immunoprecipitation of US28 with GASP-1 and GASP-2 in HEK293 cells. HEK293 transiently transfected with pcDNA3.1 (pcDNA), Flag-US28 (US28), Flag-DOP (DOP) or Flag- β_2 AR (β_2 AR) were lysed and receptors immunoprecipitated with Flag-antibody sepharose beads (IP). Precipitates were resolved by SDS/PAGE, transferred to PVDF membranes and immunoblotted for GASP-1 and GASP-2 (first panel) and receptor levels (second panel). Lysates were probed for GASP (third panel) and β -actin (fourth panel) levels. As observed before (14), native GASP proteins (C) run at higher molecular weight levels than recombinant proteins (A and B), likely because of post-translational modifications. D) Quantification of multiple experiments performed in C. Data are means of two to four experiments $\pm$ SD, * $p < 0.05$, ** $p < 0.01$.

a prominent redistribution to vesicles close to the cell membrane (Figure 2H, white arrows), with a close resemblance to the receptor localization observed in cells overexpressing cGASP-1 (compare Figure 2D and 2H, respectively), and no colocalization with lysosomes (Figure 2H, red).

To rule out the possibility that shRNA knock-down of GASP-1 exerts off-target effects on lysosomal function *per se*, we examined whether ligand-induced proteolysis of the epidermal growth factor receptor (EGFR) was intact in cells infected with shScr or shGASP-1 virus. EGFR is a receptor tyrosine kinase endogenously expressed in HEK293 cells and traffics to lysosomes after prolonged agonist treatment (25). Moreover, we have previously shown that the expression of the dominant negative cGASP-1 did not interfere with EGFR degradation in HEK293 cells (14), suggesting that its degradation is not GASP-1 dependent. Indeed, the degradation of EGFR was not impaired in cells depleted of GASP-1 by shRNA (Figure 2F, top panel). Furthermore, the knock-down of

GASP-1 does not alter the expression levels of US28 (Figure 2F, 42-kDa bands).

In order to identify the vesicles to which US28 is redistributed in cells overexpressing cGASP-1 (Figure 2D) and in shGASP-1 infected cells (Figure 2H) we tested whether these vesicles belonged to the early endosomes, defined by colocalization with the early endosomal antigen 1 (EEA.1) or to the recycling endosomal pathway defined as colocalization with transferrin.

US28 partially colocalizes with EEA.1 in HEK293 cells (Figure 3A, see insert). However, in cells overexpressing GFP-cGASP-1 (Figure 3B, insert), no colocalization with EEA.1 was detectable in the US28-containing vesicles (Figure 3B, arrows). Similarly, US28 colocalizes with EEA.1 in shScr infected cells (Figure 3C, insert), whereas US28 vesicles did not overlap with EEA.1 in shGASP-1 infected cells (Figure 3D, arrows). Hence, the small US28 receptor pool detectable in early endosomes (Figure 3A,C) seems to be dependent on the presence of GASP-1.

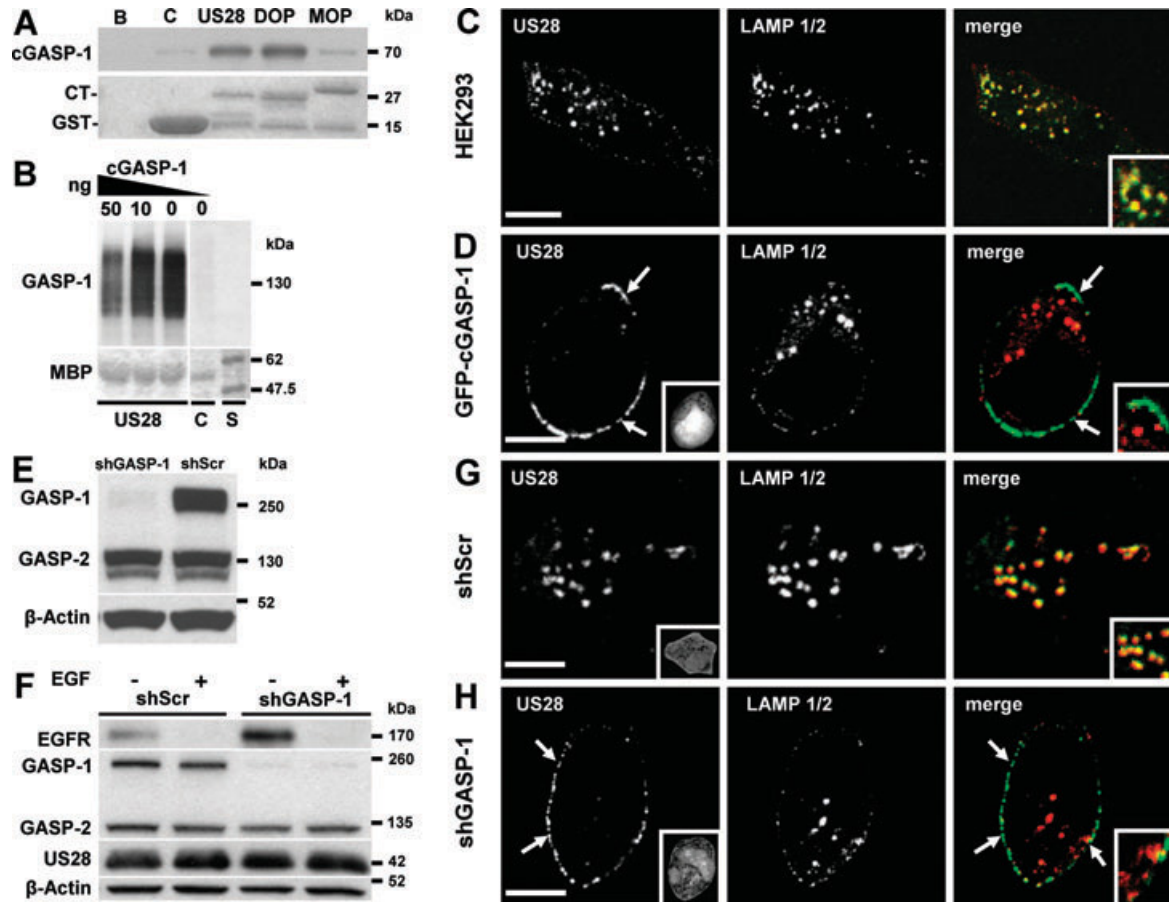


Figure 2: Post-endocytic sorting of US28 is mediated by GASP-1. A) The dominant negative cGASP-1 binds to GST-fusion proteins containing the CT of US28, the DOP, but not the GST-protein (C), beads (B) alone or the MOP. The corresponding input protein levels are shown below the radiographs. Blots are representative of at least three independent experiments. B) cGASP-1 competes with GASP-1 for the binding site on the US28-CT. MBP-US28 was incubated with increasing concentrations of purified cGASP-1 in the presence of ³⁵S-Met-labelled recombinant GASP-1. MBP-protein was used as control (C). Molecular weight marker (S). The corresponding input protein level is shown below the radiograph. C) US28 is sorted to lysosomes. HEK293 cells transiently transfected with Flag-US28 were 'fed' antibody to the extracellular Flag-tag for 45 min, then fixed and permeabilized. Receptors (green) were analysed for colocalization with the lysosomal markers LAMP1 and LAMP2 (red), merge: yellow. D) cGASP-1 alters the localization of US28. Antibody feeding experiments in HEK293 cells stably expressing GFP-cGASP-1 (see insert) and transiently expressing Flag-US28 revealed a redistribution of the receptor to vesicles close to the cell surface (see arrows). Green: Flag-US28, red: LAMP1/2, merge: yellow. E) Knock-down of endogenous GASP-1 in HEK293 cells using a shRNA-lentivirus. HEK293 cells were infected with either shGASP-1 or shScr virus. Forty-eight hours post-infection, cells were lysed, separated by SDS/PAGE and immunoblotted for GASP-1. β-actin staining served as loading control (lower panel). GASP-2 levels remained unchanged in both shScr and shGASP-1 infected cells (see 130-kDa band). F) EGF receptor degradation is not impaired by shGASP-1 knock-down. HEK293 cells infected with shScr or shGASP-1 virus were transiently transfected with Flag-US28 and either left untreated or incubated with EGF (5 μM) for 3 h. Lysates were analysed by SDS/PAGE and immunoblotted for endogenously expressed EGF receptor (first panel, EGFR), GASP-1 and GASP-2, US28 and β-actin (lower panels). G,H) shRNA knock-down of GASP-1 prevents lysosomal targeting of US28. HEK293 cells were infected with either shScr virus (G) or shGASP-1 virus (H) and transfected with Flag-US28. Antibody feeding experiments showed that US28 colocalizes with LAMP1 and LAMP2 in cells infected with scrambled virus (G), whereas the receptor is redistributed to vesicles close to the cell surface in cells infected with shGASP-1 (H, see arrows). Green: Flag-US28, red: LAMP1/2, merge: yellow, insert: EGFP-shRNA-virus. Scale bars = 10 μm.

It was previously reported that a fraction of the US28 receptor pool is found in early recycling endosomes ((5,9) and Figure 3E,G). Hence, we tested whether the US28-containing vesicles in cells overexpressing GFP-cGASP-1 or devoid of GASP-1 colocalize with the recycling endosomal marker transferrin. Indeed, we observe a

colocalization with transferrin in both, GFP-cGASP1 (Figure 3F, arrows and insert) and shGASP-1 (Figure 3H, arrows and insert) infected cells.

Together, these results suggest that GASP-1 is a key mediator in targeting US28 to lysosomes, because

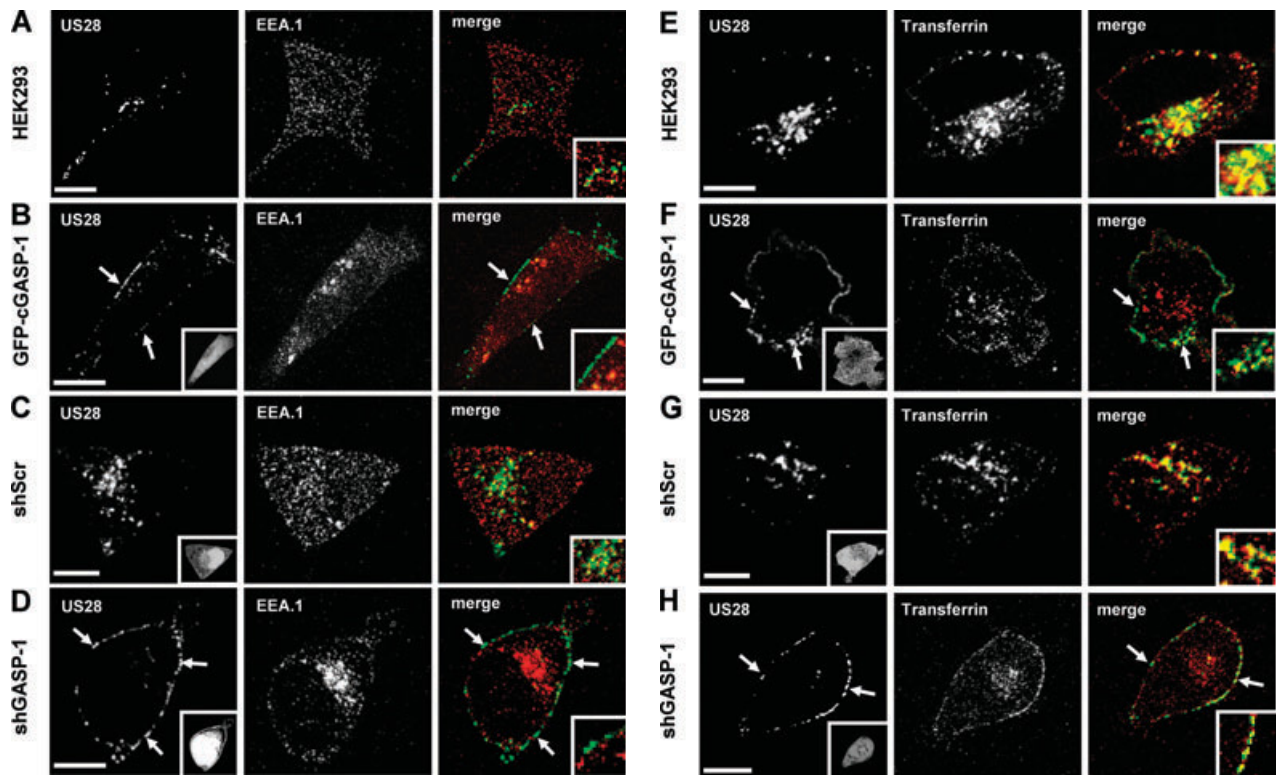


Figure 3: Colocalization of US28 with the endosomal markers EEA.1 and transferrin. A) Colocalization of US28 with EEA.1. HEK293 cells transiently transfected with Flag-US28 were 'fed' antibody to the extracellular Flag-tag for 45 min, then fixed and permeabilized. Receptors (green) were analysed for colocalization with the early endosomal marker EEA.1 (red), merge: yellow. B) US28 does not colocalize with EEA.1 in cGASP-1-overexpressing cells. Antibody feeding experiments in HEK293 cells stably expressing GFP-cGASP-1 and transiently expressing Flag-US28 revealed no colocalization of the receptors with EEA.1. Green: Flag-US28, red: EEA.1, insert: GFP-cGASP-1. C,D) Colocalization of US28 with EEA.1 in shScr and shGASP-1 infected cells. HEK293 cells were infected with either shScr virus (C) or the shGASP-1 virus (D) and transfected with Flag-US28. Antibody feeding experiments showed that US28 partially colocalizes with EEA.1 in cells infected with scrambled virus (C), whereas there was no colocalization detectable in cells infected with shGASP-1 (D). Green: Flag-US28, red: EEA.1, merge: yellow, insert: EGFP-shRNA-virus. E) US28 colocalizes with the endosomal marker transferrin. Antibody feeding experiments in HEK293 cells transiently transfected with Flag-US28 were conducted in the presence of AlexaFluor 594-conjugated transferrin (for details see *Methods*). Receptors (green) were analysed for colocalization with the endosomal marker transferrin (red), merge: yellow. F) US28 colocalizes with transferrin in GFP-cGASP-1-overexpressing cells. Antibody feeding experiments in HEK293 cells stably expressing GFP-cGASP-1 and transiently expressing Flag-US28. Green: Flag-US28, red: transferrin, merge: yellow, insert: GFP-cGASP-1. G,H) Colocalization of US28 with transferrin in shScr and shGASP-1 infected cells. HEK293 cells were transduced with either shScr virus (G) or the shGASP-1 virus (H) and transfected with Flag-US28. Antibody feeding experiments showed that US28 partially colocalizes with transferrin in cells infected with scrambled virus (G) as well as in cells infected with shGASP-1 (H). Green: Flag-US28, red: transferrin, merge: yellow, insert: EGFP-shRNA-virus. Scale bars = 10 μ m.

the disruption of GASP-1/US28 interaction prevents the sorting of US28 to lysosomes.

GASP-1 mediates degradation, but not the constitutive internalization of US28

In order to quantitatively monitor the endocytic and post-endocytic fate of US28 in the absence or presence of GASP-1, we next performed biotin protection and degradation assays. This assay allows selective monitoring of the endocytic- and post-endocytic fate of a pool of receptors that reaches the cell surface and is subsequently endocytosed and either recycled or degraded [see also (14,16,20)].

