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HCMV-encoded chemokine receptor US28 employs multiple routes for internalization

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Abstract

The human cytomegalovirus-encoded G protein-coupled receptor homologue US28 binds inflammatory chemokines and sequesters them from the environment of infected cells. Low surface deposition and endocytosis are dependent on constitutive C-terminal phosphorylation, suggesting a requirement for β-arrestin binding in receptor internalization. In this report, a US28-dependent redistribution of β-arrestin into vesicular structures occurred, although internalization of US28 was independent of β-arrestin. Internalization of US28 was dynamin-dependent, and US28 partially partitioned into the detergent-resistant membrane fraction. Endocytosis was diminished by cholesterol depletion, yet sucrose inhibition was even stronger. The relevance of the clathrin-coated pit pathway was supported by colocalization of β_2-adaptin and US28 in endocytic compartments. Exchange of the C-terminal dileucine endocytosis motif inhibited rapid endocytosis, indicating a direct interaction of US28 with the AP-2 adaptor complex. We suggest that the arrestin-independent, dynamin-dependent internalization of US28 reveals a differential sorting of β-arrestins and the virally encoded chemokine receptor homologue.

Keywords: G-protein coupled receptor; Endocytosis; Chemokine receptor; Caveolae; Lipid rafts; Clathrin-coated vesicles; Dynamin

Upon viral infections, antigen presenting cells, and immune effector cells have to be directed to the site of foreign antigen encounter, allowing the host to prevent virus damage and spreading. This process of immune cell migration is largely controlled by inflammatory chemokines. HCMV has acquired several strategies to evade immune attack, including the expression of chemokine receptor homologues [1–3]. Among the four chemokine receptor homologues encoded by HCMV, only US28 has a constitutive and ligand-induced chemokine receptor activity [4,5]. It was suggested that it may serve as a decoy for inflammatory chemokines, thereby limiting the range of secreted chemokines to attract harmful immune effector cells to the site of virus intrusion [6]. US28 has been also linked to pathogenetic processes leading to atherosclerosis and restenosis [7]. Other important functions for the viral life cycle include the augmentation of cell–cell fusion required for virus spreading in vivo. US28 undergoes rapid constitutive endocytosis and recycling [8]. In contrast to its cellular homologues, US28 was found predominantly intracellularly in perinuclear late endosomes. In our previous study, we identified that US28 is constitutively phosphorylated at its C-terminus through a G protein coupled receptor kinase (GRK)-mediated mechanism [9].

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Phosphorylated serine and threonine residues within the C-terminus determine the subcellular localization of the receptor, but also the endocytic capacity of US28.

In contrast to the human RANTES receptor CCR5, US28 was found to internalize in a β-arrestin-independent manner. Furthermore, the inhibition of the clathrin-mediated endocytosis pathway partially abrogated the endocytosis of US28 [10]. The role of β-arrestins in US28-induced signaling events was studied recently, employing C-terminal truncation mutants of US28 [11].

In general, receptor phosphorylation mediated by GRKs leads to the recruitment of cytosolic β-arrestin, the latter step disrupting the receptor-G protein association and second, downmodulation of the GPCR is facilitated by acting as an adaptor to endocytic clathrin-coated pits [12,13]. The paradigm of GRK phosphorylation and β-arrestin recruitment has been challenged by the observation that a few GPCR can internalize in an arrestin-independent manner, among them the 5-hydroxytryptamine$_{2A}$ (5-HT$_{2A}$) and the muscarinic acetylcholine receptors [14,15]. However, employing dominant-negative mutants of dynamin indicated that they still internalize via a clathrin-mediated pathway, possibly in a cell-type-dependent manner.

In the present study we have revisited receptor endocytosis. We observed a US28-induced redistribution of β-arrestin into vesicular structures. Surprisingly, US28 and β-arrestin localized to separate endocytic compartments. A requirement for dynamin was proven using the GTP-ase-deficient mutant K44A. The inhibition of the clathrin-mediated endocytosis pathway was not sufficient to block US28 sequestration completely. Highly selective site-directed dileucine, tyrosine, and cysteine mutants in combination with pharmacological inhibitors offered the advantage to assess the individual contribution of sorting signals in the cytoplasmic tail. Our data provide evidence that US28 employs constitutively multiple mechanisms to recruit the endocytic machinery.