Although our antibody feeding experiments suggest that US28 constitutively endocytoses in the absence of GASP-1, we wanted to test whether GASP-1 alters the kinetics of US28 internalization in either GFP-cGASP-1 overexpressing (Figure 4A) or in shGASP-1 (Figure 4B) infected cells. To this end, receptors that reached the cell surface of intact cells were labelled with thio-cleavable disulfide-linked biotin for 30 min in the cold to prevent internalization (corresponding to 100% in Figures 4A,B). Cells were then returned to warm media, and the biotinylated receptor pool was allowed to constitutively internalize for up to 45 min. Following this incubation, biotin on cell surface receptors was cleaved with a membrane impermeable reducing

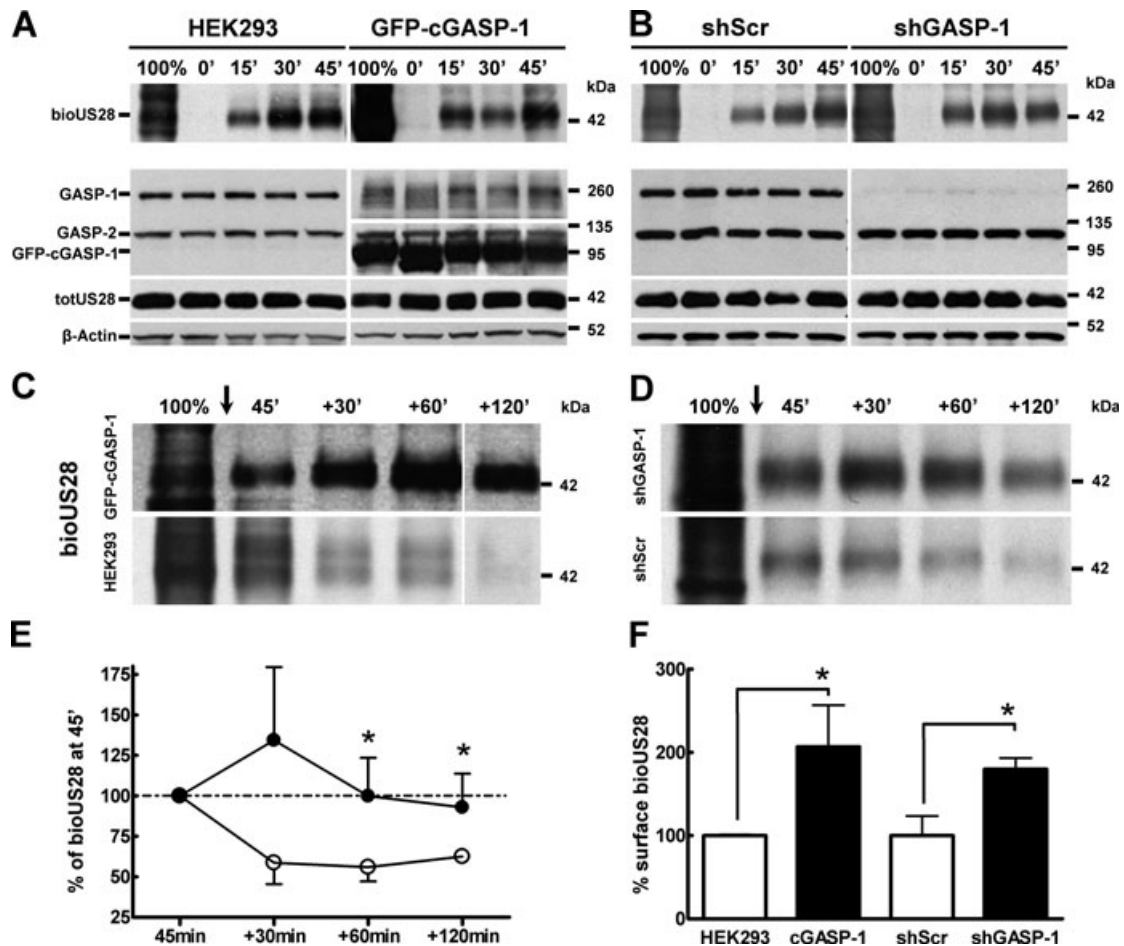


Figure 4: Disrupting the GASP-1/US28 interaction does not affect constitutive internalization of US28, but delays receptor degradation. A) Overexpression of GFP-cGASP-1 does not affect receptor internalization. HEK293 cells (upper left panel) or cells stably expressing GFP-cGASP-1 (upper right panel) were transiently transfected with Flag-US28. Internalization of biotinylated Flag-US28 (bioUS28) was monitored at the time-points indicated, before cells were stripped of surface-biotin. The total amount of biotinylated proteins before the strip is shown by 100%. The lower panels show the corresponding lysate samples immunoblotted for GASP, total receptor (totUS28) and β -actin. B) Disrupting the GASP-1/US28 interaction by shRNA does not affect receptor internalization. HEK293 cells were infected with shScr (upper left panel) or shGASP-1 virus (upper right panel) for 48 h and transiently transfected with Flag-US28. Internalization of biotinylated Flag-US28 (bioUS28) was monitored as described in A. Disrupting the GASP-1/US28 interaction delays receptor degradation. C) Overexpression of GFP-cGASP-1 delays US28 degradation. HEK293 cells stably expressing GFP-cGASP-1 (upper panel) or control cells (lower panel) were transiently transfected with Flag-US28. Biotinylated Flag-US28 (bioUS28) was allowed to constitutively internalize for 45 min (see A and B), then cells were stripped of surface-biotin (arrow). Thus, 45' indicates the internalized US28 receptor pool at 45 min. Subsequently, the degradation of US28 was monitored for additional 120 min. D) shRNA knock-down of GASP-1 delays US28 degradation. HEK293 cells were infected with shGASP-1 (upper panel) or shScr virus (lower panel) for 48 h and transiently transfected with Flag-US28. Biotinylation assays were performed as described in C. E) Quantification of biotinylation assay corresponding to D. Biotinylated receptor bands in shScr ($\circ$) or shGASP-1 ($\bullet$) cells were normalized to the total US28 receptor pool and β -actin and the 45' value was set to 100%. Data are means of five experiments $\pm$ SD, $*p < 0.05$. F) Quantification of surface-biotinylated US28. Biotinylated surface receptor bands (100% lanes) were normalized to the total US28 (totUS28) receptor pool and quantified with IMAGEJ software. Control cells (white bars) were set to 100%. In both cGASP-1 and shGASP-1 expressing cells (black bars), surface bioUS28 was significantly higher than in control cells (white bars). Data are means of two to three experiments $\pm$ SD, $*p < 0.05$.

agent. The internalized 'protected' pool of receptors was then immunoprecipitated and the remaining biotinylated receptor was visualized by streptavidin overlay (see *Methods* for details). Figure 4A,B (top panels) shows the increasing amount of internalized receptor (bioUS28) during a time-course from 0 to 45 min in HEK293 cells,

cells stably overexpressing GFP-cGASP-1 (Figure 4A) and in cells infected with shScr versus shGASP-1 (Figure 4B). US28 internalizes with similar kinetics in all conditions tested. Lysate samples were blotted for the expression of GASP-1, GASP-2, GFP-cGASP-1, Flag-US28 and β -actin (Figure 4A,B, lower panels). These data show that GASP-1

levels do not interfere with the constitutive internalization kinetics of US28. However, we consistently observed a higher amount of biotinylated surface receptor in cells overexpressing GFP-cGASP-1 or in shGASP-1 cells (compare 100% lanes in Figure 4A,B, for quantification see Figure 4F).

Next, if GASP-1 were mediating the degradation of US28, one would expect that either the overexpression of cGASP-1 or the knock-down of endogenous GASP-1 levels could delay or even inhibit receptor degradation. Hence, we next monitored the stability of the biotinylated and constitutively endocytosed US28 receptor pool. After biotinylation and 45 min in prewarmed media to allow a 'pulse' of receptor to be endocytosed, surface-biotin was stripped and cells were incubated or 'chased' for an additional 30, 60 or 120 min (see +30, +60 and +120 min lanes in Figure 4C,D) to monitor the stability of the protected pool. At the end of the experiment, US28 receptors were immunoprecipitated and remaining biotinylated receptor was visualized by streptavidin overlay.

In cells overexpressing GFP-cGASP-1 ~100-fold (see Figure S1), the degradation of biotinylated and internalized US28 was delayed (Figure 4C, upper panel) when compared to control cells (Figure 4C, lower panel). Likewise, in HEK293 cells infected with shGASP-1 lentivirus (Figure 4D, upper panel), internalized and

biotinylated US28 receptor was also significantly more stable than in cells infected with shScr (Figure 4D, lower panel, compare also shGASP-1 (●) and shScr (○) in Figure 4E).

Taken together, these results indicate that the interaction between US28 and GASP-1 is crucial for the sorting of the receptor to the degradative pathway.

US28 recycles in the absence of GASP-1

We next examined whether disruption of the US28 GASP-1 interaction not only blocked receptor degradation but also facilitated recycling. Because US28 has not only been reported to constitutively internalize, but also constitutively recycle in some circumstances [(9) and Figure 3E–H], we anticipated this might be the case.

To assess recycling, cells transiently transfected with Flag-US28 alone (Figure 5A) and overexpressing GFP-cGASP-1 (Figure 5B) were fed M1-antibody for 45 min to allow for receptor internalization. Then cells were stripped of the M1-antibody (Figure 5A,B, strip) and resurfacing (recycling) of the receptors was monitored for 15 and 30 min. Cells were then fixed and the recycled receptor was detected by staining with a fluorescent antibody targeting the M1-antibody under non-permeabilizing conditions. Control cells show weak surface staining of US28 (Figure 5A,C, 45 min), which is consistent

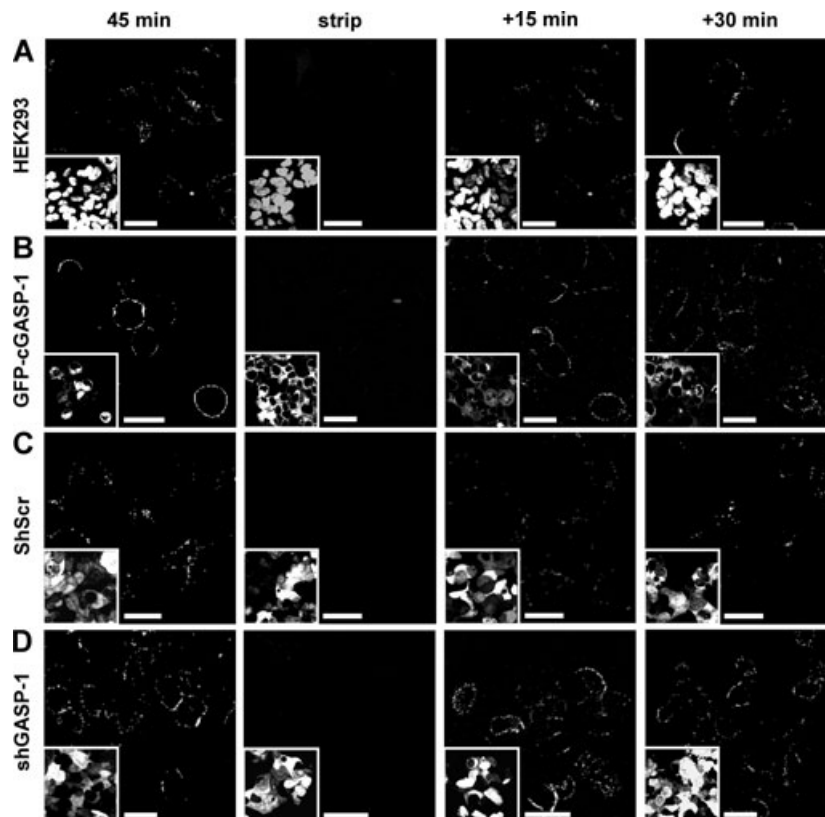


Figure 5: Constitutive recycling of US28 is not dependent on GASP-1. A,B) Recycling of US28 is not impaired in cells overexpressing GFP-cGASP-1. HEK293 cells (A) or cells stably expressing GFP-cGASP-1 (B) transiently transfected with Flag-US28 were 'fed' a Ca^{2+} -sensitive antibody to the extracellular Flag-tag for 45 min. Then the cells were stripped with PBS-EDTA (strip) and warm growth medium was added. After 15 or 30 min the cells were fixed and the recycled receptor was stained under non-permeabilizing conditions. *Inserts:* DAPI (A) and GFP-cGASP-1 (B). C,D) Recycling of US28 is not impaired in shScr or shGASP-1 infected cells. HEK293 cells infected with shScr virus (C) or shGASP-1 virus (D) were transiently transfected with Flag-US28. Recycling experiments were performed as in A and B. *Inserts:* GFP-shRNA lentivirus. Scale bars = 20 μm .

with ~20% of surface expression of the total US28 receptor pool reported previously (5,9). Nevertheless, recycling US28 was detected as early as 15 min after stripping (Figure 5A,C, +15 min panel). However, in cells overexpressing GFP-cGASP-1 (Figure 5B, 45 min) or shGASP-1 cells (Figure 5D, 45 min), significantly more US28 surface staining was visible than in control cells. Similarly to the control cells, recycling US28 was detectable in cells as early as 15 min after stripping (Figure 5B,D, +15 min panels) and US28 surface staining was detectable up to +30 min. These data are also consistent with the increased surface expression of US28 in shGASP-1 and GFP-cGASP-1 overexpressing cells detected in the biotinylation assays (see Figure 4F).

GASP-1 alters the constitutive $G\alpha_q$ -mediated signalling capacity of US28

The sorting of individual receptors between recycling and degradative pathways is a fundamental process that can regulate the signalling capacity of a receptor. Hence, we set out to elucidate the implications of the GASP-1-mediated post-endocytic sorting on the signalling capacity of US28.

US28 has been shown to regulate the $G\alpha_q$ /phospholipase C (PLC) pathway both constitutively and in a ligand-dependent manner (3,4,26,27). Here we set out to examine the ability of US28 to mediate the $G\alpha_q$ /PLC-dependent accumulation of inositol phosphate (IP) in cells where the interaction with GASP-1 had been altered. As we found that GASP-1 facilitates the degradation of US28 after endocytosis, we anticipated that disruption of the US28/GASP-1 interaction might enhance constitutive signalling of US28. Surprisingly, an ~0.5-fold overexpression of GASP-1 (Figure 6B, upper panel) significantly increased the IP turnover by US28 (Figure 6A; compare black versus striped bars). In contrast, overexpression of cGASP-1 (Figure 6B, lower panel, and Figure S2B) significantly reduced the IP turnover (Figure 6A; compare black versus grey bars). These effects were not attributable to different total expression levels of US28 in the respective assays, as verified by enzyme-linked immunosorbent assay (ELISA) (Figure 6C). Next, we examined the IP turnover in cells in which GASP-1 has been knocked down with the shGASP-1 virus. In agreement with our previous findings, the IP turnover was significantly reduced in shGASP-1 infected cells (Figure 6D, compare black versus striped bar).

To confirm that the decreased signalling in shGASP-1 infected cells was because of the knock-down of GASP-1, we conducted rescue experiments with a shRNA-insensitive (rescue)GASP-1 (for details see *Methods*). Transfection of shGASP-1 infected cells with rGASP-1 resulted in a replenished GASP-1 pool (Figures 6E and S2B, compare grey versus black bars). Furthermore, we confirmed that rGASP-1 bound to the C-terminus of US28 *in vitro* (Figure S3). Indeed, as Figure 6D (compare striped versus grey bars) shows, the decreased IP turnover

in shGASP-1 infected cells could be restored by the re-expression of rGASP-1. US28 receptor levels were comparable in all assays (Figure 6F).

In summary, these data show that GASP-1 modulates the constitutive $G\alpha_q$ /PLC/IP turnover mediated by US28.

The activation of transcription factors by US28 is impaired in cells deprived of GASP-1

US28 has been reported to activate downstream transcription factors such as NF- κ B, CREB (3,4,28), NFAT (22) and SRF (23). Following our findings that GASP-1 levels alter the ability of US28 to activate the $G\alpha_q$ /PLC/IP pathway, we examined whether this effect would also translate to an altered downstream signal transduction profile. Therefore, we tested the impact of altered GASP-1 levels on the US28-mediated activation of the transcription factors NF- κ B and CREB. Indeed, transient expression of US28 and overexpression of GASP-1 in HEK293 cells resulted in a significant increase of both NF- κ B and CREB-luciferase activity (Figure 7A,B, respectively, compare black and striped bars). Next, we disrupted the interaction of US28 and endogenous GASP-1 by overexpression of cGASP-1. cGASP-1 blocked the downstream activation of both NF- κ B and CREB-luciferase activity (Figure 7A,B, compare black versus grey bars). Receptor expression levels were similar in all experiments (Figure 7C,D).

To further test the hypothesis that GASP-1 is involved in the regulation of the downstream signal transduction pathways of US28, we performed the NF- κ B and CREB-luciferase assays in cells infected with the shGASP-1 and shScr virus. Indeed, the activation of transcription factors is significantly decreased in shGASP-1 infected cells in comparison to shScr infected cells (Figure 8A,B, compare black bars versus grey bars). To confirm that the reduced NF- κ B and CREB activation in shGASP-1 infected cells was because of the knock-down of GASP-1, we reintroduced GASP-1 into shGASP-1 cells by transfection with rGASP-1. Again, rGASP-1 'rescued' the activation of transcription factors via US28 in shGASP-1 infected cells (Figure 8A,B, compare grey versus striped bars). Receptor expression levels were similar in all experiments (Figure 8C,D).

In summary, our data suggest that GASP-1 not only modulates the ability of US28 to activate $G\alpha_q$ /PLC/IP-dependent signalling, but is also a crucial component in activating further downstream signalling events, such as the transcription factors NF- κ B and CREB.

Discussion

Here we show that the sorting protein GASP-1 is an important cellular determinant in regulating the post-endocytic trafficking and constitutive signalling capacity of the viral receptor US28. Our data suggest that US28 is targeted for degradation via a direct protein–protein interaction with the sorting protein GASP-1 (Figure 1). Disruption of this

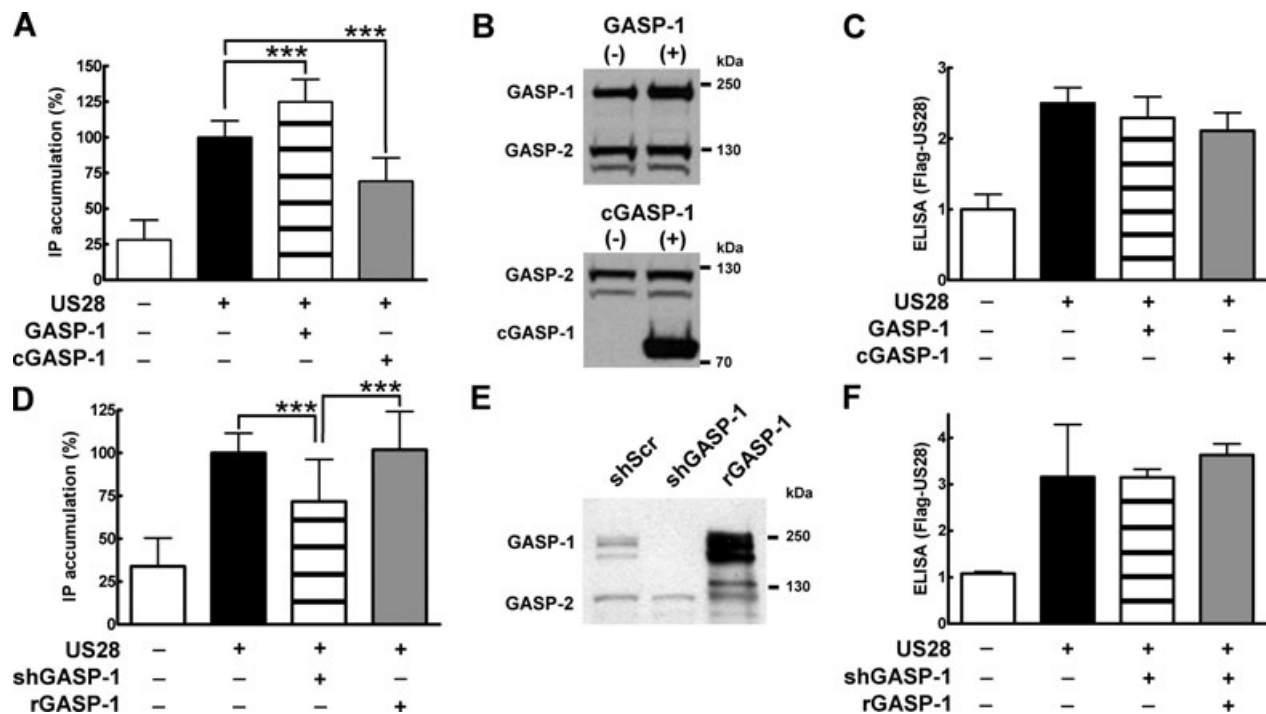


Figure 6: GASP-1 modulates US28-mediated IP accumulation. A) Overexpression of GASP-1 and cGASP-1 alters US28-mediated IP turnover. HEK293 cells were transiently transfected with Flag-US28 (75 ng/well) and 100 ng/well of either pcDNA3.1 (black bar), HA-GASP-1 (striped bar) or HA-cGASP-1 (grey bar). Controls were transfected with pcDNA3.1 (175 ng/well, white bar). IP production mediated by US28 was allowed to accumulate for 45 min. Values were normalized to the cell number, and 100% corresponds to the basal activity of US28 (black bar). Data are means of six experiments $\pm$ SD carried out in quadruplicates, *** p < 0.001. B) Expression levels of HA-GASP-1 and HA-cGASP-1 in HEK293-US28 cells. HEK293 cells were transiently transfected with Flag-US28 in combination with pcDNA3.1 and HA-GASP-1 (upper panel) or with HA-cGASP-1 (lower panel). The cells were lysed, separated by SDS/PAGE and blotted with GASP antibody after 24 h. Overexpression of HA-GASP-1 or HA-cGASP-1 does not alter GASP-2 levels. C) US28 levels in HEK293 cells overexpressing GASP-1 and cGASP-1. HEK293 cells were transfected as in A. The expression levels of US28 were assayed by ELISA against the Flag-epitope tag of US28. Values were normalized to the cell number. Data are means $\pm$ SD of one representative experiment carried out in quadruplicates. The control (white bar) was set to one and the other values were normalized accordingly. D) Reduced IP accumulation in shGASP-1 infected cells is restored by overexpression of rGASP-1. HEK293 cells were infected with shScr (black bar) or the shGASP-1 virus (striped bar and grey bar). Then cells were transiently transfected with Flag-US28 (75 ng/well) and either 100 ng/well pcDNA3.1 (black and striped bars) or 100 ng/well HA-rGASP-1 (grey bar). Controls were transfected with pcDNA3.1 (175 ng/well, white bar). The IP accumulation assay was conducted as described in A. Values were normalized to the cell number, and 100% corresponds to the basal activity of US28 in shScr infected cells (black bar). Data are means of four experiments $\pm$ SD carried out in quadruplicates, *** p < 0.001. E) GASP-1 levels in shGASP-1 infected cells can be restored with HA-rGASP-1. ShScr infected cells were transfected with Flag-US28 and pcDNA3.1. shGASP-1 infected cells were transfected with either pcDNA3.1 or HA-rGASP-1. The cells were lysed, separated by SDS/PAGE and blotted with the GASP antibody after 24 h. Blot shows one representative of at least three independent experiments. F) US28 levels in shScr and shGASP-1 infected cells. HEK293 cells were infected and transfected (see D) and ELISA was performed as described in C.

process results in a vesicular redistribution of US28 close to the plasma membrane (Figure 2D,H). These vesicles colocalize with transferrin (Figure 3F,H) but do not co-stain for the early endosomal marker EEA.1 (Figure 3B,D). Importantly, the constitutive internalization kinetics of US28 are not altered when the interaction of US28 and GASP-1 is disrupted (Figure 4A,B). The degradation of US28 is significantly delayed in cells knocked down for GASP-1 expression (Figure 4D,E) and also slowed in cells overexpressing the dominant negative cGASP-1 (Figure 4C). In addition, recycling of US28 is enhanced when the US28–GASP-1 interaction is disrupted (Figure 5)

and results in a higher surface expression of US28 in these cells (Figure 4F).

In addition, we show that GASP-1 is an important regulator of signalling pathways activated by US28. US28 has previously been reported to constitutively activate the G_{α_q} /PLC/IP pathway and the transcription factors NF- κ B and CREB (3,4,28). Here we show that GASP-1 is crucial for US28-mediated IP production, i.e. overexpression of GASP-1 enhances IP turnover, whereas competition with cGASP-1 (Figure 6A) or knock-down of GASP-1 expression (Figure 6D) decreases IP accumulation. In addition, the

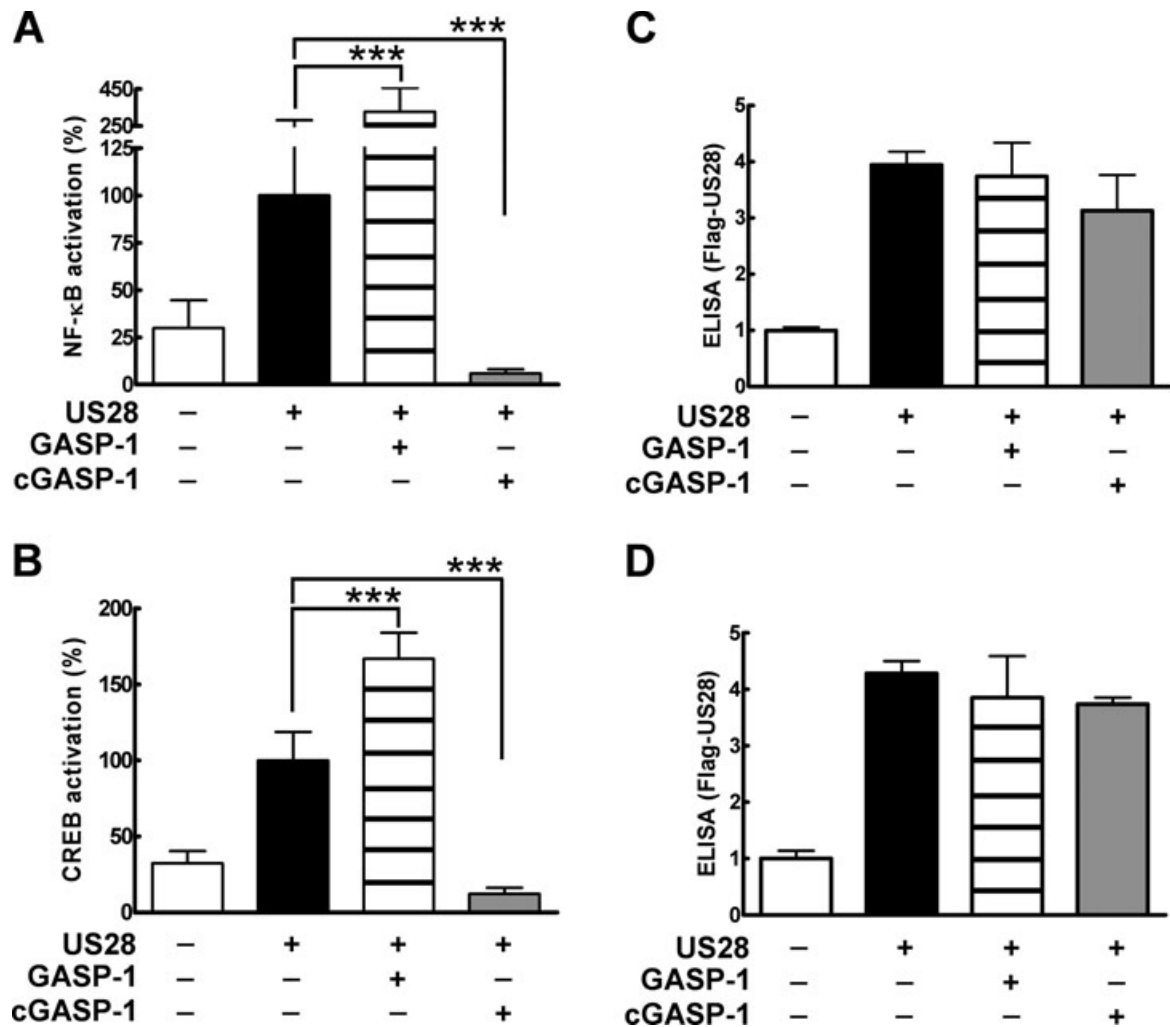


Figure 7: US28-mediated NF- κ B and CREB transcription is modulated by GASP-1. A, B) US28-mediated transcription factor activation is altered upon overexpression of GASP-1 and cGASP-1. HEK293 cells were transiently transfected with Flag-US28 (50 ng/well) in combination with 100 ng/well pcDNA3.1 (black bar), HA-GASP-1 (striped bars) or HA-cGASP-1 (grey bars) and the respective cis-reporter luciferase plasmid for either NF- κ B (50 ng/well, A) or CREB (200 ng/well, B). Controls were transfected with pcDNA3.1 (white bars). A luciferase reporter assay was conducted 24 h post-transfection. Values were normalized to the cell number, whereby 100% corresponds to the basal activity of US28 (black bar). Data are means of four experiments $\pm$ SD carried out in quadruplicates, *** p < 0.001. C, D) US28 levels in HEK293 cells overexpressing GASP-1 and cGASP-1. HEK293 cells were transiently transfected as described in A and B. The expression levels of US28 were assayed by ELISA as described before.

activation of the transcription factors NF- κ B and CREB via US28 is also dependent on GASP-1 levels (see Figures 7 and 8). Hence, GASP-1 is a key component in the regulation of both, the post-endocytic sorting and signalling properties of US28.

As mentioned before, GASP-2 is the closest homologue to GASP-1, and although the C-terminus of US28 binds GASP-2 *in vitro* (Figure 1B), we do not observe any regulatory and/or compensatory effects of GASP-2 in any of our functional assays. GASP-2 levels remain unchanged in cells either infected with shGASP-1 lentiviruses (see Figure 2E) or when co-transfected with cGASP-1 (Figure 4A, 95-kDa marker, or Figure 6B, lower panel).

The sorting of individual receptors to either the recycling or degradation pathways is a tightly regulated process that is of fundamental importance for the regulation of 7TM/ GPCR signalling. It is well established that 7TM/GPCRs mainly transmit their signals through G-proteins from the cell surface. Following their activation, GPCRs are often desensitized and then endocytosed. After endocytosis, receptors can be either recycled to the plasma membrane or targeted to lysosomes for degradation. However, there is accumulating evidence that the relationship between receptor trafficking and signalling is more complex than this. In particular, endosomes have been implicated as important sites for receptor-initiated signalling, as they contain a wide range of signalling molecules in their

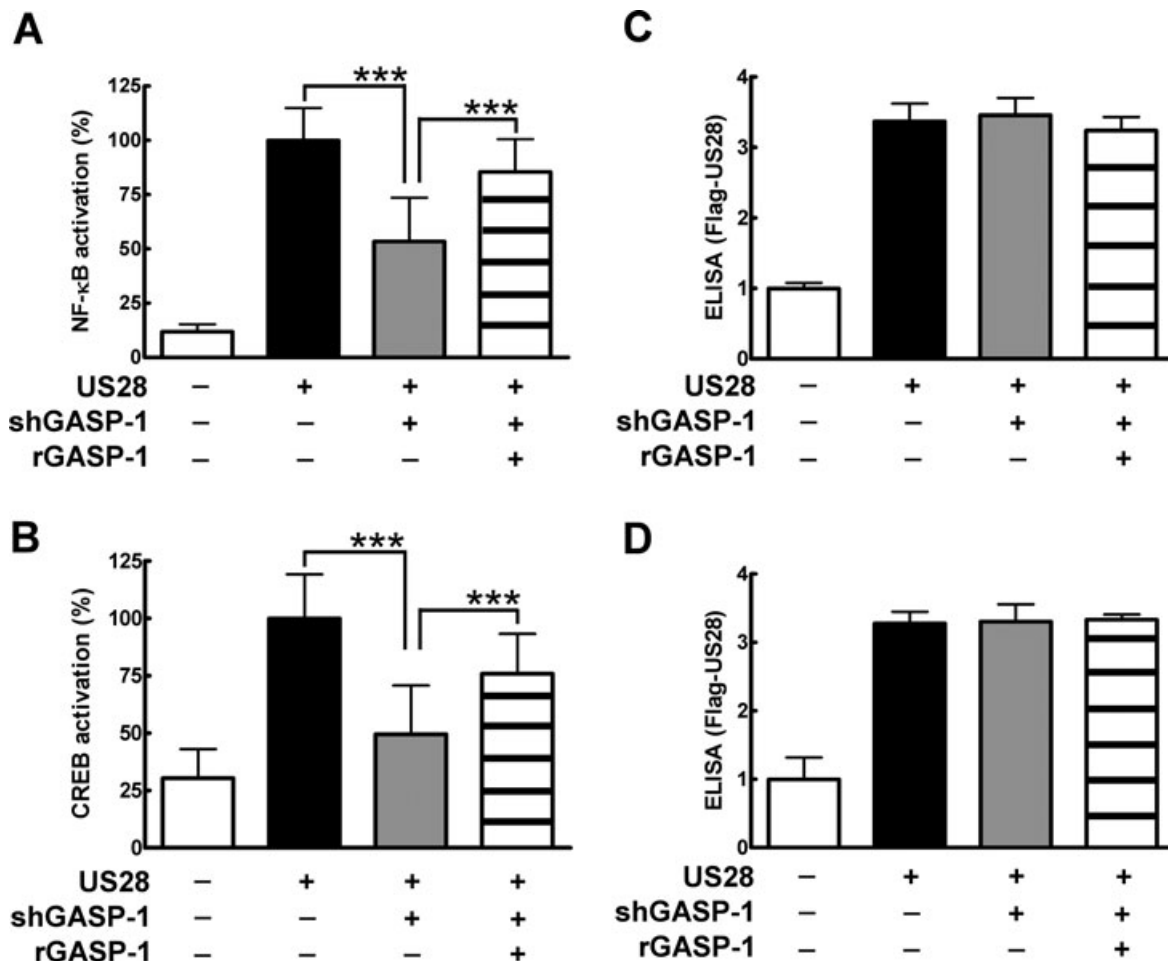


Figure 8: Reduced NF- κ B and CREB activation can be restored by overexpression of rGASP-1. A,B) US28-mediated transcription factor activation is rescued upon overexpression of rGASP-1. ShScr infected cells (black bars) and shGASP-1 infected cells (grey and striped bars) were transfected with Flag-US28 (50 ng/well) in combination with 100 ng/well of pcDNA3.1 (black bar and grey bars) or HA-rGASP-1 (striped bars) as well as the respective cis-reporter plasmids for either NF- κ B (50 ng/well, A) or CREB (200 ng/well, B). Controls were transfected with pcDNA3.1 (white bars). A luciferase reporter assay was conducted 24 h post-transfection. Values were normalized to the cell number, whereby 100% corresponds to the basal activity of US28 (black bar). Data are means of four experiments $\pm$ SD carried out in quadruplicates, *** p < 0.001. C,D) US28 levels in shGASP-1 infected and rGASP-1 transfected HEK 293 cells. HEK293 cells were infected and transiently transfected as described in A and B. The expression levels of US28 were assayed by ELISA as described before.

membranes and are able to recruit scaffolding proteins and signalling mediators. Furthermore, recent findings indicate that, in some cases, endocytosis might even be required as it can enhance receptor-mediated signalling for some pathways [for reviews see (29–31)].