Materials and methods

Antibodies. Mouse monoclonal antibodies (mAb) Tub-6 and Tub-45, specific for US28, have been described previously [9], anti-CCR5 R22/7 antibody was a kind gift from Dr. M. Oppermann (Göttingen, Germany) [16], CCR5 antibody for immunofluorescence clone 45523.111 was from R&D Systems GmbH (Wiesbaden, Germany), and rabbit anti-caveolin 1 antibody was from Santa Cruz Biotechnology (Heidelberg, Germany). Anti-Flag antibody M2 was from Sigma (Taufkirchen, Germany). Anti-human MHC class I heavy chain mAb HC10 and W6/32HL mAb were purified from hybridoma supernatant. Secondary antibodies used for immunofluorescence were rat anti-mouse CY3, donkey anti-rabbit CY3 antibody, and goat anti-mouse biotin (Jackson Immunoresearch Laboratories, Hamburg, Germany). Streptavidin-Alexa Fluor 488 and 568 were from Molecular Probes (Leiden, The Netherlands). Horseradish peroxidase-conjugated goat anti-mouse antibody was from Southern Biotechnology (Eching, Germany).

Cell line. HEK293 cells were cultured in Dulbeccos's modified Eagle's medium supplemented with 10% fetal calf serum, penicillin (100 U/ml), streptomycin (100 μg/ml), and 2 mM glutamine at 37 °C in humidified air with 5% CO_2.

Plasmid constructs. Plasmids encoding US28 wild type (wt), US28 S1-12A, and US28 ST/A have been described previously [9]. CCR5 was subcloned into pcDNA3.1(−) by PCR, employing a CCR5-Flag plasmid as a template (provided by Dr. M. Oppermann). Plasmids encoding β-arrestin 1, β-arrestin 2, β-arrestin 1-GFP, β-arrestin 2-GFP, the dominant negative arrestin mutant V53D, wt dynamin, and dynamin dominant negative mutant K44A were a generous gift from Dr. M. Caron (Durham, NC). A truncated β-arrestin 319–418 mutant in pcDNA3.1(−) was generated by PCR mutagenesis using the β-arrestin 1 plasmid as a template and site-specific primers. A β_2-adaptin flag plasmid has been described previously [17]. The HA-tagged pCMV-PI3Kp110γ wild type (PI3Kγ) and HA-tagged pCMV-PI3Kp110γ dominant negative mutant (ΔPI3Kγ; Δ942–981, deletion in ATP binding site, in the following referred to as PI3K −/−) were a generous gift from Dr. C.S. Abrams (Philadelphia, PA).

The US28 site-directed mutants R129A, C304A, C347A, C304/347A, Y208A, Y321A, and LL305/6AA were constructed by site-directed mutagenesis employing PCR and site specific primers (Supplementary information I). The mutation of the tyrosine motifs in the second intracellular loop resulted in a mutant where tyrosine 130 was replaced by proline, tyrosine 131 was deleted, and tyrosine 138 was changed to alanine (in the following referred to as ΔY130-138). All constructs were cloned into pcDNA3.1(−) and verified by cDNA sequencing.

Immunofluorescence and laser scanning microscopy. HEK293 cells were transfected using either calcium phosphate method or Fugene 6 (Roche, Mannheim, Germany). Thirty-six to forty-eight hours after transfection, cells were washed with serum free medium, labeled with 30 nM RANTES (R&D) for 1 h at 4 °C or left untreated as indicated, and incubated at 37 °C for 30 min. Staining of cells was done essentially as described [8]. Where indicated, streptavidin-conjugated Alexa Fluor TM 488 or Alexa Fluor 568 was added. Slides were analyzed by confocal microscopy on a Zeiss LSM510 inverted laser scanning microscope (Carl Zeiss, Göttingen, Germany). All images were processed in Adobe Photoshop.

Flow cytometric analysis. Immunofluorescence staining was done essentially as described [9]. Flow cytometric analysis was carried out on a FACSCalibur (Becton–Dickinson, Heidelberg, Germany), and 4 μM propidium iodide (Sigma) was used to exclude dead cells from analysis.

Endocytosis assay. Endocytosis assays were performed exactly as described [9]. Endocytosis inhibitors filipin III (5 μg/ml) (Sigma), methyl-β-cyclodextrin (5 mM), and sucrose (0.4 M) were added prior to radioligand exposure, as indicated in the figure legends. The proportion of internalized radioligand was calculated by dividing the acid-resistant activity by the total cell-associated activity.