Our data suggest that the sorting protein GASP-1 is crucial for the constitutive signalling activity of US28. Hence, GASP-1 may be one such 'signal mediator' for US28 once it is present in early sorting endosomes. We can only speculate on the reasons for the decreased ability of US28 to activate $G\alpha_q$ /PLC-mediated signalling in cells where the US28–GASP-1 interaction is disrupted (see Figure 6). One possible explanation would be the changed localization of the receptor in the absence of GASP-1 (see Figures 2

and 3). If US28 transmits its signals from the cell surface, this process might be disturbed when the receptor is localized in vesicles close to the cell membrane, such as that observed in cells where GASP-1 expression has been knocked down or in cells overexpressing the dominant negative cGASP-1. For instance, US28 cannot be found in EEA.1 positive early endosomes in cells devoid of GASP-1 or in the presence of dominant negative cGASP-1 (Figure 3); hence, one might speculate that these vesicles represent the 'signalosome' for US28. On the other hand, overexpression of GASP-1 appears to upregulate signalling from US28, suggesting that GASP-1 plays a direct role in signal transduction. Thus, one could speculate that GASP-1 enhances signalling because of a conformational change of the receptor to a more active state upon

interaction with GASP-1. But likewise, it could be possible that GASP-1 is necessary for targeting the receptor to a particular compartment for signal transduction, especially because only a minor fraction of this receptor is typically found on the cell surface (5,9). Importantly, a recent report shows that HCMV acquires its final envelopment on cytoplasmic membranes containing, among others, endosomal markers such as EEA.1, transferrin and the closely related UL33 receptor (11). Hence, GASP-1 may target the viral receptors to endosomes necessary for virion formation. Further experiments will be necessary to determine this.

Such interplay between the sorting of US28 and its constitutive signalling capacity might also be of clinical relevance. Human cytomegalovirus is a widespread pathogen that has been shown to be present in various malignancies, including breast cancer (32), colon cancer (33) or malignant glioblastoma (34), and US28 was recently shown to induce tumours in an NIH3T3 nude mouse model (35). Furthermore, US28 is able to upregulate several proteins involved in oncogenesis, especially cyclooxygenase-2 (COX-2) a transcriptional target of NF- κ B, suggesting a possible role for US28 in tumorigenesis (36). By identifying the sorting protein GASP-1 as a key regulator of not only the trafficking, but also, importantly, the signalling of US28, we may be one step closer to gaining a better understanding of this viral receptor and its significance in the pathogenesis implicated by HCMV.

Materials and Methods

Reagents

Mouse M1 and M2 monoclonal antibody, ANTI-Flag M2 Affinity Gel, Albumin from bovine serum, L-Glutathione, Iodoacetamide, gelatine from bovine skin Type B, Triton X-100, Kodak BioMax light films, 3, 3', 5, 5'-Tetramethylbenzidine Liquid substrate system and Tween-20 were purchased from Sigma Aldrich. Tris-Glycine gradient gels (4–20%), cell culture media (DMEM), foetal bovine serum (FBS), Dulbeccos phosphate-buffered saline (DPBS), ViraPowerTM Lentiviral Expression Systems and Lipofectamine 2000, Alexa Fluor 647 nm conjugated IgG2b, 594 nm conjugated IgG1a, 594 nm conjugated IgG2b, 594 nm conjugated donkey anti-rabbit IgG and 594 nm conjugated transferrin were purchased from Invitrogen. QuickTiterTM Lentivirus Quantification Kit (HIV p24 ELISA) was purchased from Cell Biolabs (THP). Horseradish peroxidase (HRP)-conjugated anti-rabbit antibody and HRP-conjugated anti-mouse antibody were from Jackson Immuno Research (Dianova). Generation of anti-GASP1 antibodies has been described previously (14). Mouse monoclonal LAMP1 and LAMP2 antibodies were from the Developmental Studies Hybridoma Bank developed under the auspices of the NICHD and maintained by the University of Iowa, Department of Biological Sciences, Iowa City, IA 52242. Complete Protease inhibitor cocktail tablets were from Roche. Immobilon-P Transfer Membrane was purchased from Millipore. Enhanced chemiluminescence (ECL) Western Blotting Substrate was from Pierce (THP). Vectashield mounting medium and Vectastain ABC Kit were purchased from Vector Laboratories (Szabo-Scandic). The white 96-well culture plates and the Steadylite Plus Assay Kit, high-sensitivity luminescence reporter gene assay system, [^{3} H]myo-inositol and EasyTag ^{35} S-methionine were purchased from PerkinElmer. Yttrium silicate (YSi) SPA beads were from GE Healthcare, EZ-Link Sulfo-NHS-SS-Biotin was from Thermo Scientific (Histocom). NaOH, KCl and CaCl $_2$ * 2H $_2$ O were obtained from Merck, NaCl, Tris and Formaldehyde were from Roth

(Lactan). TNT T7 Coupled Reticulocyte Lysate System were purchased from Promega and PAGEBlue from Fermentas. Goat antibodies to EGFR and HRP-conjugated secondary anti-goat antibody were from Santa Cruz Biotechnology (Szabo-Scandic).

Polyclonal rabbit anti-EEA.1 was purchased from Calbiochem (Merck). Human EGF was from Peprotech.

G418 sulphate solution was purchased from PAA Laboratories.

DNA constructs

A Flag-tagged version of US28 (Flag-US28) was cloned by using the BamHI and XbaI restriction sites in the SS-Flag pcDNA3.1 zeo (+) vector (16). The cloning of HA-tagged GASP-1 (HA-GASP-1), cGASP-1 (HA-cGASP-1), GFP-tagged cGASP-1 (GFP-cGASP-1), the Flag-DOP-R and Flag- β 2AR were described previously (14,18). Oligonucleotides for shRNA were constructed using AAG(N18)TT criteria to select an appropriate sequence. The oligonucleotides were annealed and cloned into the lentilox vector pLL3.7 (ATCC) using the XhoI and HpaI restriction sites. The target sequence for GASP-1 (GTACCTGTCTGAGG ATAGA) and the scrambled shRNA (GTAAAGGTTCCGGCAGCA) were synthesized by Operon. Lentiviruses encoding for either shRNA against GASP-1 (shGASP-1) or scrambled shRNA (shScr) were produced in HEK293FT cells. The generation of the GST-fusion protein constructs containing the 59 carboxy-terminal amino acids of US28 (GST-US28) (24), the carboxy-terminal amino acids of the delta opioid receptor (GST-DOP), the mu opioid receptor (GST-MOP) and the carboxy-terminal part of GASP-1 (GST-cGASP-1) were described elsewhere (14). The maltose binding (MBP) fusion protein MBP-US28 was generated by subcloning the 59 C-terminal amino acids of US28 into the pMALc2 expression vector (New England Biolabs) using the BamHI and XhoI restriction sites. For rescue experiments we designed a rescue full-length GASP-1 (rGASP-1). To inactivate the shRNA recognition site we performed targeted mutagenesis (QuikChange Site-Directed Mutagenesis Kit, Stratagene) according to the instruction manual. Three point mutations were introduced without altering the protein sequence by the following oligonucleotide primers (mutations are bold and underlined): F-ctgtcttgggtgctaaacaaagta**ctctgcgag** gacagagaactggtaatac, R-gtattaaccagttctct**gtcctcgg** acagatacttggtttagcaaccaagacag. All DNA constructs were verified by sequencing.

GST-fusion protein-binding assay

GST-fusion protein-binding assays were performed essentially as described in (14,24). In brief, GST-fusion proteins were expressed in XL BLUE Top 10 *Escherichia coli* and bound to glutathione-agarose. Fusion proteins on beads (~3 μ g) were incubated in blocking buffer (20 mM Hepes, pH 7.4, 100 mM NaCl, 2 mM MgCl $_2$, 0.1% Triton X-100, 5% BSA) overnight at 4°C. Subsequently, fusion proteins were incubated with 2 μ L of *in vitro* translated ^{35} S-methionine-labelled HA-GASP-1, HA-cGASP-2 or HA-rGASP-1 (TNT T7 Coupled Reticulocyte Lysate System) in wash buffer (20 mM HEPES, pH 7.4, 100 mM NaCl, 2 mM MgCl $_2$, 0.1% Triton X-100) under vigorous shaking for 1 h at 4°C. Probes were then washed five times in ice-cold wash buffer, eluted in sample buffer and resolved by SDS-polyacrylamide gel electrophoresis (SDS/PAGE). Gels were stained with PAGEBlue, dried for 3 h in a gel drier and exposed to X-ray films.

MBP competition binding assay

MBP-US28 and MBP alone (control) were expressed and purified on amylose resin. ^{35} S-methionine-labelled HA-GASP-1 was generated as described above and incubated with 15 μ g MBP-US28 or MBP. For competition assays, GST-cGASP-1 was bacterially expressed and purified on glutathione-agarose, and the purified protein was eluted with 10 mM glutathione. Increasing concentrations of eluted GST-cGASP-1 were added to the MBP-US28 or MBP (control) slurry just before addition of ^{35} S-methionine-labelled HA-GASP-1. Probes were further processed and analysed as described above.

Cell culture

HEK293 cells were maintained at 5% CO $_2$ at 37°C in DMEM supplemented with 10% FBS. The construction of HEK293 cells stably expressing

GFP-cGASP-1 was described in (14), cells were maintained as described above plus 0.2 mg/mL G418.

Co-immunoprecipitation

HEK293 cells were transfected with 20 µg/10 cm dish of pcDNA3.1, Flag-US28, Flag-DOP or Flag-β₂AR using Lipofectamine 2000. Forty-eight hours post-transfection, co-immunoprecipitation of tagged receptors and endogenous GASP-1 and GASP-2 was performed essentially as described in (14,16). In brief, cells were washed twice with PBS and lysed in 0.1% Triton X-100, 150 mM NaCl, 25 mM KCl, 10 mM Tris-HCl pH 7.4, 1 mM CaCl₂ supplemented with complete protease inhibitors. Cleared lysates were incubated with 20 µL of M2 monoclonal antibody affinity matrix overnight at 4°C. After extensive washing, precipitates were resolved on a 4–20% Tris-Glycine gradient gel and then transferred to Polyvinylidene difluoride (PVDF) membrane. The blots were cut below the 70-kDa marker band and separately immunoblotted for either receptor or β-actin (lower panels) or GASP-1 and GASP-2 (upper panels). GASP blots were incubated with the rabbit anti-GASP serum recognizing both GASP-isoforms (1:1000) and visualized with a HRP-conjugated anti-rabbit antibody (1:4000). Receptor blots were incubated for 2 h with M2 monoclonal antibody (1:500) followed by 2 h incubation with HRP-conjugated anti-mouse antibody (1:4000). β-actin blots were incubated for 2 h with β-actin monoclonal antibody (1:4000) followed by 2 h incubation with HRP-conjugated anti-mouse antibody (1:4000). Blots were visualized with ECL western blotting substrate.

Lentivirus production and shRNA knock-down of endogenous GASP-1

The Lentiviruses with scrambled or GASP-1 shRNA were produced in HEK293FT cells. 2×10^7 HEK293FT cells were grown in DMEM supplemented with 10% FBS and transfected with 7 µg of scrambled shRNA LentiLox or GASP-1 shRNA LentiLox vectors and 20 µg of Virapower packaging mix. Forty-eight hours post-transfection, viruses were harvested and purified by ultracentrifugation (40000 g, 90 min, 4°C). The virus pellets were resuspended in 25 µL salt-free PBS, frozen in liquid nitrogen and stored at –80°C. Virus titre was estimated by ELISA using the QuickTiter™ Lentivirus Quantification Kit. For the knock-down of endogenous GASP-1, HEK293 cells were infected with 10 MOI (multiplicity of infection) of Lenti-shGASP-1 (shGASP-1) or Lenti-shScrambled (shScr) virus and incubated at 37°C and 5% CO₂ for 48–72 h. Successful Lenti-shRNA infection was monitored by immunofluorescence of EGFP, which is under the control of a CMV promoter following the LoxP site of the LentiLox 3.7 vector. GASP-1 knock-down was verified by western blot analysis of GASP-1 protein levels.

EGFR western blot

HEK293 cells infected with shScr or shGASP-1 virus were transiently transfected with Flag-US28 and treated with 5 µM EGF in OptiMeM or OptiMeM alone for 3 h. Cells were washed with PBS and lysates were separated by SDS/PAGE. Finally, samples were immunoblotted with goat antibodies to EGFR (1:500), followed by HRP-conjugated secondary anti-goat (1:4000) antibody and development with ECL substrate.

Immunocytochemistry

Antibody feeding experiments

Wild-type HEK293 cells or HEK293 cells stably expressing GFP-cGASP-1 were grown on gelatine-coated cover slips and transfected with Flag-US28 (4 µg/coverslip). Twenty-four hours post-transfection, antibody feeding experiments were conducted essentially as described before (16). In brief, living cells were incubated with 1:500 anti-Flag M1 antibody for 45 min, fixed with 3.7% formaldehyde in PBS and permeabilized in blotto (50 mM Tris-HCl, pH 7.5, 1 mM CaCl₂, 0.1% Triton X-100 and 3% milk). After colocalization staining (see below), all cells were mounted with Vecta shield mounting medium.

Colocalization experiments with LAMP1 and LAMP2

Following the feeding experiments described above, cells were incubated with a monoclonal antibody directed against LAMP 1/2 (IgG1 1:500) for 60 min. Subsequently, the cells were stained with the subtype-selective antibodies AlexaFluor 647-conjugated IgG2b against the Flag-tag (1:1000, 20 min) and AlexaFluor 594-conjugated IgG1a against LAMP 1/2 (1:500, 20 min).

Colocalization experiments with EEA.1

Following the feeding experiments described above, cells were incubated with a polyclonal antibody directed against EEA.1 (IgG, 1:500) for 60 min. Cells were stained with the subtype-selective antibodies AlexaFluor 647-conjugated IgG2b against the Flag-tag (1:1000) and the AlexaFluor 594-conjugated goat-anti-rabbit IgG (1:1000) for 20 min.

Colocalization experiments with transferrin

For colocalization studies with transferrin, cells were serum-starved for 60 min prior to incubation with M1 antibody directed against the Flag-epitope (1:1000, 45 min). Then cells were labelled with AlexaFluor 594-nm-conjugated transferrin (1:500) for the last 30 min. After fixation and permeabilization, the cells were stained with the subtype-selective antibody AlexaFluor 647 nm conjugated IgG2b against the Flag-tag (1:1000, 20 min).

Antibody feeding experiments in shScr and shGASP-1 infected cells

Cells infected with either shScr or shGASP-1 virus for 48 h were transiently transfected with 4 µg/coverslip Flag-US28. Twenty-four hours post-transfection, antibody feeding and colocalization experiments were performed as described above.

Microscopy

Cells were analysed with a Zeiss LSM 510 META confocal microscope. To determine the exact localization of the receptor, Z-stacks of representative cells were taken and the pictures of the middle of the cells are shown.

Biotin internalization and biotin protection/degradation assays

HEK293 cells, HEK293 cells stably expressing GFP-cGASP-1 or HEK293 cells infected with either shScr or shGASP-1 virus for 48 h were seeded on gelatine-coated 10-cm dishes and transiently transfected with 20 µg of Flag-US28. Biotin assays were performed 24 h post-transfection essentially as previously described (14,16). In brief, cells were washed in PBS, treated with 3 µg/mL disulfide cleavable biotin for 30 min at 4°C and washed in TBS (25 mM Tris base, 135 mM NaCl, 2.5 mM KCl, 1 mM CaCl₂ * 2H₂O, pH 7.4). Cells labelled 100% biotinylated were left on ice in cold medium. For *internalization assays* cells were placed in prewarmed medium for 0–45 min to allow constitutive receptor endocytosis. The cells were then washed in PBS, and the remaining cell surface-biotinylated receptors were stripped in stripbuffer (50 mM glutathione, 75 mM NaCl, 10% fetal bovine serum) at 4°C for 30 min (except 100% plates) and quenched with PBS containing 50 mM iodoacetamide and 1% bovine serum albumin. The cells were then washed in PBS and cell lysates were prepared with 0.1% Triton X-100, 150 mM NaCl, 25 mM KCl, 10 mM Tris-HCl, 1 mM CaCl₂ and 5 mM iodoacetamide, pH 7.4, with protease inhibitors. For *protection/degradation assays* biotinylated cells were placed in prewarmed medium for 45 min to allow constitutive receptor endocytosis. The cells were then washed in PBS, and the remaining cell surface-biotinylated receptors were stripped in stripbuffer at 4°C for 30 min (except 100% plates) and quenched with PBS containing 50 mM iodoacetamide and 1% bovine serum albumin. The cells were then washed in PBS and placed in prewarmed medium for the indicated time-points to allow receptor degradation, whereby the 100% plates remained at 4°C. Finally, the plates were washed in PBS and cell lysates were prepared. Samples of cleared lysates were taken for control

western blots (anti-GASP 1:1000; anti β -actin 1:4000, anti-Flag M2 1:500), and the remaining lysates were immunoprecipitated with anti-Flag affinity matrix overnight at 4°C. Precipitates were resolved by SDS/PAGE, and visualized by incubating with Vectastain ABC immunoperoxidase reagent. Blots were scanned and quantified using IMAGEJ Software.

Recycling experiments

Wild-type HEK293 cells, cells stably expressing GFP-cGASP-1 or cells infected with either shScr or shGASP-1 were grown on gelatine-coated coverslips. Subsequently, the cells were transiently transfected with Flag-US28 (4 μ g/coverslip). Twenty-four hours post-transfection, recycling experiments were performed essentially as described in (16). In brief, living cells were fed with the Ca^{2+} – sensitive M1 antibody directed against the Flag-epitope (1:500, 45 min). Then residual surface receptors were stripped of antibody by washing twice with cold 0.04 mM PBS–EDTA solution. To determine the constitutive recycling of the receptor, warm medium was readmitted to the cells. After 15 or 30 min the medium was aspirated and the cells were fixed with 3.7% formaldehyde in PBS. Cells were stained with the subtype-selective antibody AlexaFluor 594-conjugated IgG2b against the Flag-tag (1:1000, 20 min) under non-permeabilizing (minus Triton-X) conditions. Finally, the cells were mounted with Vecta shield mounting medium.