Isolation of detergent-resistant membranes. Isolation of detergent-resistant membrane fractions was done exactly as described [18]. Briefly, HEK293 cells were transiently transfected with US28 and starved in methionine and cysteine-free medium for 45 min. Cells were labeled with 200 μCi [^{35}S]methionine/cysteine and lysed in TX-100 containing lysis buffer (25 mM Pipes, pH 6.5, 150 mM NaCl, 1% TX-100, and 1 mM PMSF) on ice for 30 min. Cells were centrifuged at 13,000 rpm for 10 min. Supernatant was transferred into a new tube, and the pellet, corresponding to the detergent-resistant membranes, was redissolved in PBS/1% SDS and boiled for 10 min. DNA was sheared by passing the lysates through a 25 gauge needle several times. Subsequently, US28 was immunoprecipitated with mAb Tub-45 and MHC class I molecules were precipitated with mAb HC10 or mAb W32/HL, respectively. Samples were analyzed by SDS–PAGE (12.5%).

Palmitoylation of US28. HEK293 cells were transiently transfected with US28 wt or CCR5. Forty hours later cells were labeled with serum-free culture medium containing 0.1% fatty acid free BSA and [^{3}H]

palmitic acid (400 μCi/ml, Perkin–Elmer-NEN) at 37 °C for 4 h. Cells were lysed in PBS, 1% Triton X-100, 5 mM EDTA, and 1 mM PMSF for 1 h at 4 °C. US28 was immunoprecipitated from precleared cell lysates with mAb Tub-45, whereas CCR5 was recovered using mAb R22/7. Immunoprecipitates were analyzed by 13% SDS–PAGE under non-reducing conditions. To assess receptor protein loads, aliquots of each cell lysate were analyzed by immunoblotting, as described [9]. Bound antibody was visualized by chemoluminescence (ECL kit, Amersham Biosciences).

Results

US28 and β-arrestin reside in separate endocytic vesicles

Since receptor phosphorylation determines the subcellular distribution of US28, we examined the agonist-independent distribution of US28 and β-arrestin 2 GFP in transiently transfected HEK293 cells. The cellular homologue of US28, the human CCR5 receptor (Figs. 1A and D), induced a redistribution of β-arrestin 2-GFP to cytoplasmic punctate structures only upon ligand challenge (Fig. 1E), but not in the absence of RANTES (Fig. 1B). A specific colocalization of both molecules, visible as yellow structures in the merged image, was observed as expected (Fig. 1F). In agreement with our previous report, US28 was located predominantly intracellularly in punctate areas (Fig. 1G). Similar to the coexpression of CCR5 after ligand stimulation, β-arrestin 2-GFP was internalized in endocytic vesicles when US28 was present in the cell (Fig. 1H), however, upon careful examination a colocalization with US28 in overlapping vesicular structures was not seen (Fig. 1I). Instead, β-arrestin 2-GFP preferentially accumulated in most of the cells in an expanded compartment, where US28 was essentially absent (Fig. 1H).

The phosphorylation-deficient US28 S1-12A mutant failed to induce β-arrestin 2-GFP redistribution (Figs. 1J, K, and L), as could be expected if β-arrestins were involved in US28-endocytosis [9]. An alternative β-arrestin binding site in the second intracellular loop, the DRY motif, could also play a role in the recruitment of β-arrestins to the plasma membrane [19]. The signaling deficient US28 mutant R129A [20] was cotransfected with β-arrestin 2-GFP into HEK293 cells and analyzed by immunofluorescence and confocal microscopy (Figs. 1M and N). Mutation of a single amino acid, R129A, in the US28 receptor was sufficient to prevent β-arrestin redistribution into vesicles. In contrast to the altered arrestin localization the distribution of the R129A receptor mutant itself was largely identical to that seen for the US28 wt receptor (Figs. 1M and G). To strengthen our contention that US28 and β-arrestin employ the same endocytic machinery, but separate endocytic routes, we determined the colocalization of US28 and β-arrestin 2-GFP with the AP-2 adaptor complex. The AP-2 adaptor is composed of a heterotetrameric protein

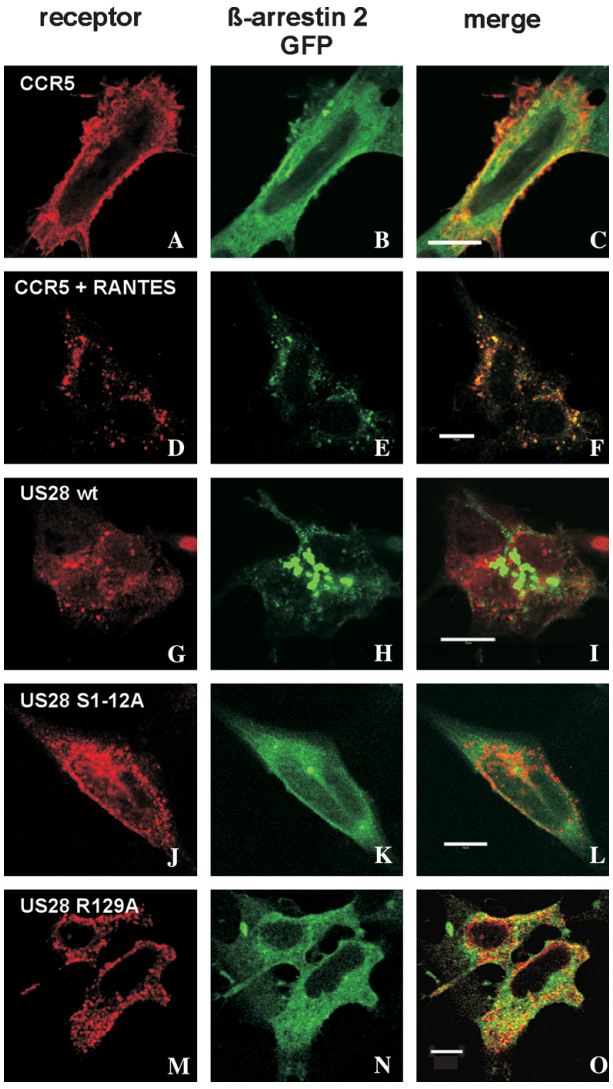

Fig. 1. US28 induces redistribution of β-arrestin-GFP to the plasma membrane, but they do not colocalize. (A–F) CCR5 and β-arrestin 2-GFP colocalize after ligand stimulation in HEK293 cells. HEK293 cells were cotransfected with CCR5 and β-arrestin 2-GFP. Cells were left untreated (A, B, and C) or were treated with 30 nM RANTES (D, E, and F), followed by transfer to 37 °C to stimulate endocytosis. After 30 min, cells were fixed, permeabilized, and stained with anti-CCR5 antibody (A,D). Antibody staining was visualized using a biotinylated goat anti-mouse IgG-antibody and streptavidin-conjugated AlexaFluor 568 (red). Overlay (F). (G–O) HEK293 cells were cotransfected with US28 wt (G), US28 S1-12A (J) or US28 R129A (M), and β-arrestin 2-GFP (H, K, and N), respectively. US28 was immunostained with mAb Tub-45 (red), as described in (A), but without RANTES treatment. US28 wt was found predominantly in punctate cytoplasmic structures (G), and β-arrestin 2-GFP staining was observed in vesicular structures (H), similar to the coexpression with CCR5 (E), but also in an expanded cytoplasmic compartment. In the overlay (I), colocalization was not seen. The phosphorylation-deficient mutant US28 S1-12A (J) failed to recruit β-arrestin 2-GFP to the plasma membrane (K). Likewise, the DRY-motif mutant US28 R129A (M) did not induce redistribution of β-arrestin 2-GFP (N) into vesicular compartments. For both mutants, in the merged images (L,O) colocalization with β-arrestin 2-GFP in the same compartment was not seen. All cells were analyzed by confocal microscopy. Scale bar, 10 μm.

complex, among them the β_2-adaptin subunit. Functionally, this complex serves to connect physically cargo molecules destined for endocytosis with the polymeric components of the clathrin coat. In comparison to the receptor-β_2-adaptin colocalization, β-arrestin 2-GFP colocalized with β_2-adaptin separate from US28 and β_2-adaptin (see Supplementary information II).

Dynamin regulates internalization and surface expression of US28

Concomitant expression of US28 wt receptor with either of two dominant-negative arrestin mutants failed to inhibit endocytosis in HEK293 cells (see Supplementary information IV A, [10]). To explore alternative endocytic key molecules, we made use of a dominant-negative mutant of the large GTP-ase dynamin, dynamin K44A. Dynamin has been implicated in the scission of clathrin-coated vesicles from the plasma membrane. HEK293 cells were cotransfected with receptor and the dominant negative dynamin mutant K44A (Fig. 2A).

In average, transfection rates for all endocytosis assays varied between 20% and 36% of all cells, as determined by flow cytometry. In agreement with published reports [8,9], surface deposition of US28 was always much lower than internal receptor expression (see Supplementary information III).