Reporter gene assays

Transcription factor luciferase assays were performed essentially as described in (4). In brief, HEK293 cells (40 000 cells/well) were seeded on gelatine-coated 96-well plates. For GASP-1 knock-down experiments, HEK293 cells were infected with either shScr or shGASP-1 virus 48 h before transfection. Cells were transiently transfected with 50 ng/well Flag-US28 and the cis-reporter plasmid for CREB (200 ng/well) or NF- κ B (50 ng/well). Depending on the experiment (indicated at the figure legends) US28 was co-transfected with 100 ng/well pcDNA3.1, HA-GASP-1, HA-cGASP-1 or HA-rGASP-1. The amount of DNA was kept constant for all samples. In parallel to each assay, an additional plate was prepared for ELISA (see below) to control for receptor expression levels. The luciferase reporter gene assay was conducted 24 h post-transfection according to the manufacturer's guidelines. In brief, cells were washed twice with PBS and the cell number was determined by optical density in a FlexStation II Device (Molecular Probes, Endpoint Measurement). Then 100 μ L/well Steadylight luciferase assay reagent were added to 100 μ L/well PBS. Following a 10-min incubation period, luminescence was measured using a TopCounter Device (TopCount NXT, PerkinElmer).

Enzyme-linked immunosorbent assay

On the same day as the respective signalling assay (see above), cells were fixed with 3.7% formaldehyde in PBS and permeabilized in blotto (50 mM Tris–HCl, pH 7.5, 1 mM CaCl_2 , 0.1% Triton X-100 and 3% milk) for 1 h. Next, the cells were incubated with 1:500 anti-Flag M1 antibody overnight at 4°C. On the next day, the cells were washed with TBS (25 mM Tris base, 135 mM NaCl, 2.5 mM KCl, 1 mM $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$, pH 7.4) and incubated with HRP-conjugated anti-mouse antibody (1:2500) in blotto (50 mM Tris–HCl, pH 7.5, 1 mM CaCl_2 and 1.5% milk) for 2 h at room temperature. After three washes with TBS, the cell number was determined by optical density in a FlexStation II Device. Then 100 μ L/well TMB (3, 3', 5, 5'-Tetramethylbenzidine) substrate was added, and the colouring reaction was stopped by the addition of 50 μ L/well 0.5 M sulfuric acid after 2 min at room temperature. Receptor levels were measured at the optical density of 450 nm in a BioRad xMark Microplate Spectrophotometer.

Inositol phosphate accumulation assay

HEK293 cells were seeded on gelatine-coated 96-well plates (40 000 cells/well). For GASP-1 knock-down experiments, HEK293 cells were infected with either shScr or shGASP-1 virus 48 h before transfection. Cells were transfected with 75 ng/well Flag-US28 and depending on the experimental setting indicated in the figure legends, co-transfected with

100 ng/well of either pcDNA3.1, HA-GASP-1, HA-cGASP-1 or HA-rGASP-1 (rescue experiments). Controls were transfected with 175 ng/well pcDNA3.1 in the absence of US28. Twenty-four hours post-transfection, the cells were loaded with 1 μ Ci/well [^3H]myo-inositol in 100 μ L/well Optimem and incubated overnight at 37°C and 5% CO_2 . The next day, the labelling medium was aspirated, 100 μ L/well Hanks' balanced salt solution (HBSS) buffer (including CaCl_2 and MgCl_2) containing 10 mM LiCl was added and the cells were incubated for 45 min at 37°C and 5% CO_2 . The reaction was terminated by aspiration and the cell number was determined by optical density in a FlexStation II Device. Subsequently, cells were lysed with 50 μ L/well 10 mM formic acid for 90 min on ice. The resulting cell extract of about 40 μ L was transferred to 160 μ L of YSi-SPA beads (12.5 mg/mL) and shaken for 90 min at 4°C. The plates were then stored at 4°C and the accumulation of IP was counted in a TopCount microplate scintillation counter (TopCount NXT, PerkinElmer) 24 h later.

Statistical analysis

Statistical analyses were performed using ANOVA analysis of variance for comparisons between multiple groups, followed by a Bonferroni's post hoc analysis and *t*-tests using GRAPHPAD PRISM software.

Acknowledgments

We would like to thank Tatjana Kueznik for her excellent support using the ZEISS confocal microscope at the ZMF at the Medical University of Graz, and Rufina Schuligoi and Gunther Marsche for critically reading this manuscript. This work was supported by grants from the Austrian Science Fund (P18723 to MW, P15591 and P18630 to HS), the Jubiläumsfonds of the Austrian National Bank and the Lanyar Stiftung Graz (all to MW), the 'Molecular Medicine Ph.D. program' from the Medical University of Graz, Austria (to PMT and EM) and the BaCaVisiting Scientists program (to PMT). M. J. S. and H. F. V. were supported by the Dutch Organization for Scientific Research NW (Vidi grants 700.54.425 to MJS and 700.55.403 to HFV). D. T., L. M. and J. L. W. were supported by funds provided by the State of California for medical research through the University of California San Francisco (to J. L. W.), and by the National Institute of Mental Health Grant R01 MH68442 (to J. L. W.). L. M. was supported by a grant from the Lundbeck Foundation, Denmark.

Supporting Information

Additional Supporting Information may be found in the online version of this article:

Figure S1: Overexpression of GFP-cGASP-1 in HEK293 cells. Serial dilutions of lysates from a HEK293 cell line stably expressing GFP-cGASP-1 (14) on immunoblot show that cGASP-1 is overexpressed by at least 100-fold compared to endogenous GASP-1.

Figure S2: Quantification of western blots. A) Quantification of the GASP-1 levels in cells overexpressing HA-GASP-1. HEK293 cells transfected with Flag-US28 in combination with either pcDNA3.1 (white bar) or HA-GASP-1 (black bar) were analysed by western blot. The GASP-1 levels corresponding to western blots in Figure 6A, upper panel, were compared and quantified with IMAGEJ software. Finally, the GASP-1 level in the control (white bar) was set to 100% and the overexpression of GASP-1 was normalized accordingly. B) Quantification of the restored GASP-1 levels in shGASP-1 infected cells. HEK293 cells infected with shScr virus were transfected with Flag-US28 and pcDNA3.1 (white bar). shGASP-1 infected cells were transfected with Flag-US28 and either pcDNA3.1 (grey bar) or HA-rGASP-1 (black bar). The GASP-1 levels corresponding to western blots in Figure 6E were analysed by western blot and quantified with IMAGEJ software. Finally, the GASP-1 level in shScr infected cells (white bar) was set to 100% and the other values were normalized accordingly.

Figure S3: US28 interacts with rGASP-1 *in vitro*. *In vitro* translation assay of GASP-1 binding. ^{35}S -methionine labelled recombinant rGASP-1 binds to GST-fusion proteins containing the CT of US28 and the DOP, but not the GST-protein (C), beads (B) alone or the MOP. The

corresponding input protein levels are shown below the radiograph. Blots are representative of at least three independent experiments.

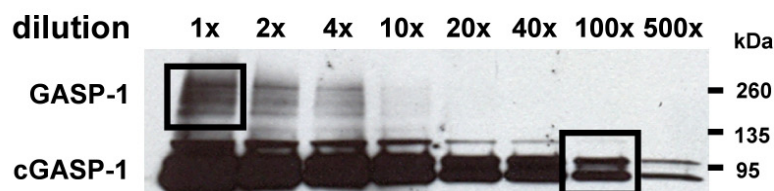
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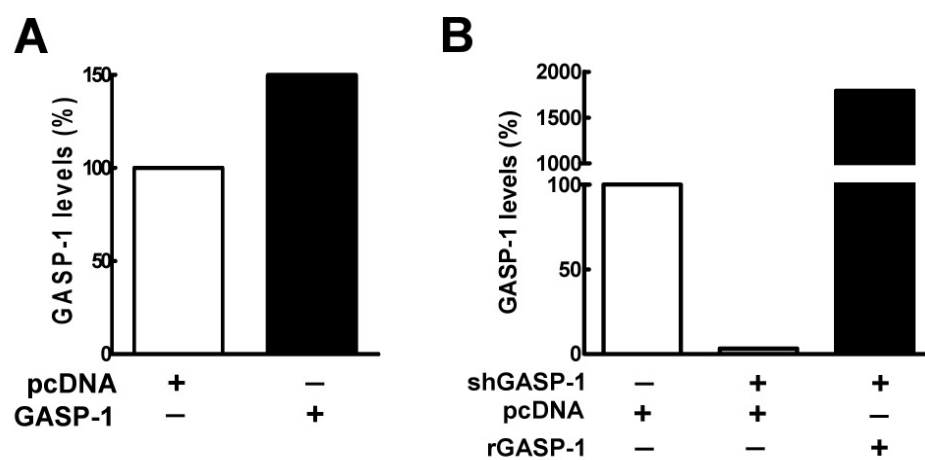
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Supplemental Figures

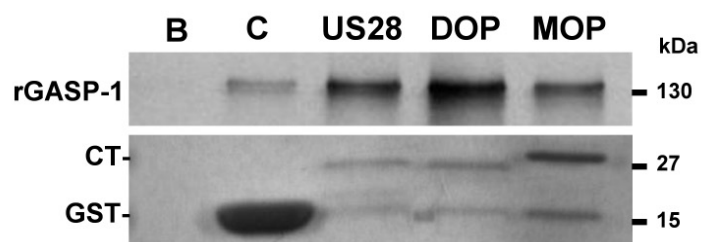
Supplemental Figure 1



Supplemental Figure 2



Supplemental Figure 3



Appendix

Appendix II

G protein Coupled Receptor Associated Sorting Protein 1 (GASP-1) regulates the post-endocytic sorting of 7TM/GPCRs

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Keywords: GASP-1, GASP-2, post-endocytic receptor sorting, 7TM/GPCR, trafficking, degradation, receptor downregulation, adaptor protein

Summary

The largest superfamily of membrane proteins that translate extracellular signals into intracellular messages are the 7 transmembrane spanning (7TM) G protein coupled receptors (GPCRs). One of the ways in which their activity is controlled is by the process of desensitization and endocytosis, whereby agonist-activated receptors are rapidly and often reversibly silenced through removal from the cell surface. Indeed, following endocytosis, individual receptors can be sorted differentially between recycling endosomes and lysosomes, which controls the reversibility of the silencing. Thus, endocytosis can either serve as a mechanism for receptor resensitization by delivering receptors back to the plasma membrane or, endocytosis can facilitate receptor downregulation by serving as the first step towards targeting the receptors to lysosomes for degradation. The sorting of receptors to the lysosomal pathway can be facilitated by the interaction with an array of accessory proteins. One of these proteins is the GPCR-associated sorting protein 1 (GASP-1), which specifically targets several 7TM/GPCRs to the lysosomal pathway after endocytosis. Furthermore, GASP-1 was recently found to directly affect the signaling capacity of a 7TM/GPCR. Importantly, the *in vivo* relevance of GASP-1-dependent receptor sorting has also begun to be verified in animal models. Here, we summarize the recent advances in elucidating GASP-1 dependent receptor sorting functions and its potential implications *in vivo*.

Introduction

Endocytosis of 7TM/GPCRs

7TM/GPCRs comprise the largest superfamily of membrane proteins that translate extracellular signals into intracellular messages and can be activated by a wide range of compounds, including odorants, photons, hormones and neurotransmitters^{1,2}. Due, likely, to their involvement in a variety of physiological and pathophysiological processes, the activity of 7TM/GPCRs is extensively regulated. One of the ways in which receptor-mediated signaling is modulated is by the process of endocytosis, whereby agonist-activated cell surface receptors are rapidly removed from the plasma membrane (Figure 1). Typically, receptor endocytosis is facilitated by phosphorylation of the receptor by G protein coupled receptor kinases (GRK) and the subsequent interaction with non-visual (beta) arrestins both of which “desensitize” signal transduction between receptor and G protein. In general, receptors can be endocytosed via clathrin coated pits, caveolae or uncoated pits³. However, most cell surface receptors are internalized by clathrin-dependent mechanisms, where the invagination and pit assembly are supported by clathrin and adaptor proteins, such as the adaptor protein 2 (AP-2)^{4,5}.

Post-endocytic sorting of 7TM/GPCRs

Following endocytosis, individual receptors can be sorted between recycling or degradative pathways (Figure 2)^{6,7}. Thus, the postendocytic fate of a 7TM/GPCR determines the role of endocytosis in signal transduction. For a recycling receptor, endocytosis can serve as a mechanism for receptor resensitization by delivering internalized receptors to endosomes, from where they can be recycled to the plasma membrane in a fully active state. Alternatively, for receptors that are degraded, rapid endocytosis can serve as a first step toward receptor downregulation by delivering the receptors to endosomes, from which they are further

targeted to the lysosomes. The cascade of events for each receptor, globally referred to as “receptor trafficking”, is thought to be initiated already at the plasma membrane, where receptors are ‘tagged’ – e.g. phosphorylated or ubiquitinated - to determine their endocytic and post-endocytic fates.

Receptor phosphorylation can occur in response to activation by an agonist and is necessary for receptor endocytosis (for reviews see ⁸⁻¹⁰). However, it has been suggested that receptor phosphorylation not only influences endocytosis, but also influences the post-endocytic fate of a receptor by regulating interactions with sorting proteins or by inducing other posttranslational modifications ^{11, 12}. Indeed, ubiquitination of 7TM/GPCRs, which involves the covalent attachment of ubiquitin to lysine residues of the cytoplasmic tail, has - in some cases - been shown to act as a ‘sorting signal’ that promotes endocytosis and lysosomal targeting ¹³⁻¹⁵. In addition, several proteins have been identified that specifically target 7TM/GPCRs to either recycling ^{11, 16-19} or degradative pathways ^{20, 21}.

Recycling of 7TM/GPCRs - Traditionally, recycling of receptors was thought to occur by default. This was based on the observations that (i) the membrane itself is continuously recycled and (ii) disruption of lysosomal sorting often results in enhanced recycling of 7TM/GPCRs ²²⁻²⁴. Intriguingly, recent findings support a model of regulated recycling of some 7TM/GPCRs that is mediated through specific recycling sequences contained in their cytoplasmic tails ^{16, 25-27}. One example for sequence-directed recycling involves the β 2 adrenergic receptor (β 2-AR). β 2-AR contains a distal sequence (DSLL) that was shown to be important for its recycling to the cell surface after endocytosis via interactions with PDZ (postsynaptic density 95/disc large/zonula occludens-1) domain-containing proteins of the NHERF (Na⁺/H⁺ exchanger regulatory factor)/EBP50 (ezrinradixin-moesin(ERM)-binding phosphoprotein-50) –family ^{16 28}. Deletion of this domain, or phosphorylation of the serine in

this domain disrupts the interaction of the β 2-AR with NHERF/EBP50, prevents receptor recycling,¹¹ and promotes receptor degradation²⁹

Lysosomal sorting of 7TM/GPCRs - The sorting of receptors to the lysosomal pathway does not occur by default, but instead appears to be facilitated by interaction with one or many of an array of sorting proteins. For ubiquitinated proteins, the highly conserved ESCRT (endosomal sorting complex required for transport) machinery directs the transport of ubiquitinated cargo for degradation via a compartment termed the multivesicular body (MVB) (reviewed in²⁵). The sorting process is initiated by a protein complex (often referred to as ESCRT-0) consisting of the hepatocyte growth factor regulated tyrosine kinase substrate (Hrs) and the signal transduction adapter molecule (STAM), which recognize ubiquitinated proteins with their ubiquitin-interaction motifs (UIMs). By interaction of Hrs with the ubiquitin E2 variant (UEV) domain of the tumor suppressor gene-101 (TSG101), the binding of ESCRT-I complex is initiated. Subsequently, ESCRT-II and -III complexes are recruited to the endosomal membrane, which deliver the cargo to the lysosomes for degradation³⁰⁻³². However, the ESCRT machinery appears to be required for degradation of membrane proteins, including some GPCRs that are not ubiquitinated as well. For example, the delta opioid receptor (DOR) is ubiquitinated, and targeted for degradation following its endocytosis. However, ubiquitination of DOR is not required for either ligand-induced endocytosis or post endocytic degradation of DOR²⁶. Together these data suggest that the ESCRT machinery in some way can recognize and select non-ubiquitinated receptors and transport them for degradation. However, it is unclear in this case what “tags” these non-ubiquitinated proteins for degradation.

One possibility is the existence of accessory sorting proteins that serve as linkers between membrane proteins and the ESCRT machinery. Sorting nexin-1 (SNX-1) is one non-ESCRT protein that is involved in targeting 7TM/GPCRs to the lysosomal pathway. Initially, SNX-1

was shown to bind to Hrs, and promote the targeting of the non-GPCR epidermal growth factor (EGF) receptor to the degradative pathway ³³. Subsequently, SNX-1 was shown to effect the down-regulation of the protease-activated receptor-1 (PAR1) ³⁴ GPCR, and is reported to bind, at least in vitro, to the c-termini of several 7TM/GPCRs, such as the virally encoded chemokine receptor US28 ³⁵ and the delta opioid receptor (DOR) ³⁵. However, not all 7TM/GPCRs that bind SNX-1 are targeted for degradation; for instance, the muscarinic receptors M1 and M4 both interact with SNX-1 ³⁵, but are still known to be efficiently recycled after agonist stimulation ^{36, 37}. More recently, dysbindin - a cytoplasmic protein encoded by DTNBP1 ³⁸ - was implicated in the post-endocytic sorting of the dopamine D2 and the DOR to the degradative pathway and was shown to co-immunoprecipitate with both Hrs proteins and another sorting protein GASP-1 ³⁹

The G protein coupled receptor associated sorting proteins - GASPs

The GPCR-associated sorting protein -1 (GASP-1) was discovered by Whistler *et al* in 2002 in a yeast two-hybrid screen with the carboxyl terminus of the DOR ⁷ and has since been reported to specifically target several diverse 7TM/GPCRs to the lysosomal/degradative pathway ^{7, 29, 40-45} (see also Table 1). Although 9 additional family members of GASP-1 (GASP-2 to GASP-10) have been suggested ⁴⁶, there is no functional evidence to date that any of these other GASP-family members are involved in the sorting of 7TM/GPCRs.

GASP-1 is a large acidic protein of 1394 amino acids that is highly expressed in brain and in a few additional tissues ⁴⁷, encoded by a gene on the X chromosome in both humans and mice ⁴⁶. The 497 amino acids COOH terminal of GASP-1 (cGASP-1) can disrupt the interaction of GASP-1 proteins with 7TM/GPCRs ^{7, 40, 42}, and when overexpressed cGASP-1 can function as a 'dominant negative competitor' for GASP. For instance, the DOR-GASP-1 interaction is disrupted by overexpression of dominant negative cGASP-1 ⁷, which inhibits the trafficking of DORs to lysosomes and promotes its recycling back to the cell surface ⁷.

GASP-2, with the closest homology to GASP-1, has been shown to directly bind the D2 dopamine receptor (D2R) and the β -2 adrenergic receptor (β 2AR)²⁹, the viral chemokine receptor US28⁴⁴ as well as huntingtin (htt), a protein playing a role in the neurodegenerative disorder Huntington's disease (HD)⁴⁸ (see also Table 2). However, to date no functional consequence was reported for protein interactions with GASP-2. Nevertheless, cGASP-1 shares 62% sequence identity with the c terminal region of GASP-2 (Figure 3). Hence it is possible that if GASP-1 and GASP-2 have similar functions, cGASP-1 will act as a dominant negative for both.

GASP-1 dependent sorting of 7TM/GPCRs

Intriguingly, GASP-1 seems to show selectivity for individual members of 7TM/GPCR sub-families. For example, the DOR, B1R (bradykinin1 receptor) and the D2 receptors are targeted for degradation by GASP-1, while their respective family members MOR (mu opioid receptor), B2R (bradykinin 2 receptor) and D1R (dopamine 1 receptor) do not interact with GASP-1 and are recycled to the plasma membrane rather than targeted for degradation^{7, 40, 49}.

A study by Thompson *et al.*²⁹ showed that the relative affinity of individual receptors for GASP-1 and other sorting proteins can regulate their post-endocytic sorting capacities. For instance, the c-terminus of wild type β 2 adrenergic receptor (β 2AR) binds to GASP-1 in GST-pull down assays. However, no direct protein-protein interaction of β 2AR with GASP-1 was detected in a HEK293 cell model, resulting in an efficient recycling of the β 2AR. Hence, it seems that recycling proteins - such as NHERF/EBP50 and N-ethylmaleimide-sensitive factor (NSF) - “win” the competition with GASP-1 for binding to this receptor. Indeed, when the PDZ domain of the β 2AR is disrupted and thus β 2AR cannot bind the recycling proteins, this mutant β 2AR associates with GASP-1 and is degraded²⁹. Another example is the MOR, where structural sequence determinants within the c-tail of the receptor seem to prevent the binding to GASP-1. Here, the truncation of the last 17 amino acids of the receptor²⁶ was

shown to enhance its interaction with GASP-1 and lead to lysosomal targeting of the mutant receptor ²⁹. In contrast, the deletion of a small motif within the cytoplasmic tail of D1R blocked the recycling of the receptor, but had no effect on the affinity of D1R for GASP-1. As a result, this mutant D1R was neither recycled, nor degraded. Hence, preventing the interaction of 7TM/GPCRs with recycling proteins alone does not promote receptor degradation, unless the receptor shows affinity for degrading sorting proteins such as GASP-1 ²⁹.

GASP-1 – a regulator of signal transduction?

The sorting of individual receptors between recycling and degradation is a tightly controlled process that is of fundamental importance for the regulation of 7TM/GPCR signaling. Until recently, the only function reported for GASP-1 was the targeting of a variety of 7TM/GPCRs to the degradative/lysosomal pathway.

Intriguingly, we recently demonstrated that GASP-1 is directly involved in modulating the signaling capacity of a 7TM/GPCR. GASP-1 targets the virally encoded 7TM/GPCR US28 to late endosomes/lysosomes ⁴⁴, a place that has been suggested to be crucial for the envelopment of viruses. In addition, we observed that the overexpression of GASP-1 in HEK293 cells significantly enhanced the Gαq/PLC/inositol phosphate (IP) turnover by US28, whereas shRNA-silencing of GASP-1 dramatically reduced IP formation in these cells ⁴⁴. Moreover, the activation of the transcription factors NF-κB (nuclear factor kappa B) and CREB (cyclic AMP responsive element binding protein) via US28 were also either enhanced or prevented by overexpression or shRNA silencing of GASP-1, respectively ⁴⁴. It is not clear yet whether the enhanced/decreased signaling capacity of US28 in the presence/absence of GASP-1 is caused by (i) a more active conformational state of the receptor upon GASP-1 binding or (ii) its re-localization into a particular endosomal compartment that is important for signal transduction ^{50, 51}. However, the capacity of GASP-1 to modulate not only the post-

endocytic sorting but also to directly influence the signaling activity of 7TM/GPCRs might point toward an important role of GASP-1 in connecting both processes.

In vivo relevance of GASP-1 dependent sorting

The first evidence suggesting that GASP could have an *in vivo* function were provided by experiments examining D2R function in the rat ventral tegmental area (VTA). VTA slices pre-treated with D2R agonist failed to recover from receptor desensitization consistent with the ability of the D2R to degrade after endocytosis. However, disrupting the D2R/GASP-1 interaction with an inhibitory antibody allowed recovery of functional D2R responses ⁴⁰. Shortly thereafter, observations that the CB1 cannabinoid receptor was sorted to lysosomes by GASP *in vitro* ⁴² led to experiments assessing the *in vivo* relevance of this finding.

Tappe Theodor *et al.* ⁴³, linked the development of analgesic tolerance to cannabinoids to the post-endocytic trafficking of the cannabinoid receptor CB1 ⁴³. In this study, mice were treated with an adenovirus expressing dominant negative cGASP-1 and were treated with chronic cannabinoid drug. Mice receiving control virus showed tolerance to the analgesic effects, while mice receiving dominant negative cGASP-1 showed significantly reduced tolerance ⁴³.

Based on the strength of these initial studies, mice with a disruption of GASP-1 were generated. Using these mice, Martini *et al.*, demonstrated that the development of tolerance to many of the physiological effects of cannabinoids were significantly reduced in GASP-1 knock out mice ⁵². Importantly, CB1 receptor levels in GASP-1 knock out mice were not altered in the spinal cord and cerebellum after repeated cannabinoid administration, whereas receptor levels were significantly decreased in the wild-type littermates.

Also using these mice Thompson *et al.* have shown that repeated treatment of wild type but not GASP-1 knock-out mice with cocaine leads to a downregulation of D2Rs in mouse striatum ⁴⁵. In addition, disruption of GASP-1 has been shown to alter behavioural responses

to cocaine. For example GASP-1 knock out mice show reduced locomotor sensitization to cocaine and reduced acquisition of cocaine self administration^{41, 45}.

Taken together, these early studies are, thus, beginning to reveal the *in vivo* relevance for GASP-1. The *in vivo* relevance of GASP-1 dependent sorting of 7TM/GPCRs including the development of tolerance or the sensitization/responsiveness to drugs might help guide new therapeutic approaches. This is especially important since the post-endocytic fate of a 7TM/GPCR target in response to drug has rarely been evaluated during preclinical development. Thus, clearly, more studies are needed to unravel the complex processes and the involved proteins and mechanisms controlling the sorting of 7TM/GPCRs.

Conclusion

Accumulating evidence suggests an important role for GASP-1 in the sorting of many 7TM/GPCRs to the degradative pathway. Several studies have now shown that lysosomal targeting involves a direct interaction of GASP-1 with the cytoplasmic tails of the receptors and that these c-termini contain specific 'target' sequences that may facilitate the interaction with GASP-1. Recently, GASP-1 was also found to be a crucial determinant in directly regulating the signaling capacity of a 7TM/GPCR, e.g. the viral receptor US28. Whether GASP-1 directly favours a more active conformation of the receptor or rather targets it to a 'signalosome' yet still remains unclear.

Importantly, the *in vitro* findings were also translated into animal models, verifying that GASP-1 dependent sorting of 7TM/GPCRs has functional consequences *in vivo*. The first *in vivo* studies identified the involvement of GASP-1 in the development of tolerance and sensitization to drugs of abuse and pain, including cocaine and cannabinoids. But there are a plethora of GPCR drugs in which the trafficking fate of the receptor remains unstudied. Hence, the identification of GASP-1 as a key player in receptor downregulation might be a

step towards a more accurate understanding of molecular mechanisms underlying 7TM/GPCR drug efficacy, side effects and tolerance.

Acknowledgements

This work was supported by grants from the Austrian Science Fund (P18723 to MW), the Jubiläumsfonds of the Austrian National Bank and the Lanyar Stiftung Graz (all to MW), the ‘Molecular Medicine Ph.D. program’ from the Medical University of Graz, Austria (to EM, JK, PMT), the BaCaVisiting Scientists program (to PT) and by funds provided by the State of California for medical research through the University of California San Francisco and the National Institute of Mental Health Grant R01 MH68442 (both to JLW).

Figure Legends

Figure 1: *β-arrestin/clathrin dependent endocytosis of 7TM/GPCRs.* Agonist binding to 7TM/GPCRs leads to the activation of heterotrimeric G proteins and initiation of a signal transduction cascade (1). Desensitization of receptors is facilitated by the phosphorylation through GRKs (2) and subsequent recruitment of β-arrestins (3). Endocytosis of receptors is finally mediated by clathrin and AP-2 (4). The vesicle abscission is facilitated by dynamin and leads to the formation of cargo containing endocytic vesicles (5).

Figure 2: *Post-endocytic sorting of 7TM/GPCRs.* Following internalization, receptors can be differentially sorted between recycling and degradative fates. Recycling of receptors can be mediated by interaction with PDZ domain-containing proteins such as NHERF/EBP50. Interaction with the ESCRT complexes, SNX-1, dysbindin or GASP-1 leads to targeting of 7TM/GPCRs to the lysosomal pathway for degradation.

Figure 3: Schematic representation of the sequence alignment of GASP-1 and GASP-2 proteins. GASP-1 and GASP-2 show a total amino acid identity of 35 %. The COOH-terminal parts of GASP-1 (= cGASP-1) and GASP-2 display a 62 % amino acid similarity.

Table 1: GASP-1 interaction partners and their associated functions.

interaction partners	interaction		function		ref.
	GST	CoIP	<i>in vitro</i>	<i>in vivo</i>	
Angiotensin II receptor type 1 (AT1)	Yes	n.d.			35
Bradykinin 1 receptor (B1R)	Yes	Yes			49
β 1 adrenergic receptor (β 1-AR)	Yes	n.d.			46
β 2 adrenergic receptor (β 2-AR)	Yes	No	competition with recycling proteins		29
B-alaR (mutant β 2-AR)	Yes	Yes	targeted degradation		29
Calcitonin receptor (CALCR)	Yes	n.d.			46
Cannabinoid 1receptor (CB1R)	Yes	Yes	targeted degradation	agonist induced receptor downregulation in the spinal cord in mice and development of antinociceptive tolerance	42, 43, 52
Dopamine 2 receptor (D2R)	Yes	Yes	targeted degradation; reduced resensitization in rat neurons	increased receptor downregulation after chronic cocaine administration in GASP-1 KO mice	7, 29, 40, 41, 45
Delta opioid receptor (DOR)	Yes	Yes	targeted degradation		7
Dysbindin	n.d.	Yes			39
Histamine 2 receptor (H2R)*	Yes	n.d.			46
5-hydroxytryptamine 7 receptor (5-HT7)	Yes	n.d.			46
Kappa-opioid receptor (KOR)	Yes	n.d.			46
Mu opioid receptor truncated (MOR-TR)	Yes	Yes	targeted degradation		29
Muscarinic receptor 1 (M1)	Yes	n.d.		increased receptor downregulation after chronic cocaine administration in GASP-1 KO mice	41, 46
Muscarinic receptor 2 (M2)*	Yes	n.d.		increased receptor downregulation after chronic cocaine administration in GASP-1 KO mice	41, 46

Opioid receptor like 1 (ORL1)	Yes	n.d.			46
ORF74	Yes	n.d.			35
Tachikinin NK1 receptor (NK1)	Yes	n.d.			35
Thromboxane A2 α receptor (TXA2 α)	Yes	n.d.			46
US28	Yes	Yes	targeted degradation; enhanced signaling		35, 44

* low affinity binding; GST - *in vitro* binding to GST-fusion proteins; Co-IP: Co-immunoprecipitation; n.d. - not determined.

Table 2: GASP-2 interaction partners and their associated functions.

interaction partners	interaction		function		ref.
	GST	CoIP	<i>in vitro</i>	<i>in vivo</i>	
β 1 adrenergic receptor (β 1-AR)*	Yes	n.d.			46
β 2 adrenergic receptor (β 2-AR)	Yes	Yes			29
B-alaR (mutant β 2-AR)	Yes	Yes			29
Calcitonin receptor (CALCR)	Yes	n.d.			46
Dopamine receptor 2 (D2R)	Yes	n.d.			29
Disease protein huntingtin (htt)	n.d.	Yes	colocalization in SH-SY5Y cells		48
Histamine H2 receptor (H2)*	Yes	n.d.			46
5-hydroxytryptamine 7 receptor (5-HT7)*	Yes	n.d.			46
Mu opioid receptor truncated (MOR-TR)	Yes	Yes			29
Muscarinic receptor 1 (M1)	Yes	n.d.			46
Muscarinic receptor 2 (M2)*	Yes	n.d.			46
US28	Yes	Yes			44

* low affinity binding; GST - *in vitro* binding to GST-fusion proteins; Co-IP: Co-immunoprecipitation; n.d. - not determined.