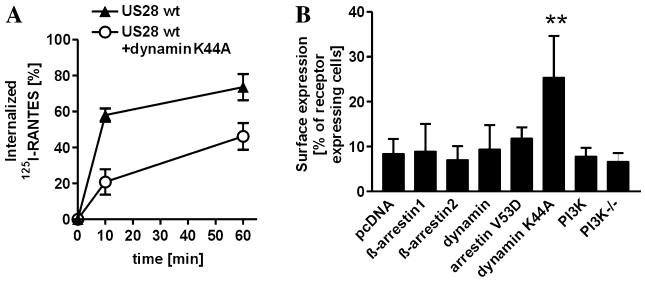

Fig. 2. Dynamin determines the internalization rate of US28 in HEK293 cells. (A) HEK293 cells were cotransfected with US28 wt and pcDNA (mock control), or US28 wt and dynamin K44A, respectively. Cells were labeled with ^{125}I-labeled RANTES for 2 h on ice, washed to remove unbound ligand, and warmed to 37 °C to allow endocytosis. At the time intervals indicated, cells were transferred on ice, and cell surface-bound ^{125}I-labeled RANTES was removed by acid wash for half of the samples (acid-resistant), followed by cell lysis and quantitation of lysate-associated activity. Internalization was calculated as percentage of acid-resistant counts (internal) to total cell-associated activity after subtraction of background activity at $t = 0$. All data points represent means ± SD of at least three independent experiments performed in duplicates. (B) Cotransfection of dominant negative dynamin K44A enhances US28 expression at the plasma membrane. HEK293 cells were cotransfected with US28 and mock vector (pcDNA), wt β-arrestin 1 and β-arrestin 2, β-arrestin V53D, wt dynamin, wt PI3K or a dominant negative mutant PI3K −/−, as indicated. 48 h after transfection, viable cells were analyzed for surface expression of US28 by flow cytometry, employing mAb Tub-6. Total cell expression of US28 was assessed after fixation and permeabilization. Data shown represent the percentage of cells with receptor surface expression in relation to total cellular US28 receptor expression. Results are expressed as means ± SD of three to five experiments. $^{**}p < 0.05$ compared to mock transfected US28 expressing cells.

The presence of the dominant negative dynamin K44A mutant reduced the internalization of the US28 receptor, suggesting the utilization of a dynamin-dependent endocytosis pathway. In agreement with the inhibition of endocytosis, we observed a significant increase or stabilization of US28 at the cell surface when dynamin K44A was coexpressed (Fig. 2B). Wt β-arrestins 1 and 2, wt dynamin, mutant β-arrestin V53D, wt and mutant PI3Kγ had essentially no effect on surface receptor expression rate.

Internalization of ^{125}I-labeled RANTES in mock-transfected cells (pcDNA3.1) was constantly negligible (see Supplementary information IV B) for all endocytosis assays.

Inhibitors of caveolae formation interfere with US28 endocytosis

The occurrence of GPCR, heterotrimeric G proteins, and other signaling molecules in specialized membrane domains that have been referred to as caveolae has been established. These specializations are found in the *trans*-Golgi network and at the plasma membrane, morphologically they are nonclathrin-coated, flask-shaped invaginations or vesicles. Operationally, they can be defined by their insolubility in non-ionic detergents and by their low-buoyant density in sucrose density gradients [21].

Since cholesterol is a major component of caveolar and lipid raft membranes, cholesterol sequestration mediated by filipin has been widely used to probe the relevance of caveolae for signaling and endocytosis [22,23]. Moreover, methyl-β-cyclodextrin (MCD) which extracts cholesterol from the membrane inhibits both caveolae and clathrin-dependent internalization [24]. Pretreatment of US28 transfected cells with filipin III affected a significant decrease in early and maximal receptor uptake (Fig. 3A). Inhibition of the clathrin-coated pit pathway by hyperosmolaric 0.4 M sucrose blocked US28 uptake at a much higher rate, although a complete inhibition was not obtained (Fig. 3A). Likewise, methyl-β-cyclodextrin (MCD) diminished internalization of the US28 receptor at early time points (10 min), whereas after 60 min endocytosis of RANTES between treated and untreated cells was indistinguishable (Fig. 3B).