Figure 1

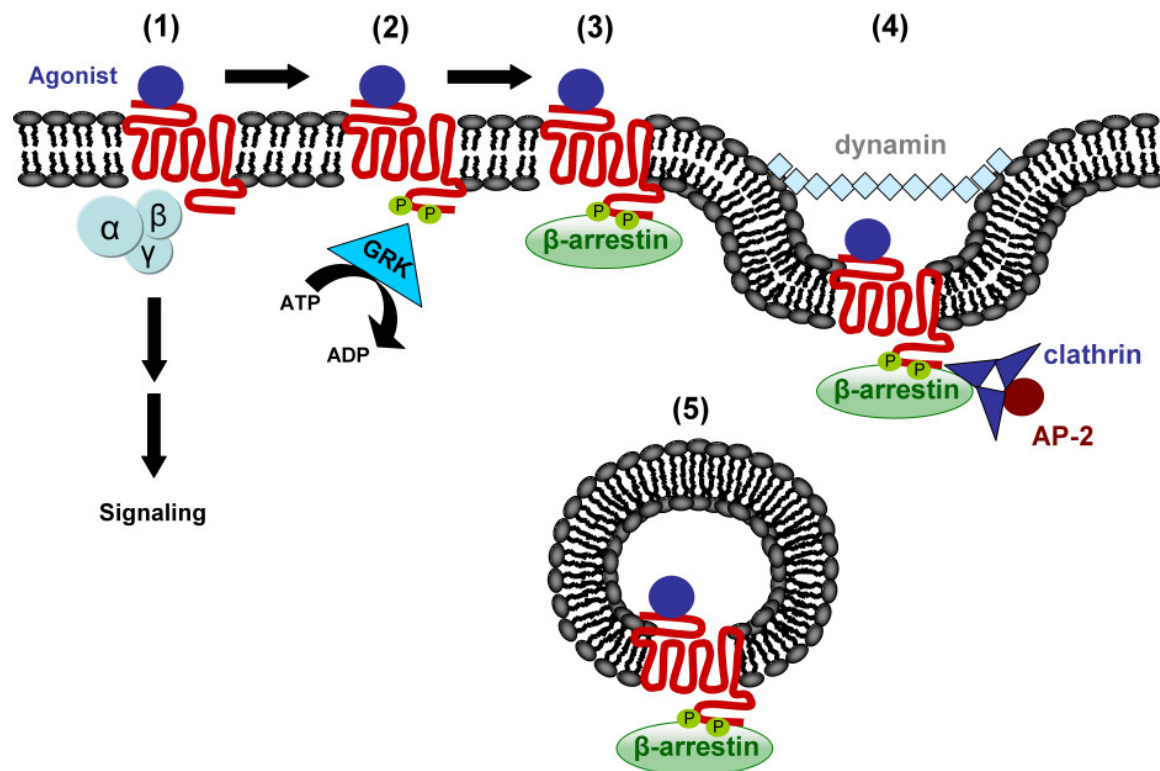


Figure 2

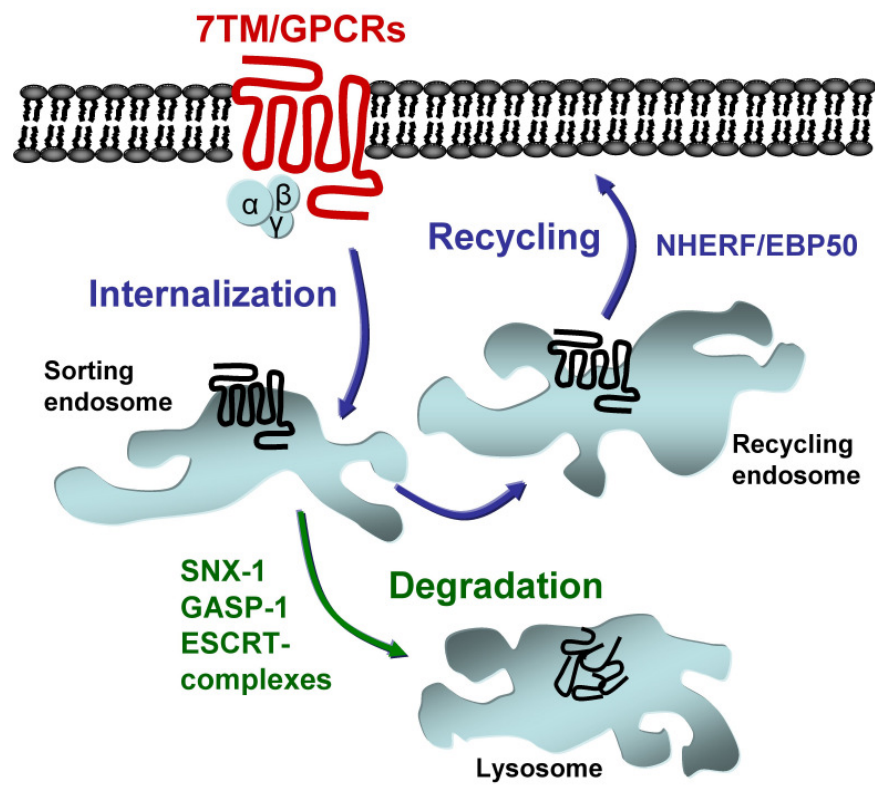
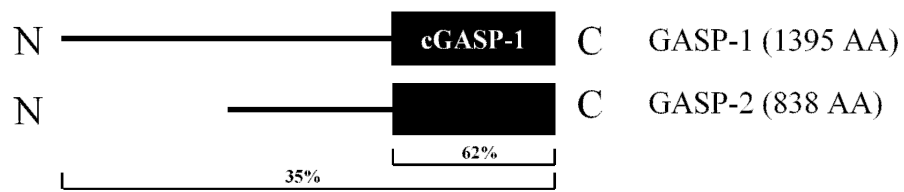


Figure 3



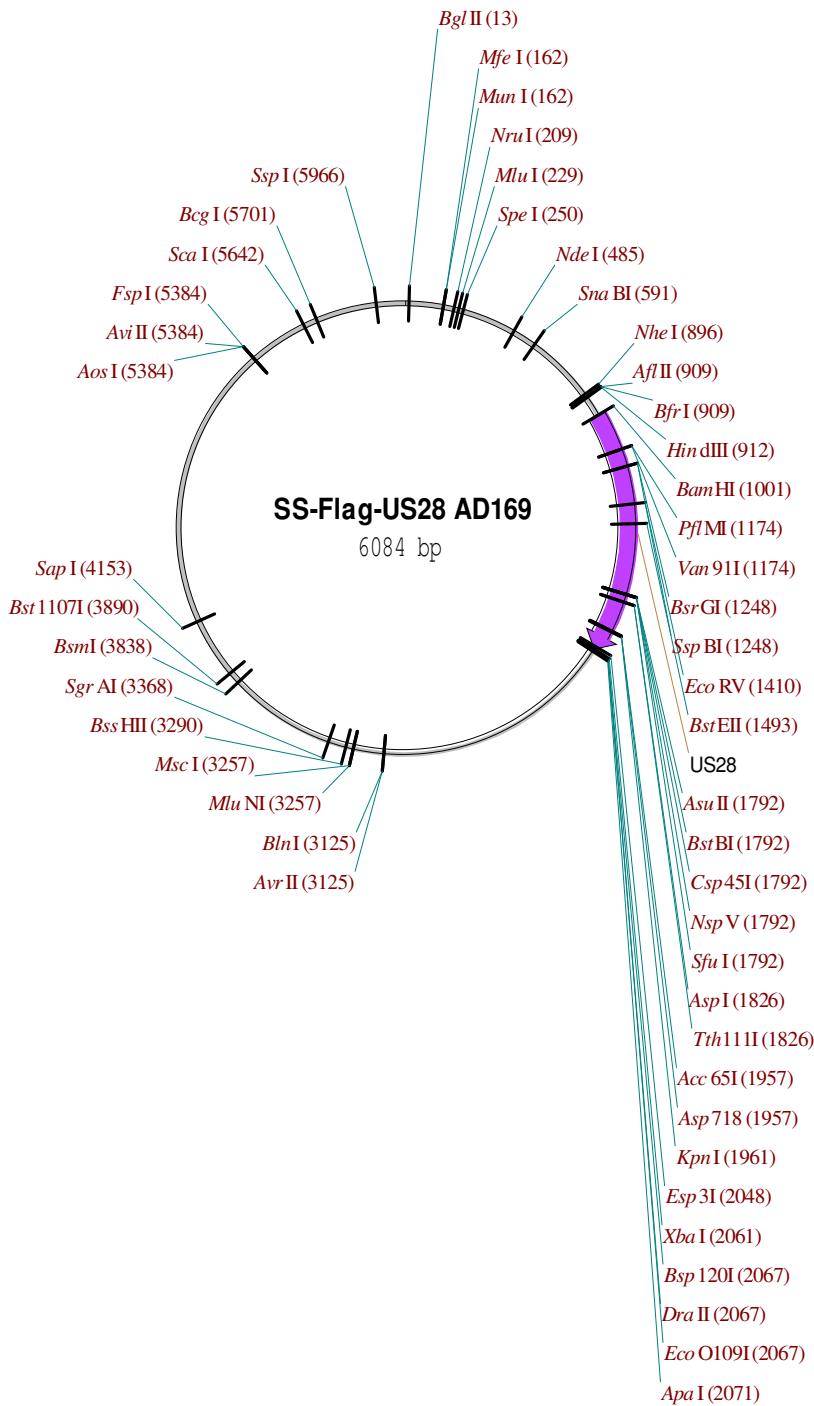
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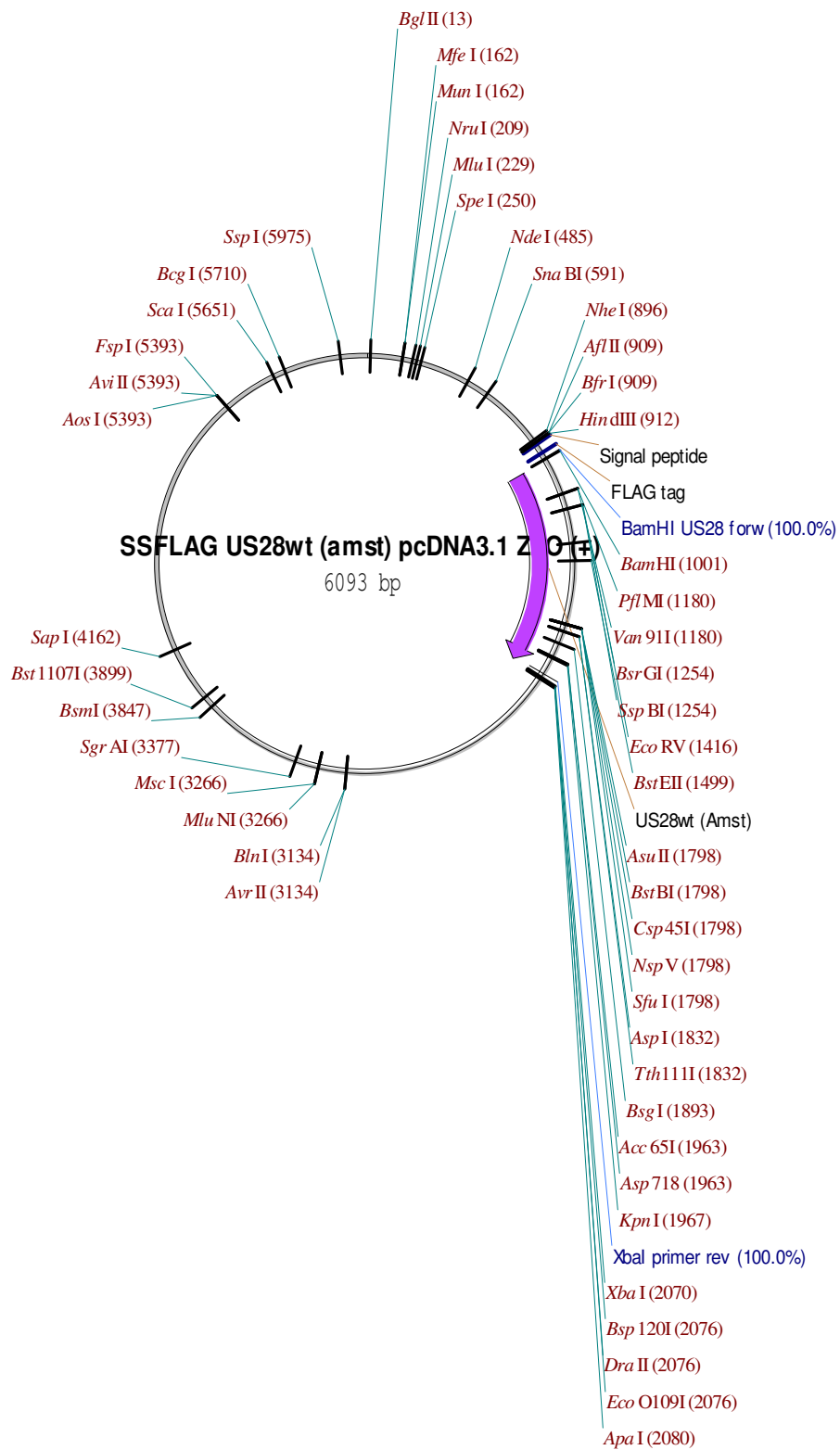
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Appendix III



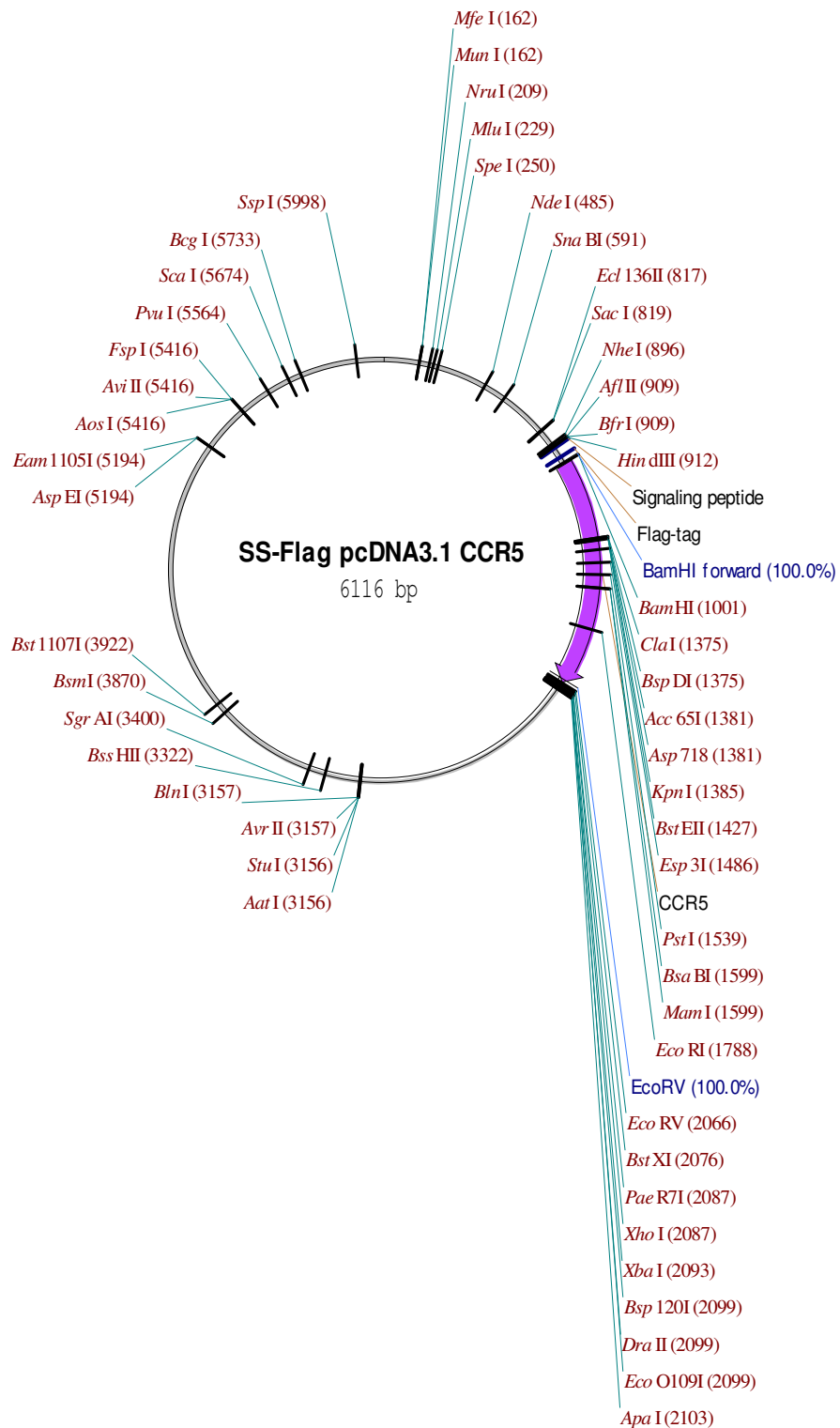
Appendix

Appendix IV



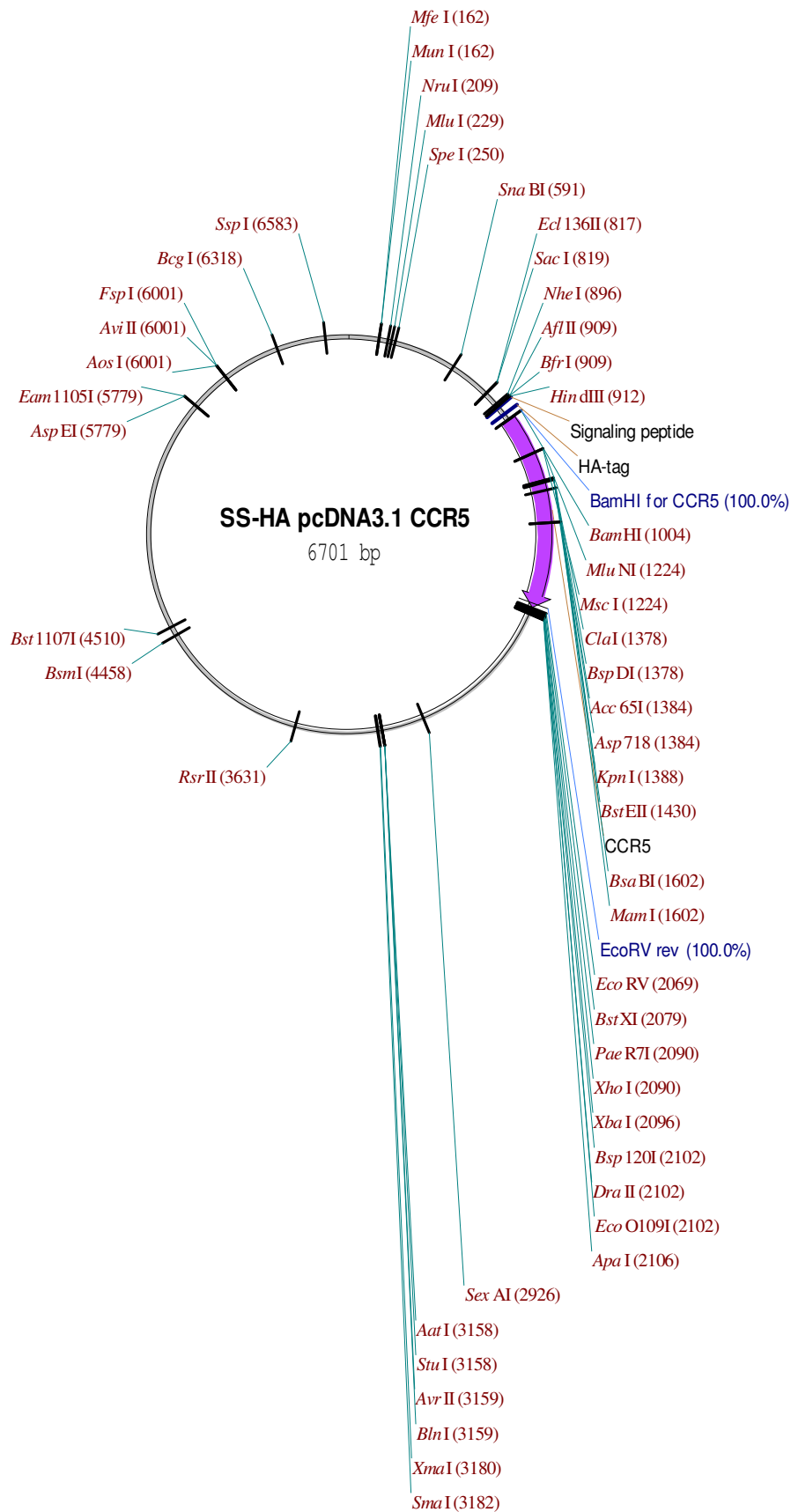
Appendix

Appendix V



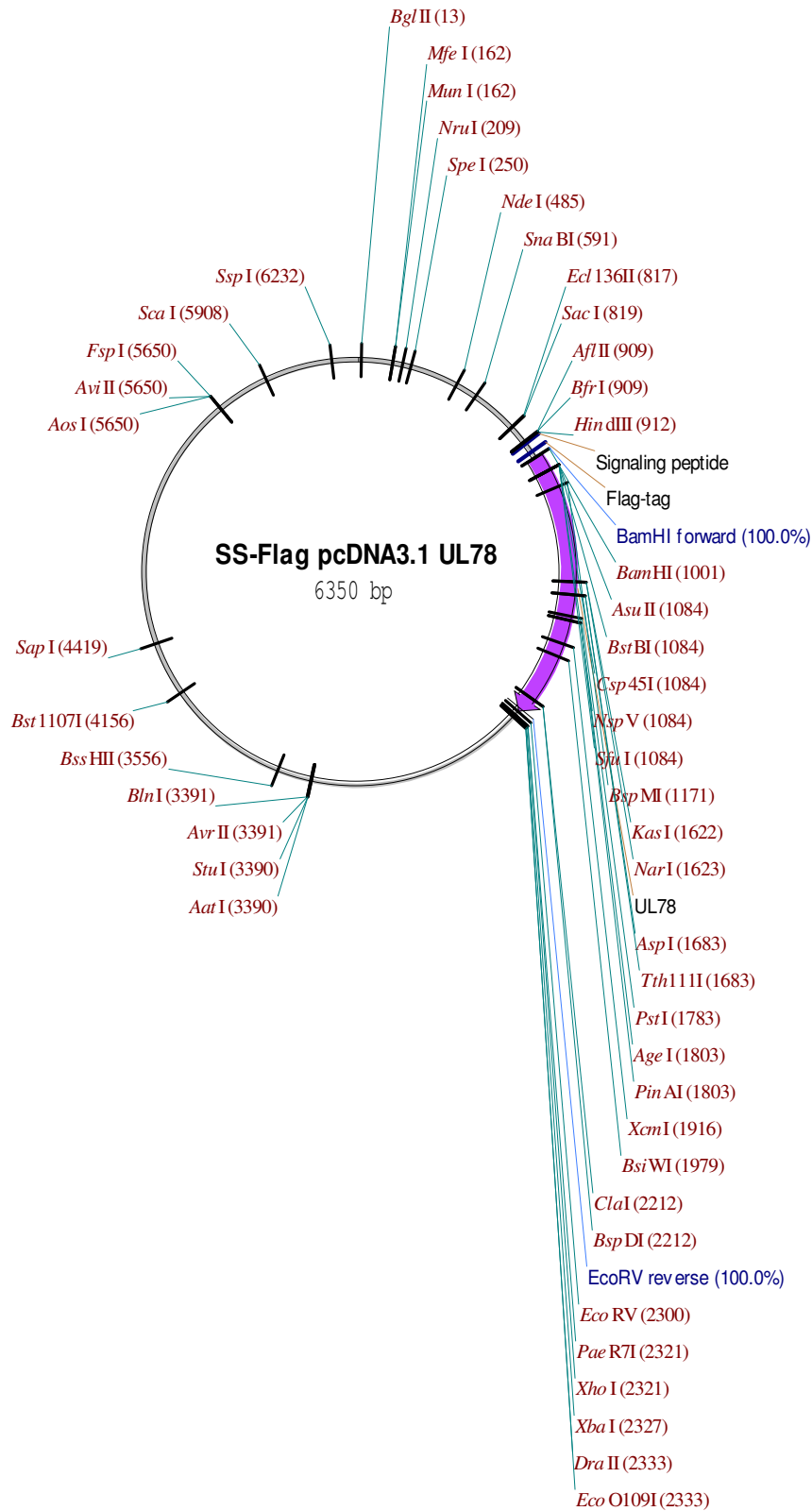
Appendix

Appendix VI



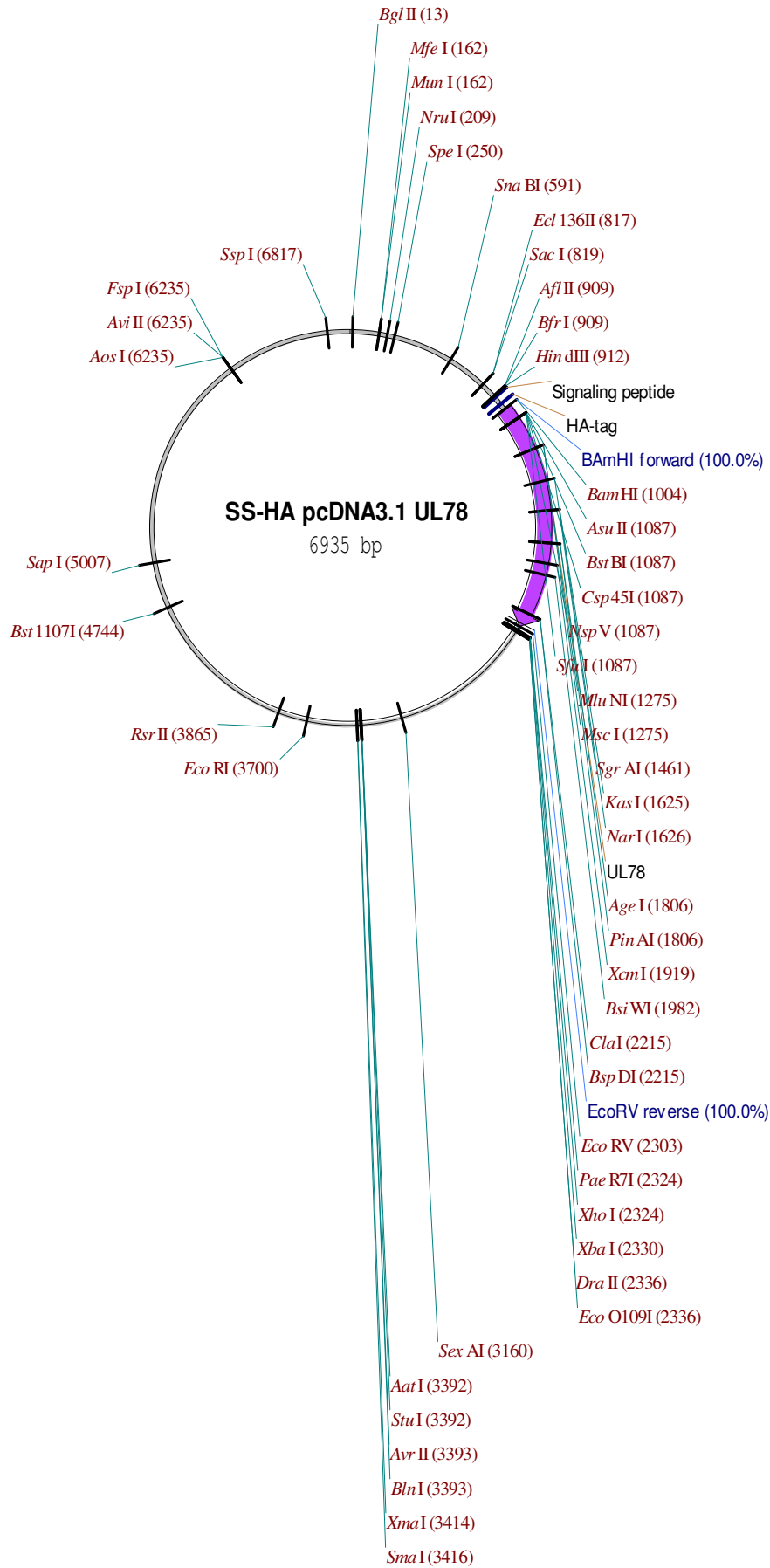
Appendix

Appendix VII



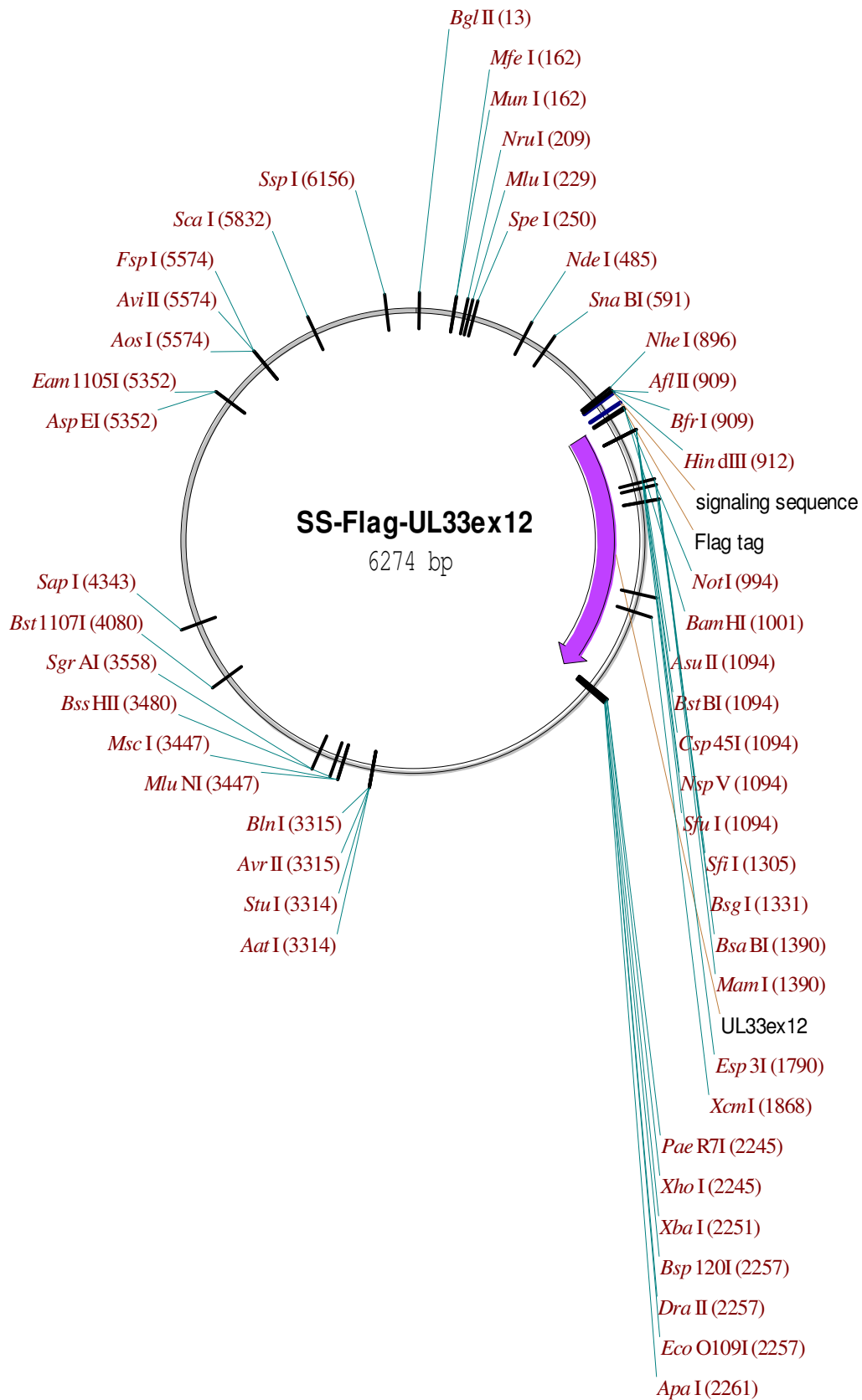
Appendix

Appendix VIII



Appendix

Appendix IX



Curriculum Vitae

- PERSONAL INFORMATION: Born on the 10th of October, 1982 in Klagenfurt (Austria)

- HIGHER EDUCATION:

2006 – 2010 **PhD** in the field of Molecular Biology at Medical University of Graz, Institute for Experimental and Clinical Pharmacology (**Austria**)

Feb. 2006 Masters Degree in Pharmaceutical Sciences, Karl-FranzensUniversity, Graz (Austria)

2001 – 2006 Studies of Pharmaceutical Sciences, Karl-Franzens University, Graz (Austria)

1993 – 2001 High school and University entrance examination, Voelkermarkt (Austria)

- SELECTED PEER-REVIEWED PUBLICATIONS

1. **Tschische P.**, Moser E., Vischer HF., Parzmair GP., Pommer V., Platzer W., Schwarzbraun T., Schaidler H., Smit MJ., Martini L., Thompson D., Whistler JL., Waldhoer M. (2010). The G protein-coupled receptor associated sorting protein GASP-1 regulates the signaling and trafficking of the viral chemokine receptor US28. *Traffic* 11:660-674
2. Moser E., Kargl J., Whistler JL., Waldhoer M., **Tschische P.** G protein coupled receptor associated sorting protein (GASP-1) regulates the post-endocytic sorting of 7TM/GPCRs. Review. *Pharmacology. in press*

- INVITED TALKS:

Oct. 2009 **University of Montreal**, Canada. GASP-1 regulates the post-endocytic sorting and constitutive signaling capacity of US28.

Sept. 2009 **University of California San Francisco (UCSF)**, USA. GASP-1 controls the trafficking and signaling properties of the HCMV encoded receptor US28.

Sept. 2008 Ernest Gallo Clinics and Research Center, **University of California San Francisco (UCSF)**, USA. The post-endocytic sorting and signaling of US28.

Sept. 2007 Ernest Gallo Clinics and Research Center, **University of California San Francisco (UCSF)**, USA. The post-endocytic trafficking of the HCMV encoded receptor US28.

- SCIENTIFIC PRESENTATIONS:

- July 2010 Poster: 16th World Congress of Basic and Clinical Pharmacology, Copenhagen, Denmark; *accepted*
- Nov. 2009 Poster: 15th Scientific Symposium of the Austrian Pharmacological Society (APHAR), Graz, Austria
- Nov. 2009 Poster: 2nd Doctoral Day of the Medical University of Graz, Austria
- Sept. 2009 Poster: SFB35 Meeting on Transmembrane proteins in health and disease, Vienna, Austria.
- June 2009 Poster: Gordon Research Conference on Molecular Pharmacology, Lucca, Italy.
- Nov. 2008 Talk: 14th Scientific Symposium of the Austrian Pharmacological Society (APHAR), Innsbruck, Austria.
- Oct. 2008 Poster: 1st Doctoral Day of the Medical University of Graz, Austria
- Sept. 2008 Poster: SFB35 Meeting on Transmembrane proteins in health and disease, Vienna, Austria.
- Nov. 2006 Poster: University Health Network (UHN) Research Day, Toronto, Canada

- RESEARCH VISITS

- Sept. 2009: Ernest Gallo Clinic and Research Center, **University of California San Francisco**, USA
- Sept. 2008: Ernest Gallo Clinic and Research Center, **University of California San Francisco**, USA
- Sept.-Oct. 2007: Ernest Gallo Clinic and Research Center, **University of California San Francisco**, USA
- Sept.–Dec. 2005: **Mahidol University**, Faculty of Pharmacy, Bangkok, Thailand

- PROFESSIONAL EXPERIENCES

- March 2006 – March 2007 Research assistant, University Health Network, Division of Cellular and Molecular Biology, **University of Toronto**, Toronto, Canada.

SCHOLARSHIPS AND AWARDS

- Jan. 2010 Scholarship for six month to support my ongoing research on heteromerization of viral receptors; awarded by the Medical University of Graz.
- Oct. 2008 Prize for *best poster presentation* at the 1st doctoral day of Medical University of Graz.
- Sept. 2007 Travel grant to support a two month research visit at the Ernest Gallo Clinic and Research Center, UCSF, USA. The scholarship was awarded by the *BA/CA Visiting Scientist Program* of the Medical University of Graz
- Jan. 2006 Scholarship to support a three month research visit at the Mahidol University in Bangkok, Thailand, provided by ASEA UNINET.
- Dec. 2005 Scholarship for excellent academic achievements (Leistungsstipendium) awarded by the Dean of the Faculty of Natural Sciences of the Karl-Franzens University Graz for the academic year 2005.