To assess the occurrence of US28 in detergent resistant membranes, we labeled HEK293 cells with [^{35}S]methionine/cysteine and performed an extraction with TX-100 containing lysis buffer. The TX-100 soluble and TX-100 insoluble fractions were then subjected to immunoprecipitation. We found substantial amounts of US28 associated with the TX-100-resistant fraction, whereas a control protein, MHC class I heavy chain, was completely absent from this fraction (Fig. 3C).

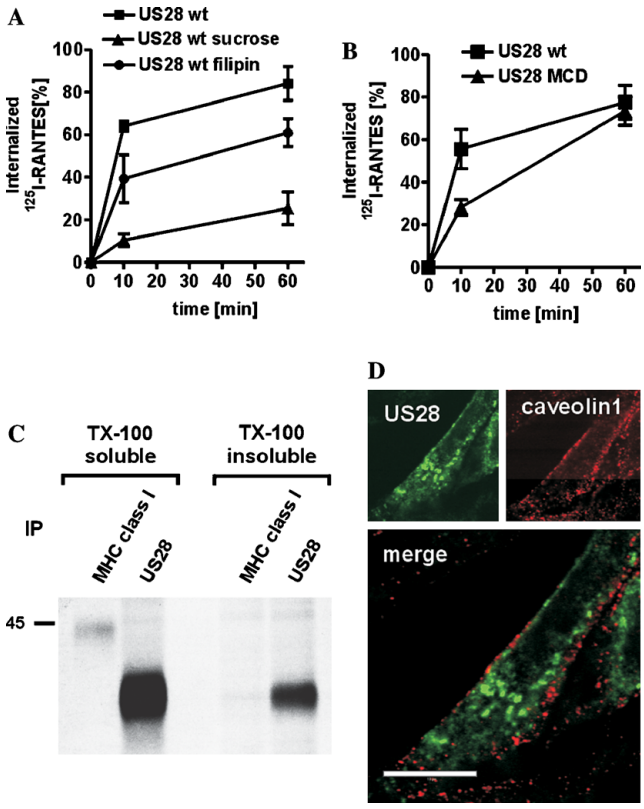

Fig. 3. US28 sequestration is sensitive to drugs that inhibit the caveolar pathway. (A) HEK293 cells were transiently transfected with US28 wt, followed by a standard internalization assay, as detailed in Fig. 2. Prior to labeling with ^{125}I-labeled RANTES, cells were washed and 5 μg/ml filipin III or 0.4 M sucrose was added for 10 min, as indicated. Cells were transferred to 37 °C to allow endocytosis, either in the presence or absence of pharmacological inhibitors. All data points represent means ± SD of at least three independent experiments performed in duplicate. (B) Cells were processed as shown in (A), except that methyl-β-cyclodextrin (MCD, 5 mM) was included prior to ligand binding for 30 min. (C) US28 is associated with non-ionic detergent-insoluble cell pellets. HEK293 cells were transiently transfected with US28. Cells were labeled for 6 h with 250 μCi [^{35}S]methionine/cysteine. Transfectants were lysed in TX-100 lysis buffer and separated into supernatant (soluble) and insoluble pellets. The pellet was re-solubilized in 1% SDS, DNA was sheared, and the extract was boiled. The SDS-concentration in the pellet fraction was adjusted to 0.1% SDS with NP-40 lysis buffer. Immunoprecipitations to recover assembled MHC class I complexes were done with mAb W6.32/HL, whereas HC10 mAb was used to immunoprecipitate nonassembled MHC class I heavy chains from the SDS-containing insoluble fraction. US28 was recovered with mAb Tub-45. Samples were analyzed on a 12.5% SDS–PAGE. (D) US28 and caveolin 1 do not colocalize. HEK293 cells were transfected with US28 wt, fixed and immunostained for US28 as described in Fig. 1. Endogenous caveolin was detected with a polyclonal rabbit anti-caveolin 1 antiserum. Analysis was done by confocal microscopy. Scale bars, 10 μm.

We scored for the colocalization of US28 with the caveolar marker molecule caveolin 1 in immunofluorescence (Fig. 3D). In confocal microscopy analysis, a colocalization of both proteins was not observed. In support of a specific association with detergent-resistant membrane fractions, a substantial amount of US28 was found in low density protein–lipid complexes, separated by sucrose density gradient centrifugation according to standard procedures [37] (Supplementary information V). Second, receptor aggregates as a cause for detergent-insolubility were not observed in confocal microscopy analysis of US28 transfected cells (Figs. 1, 3D, Supplementary information II).

A potential role of the caveolae pathway will be discussed below.

Alternative internalization signals at the cytosolic tail of US28 contribute to the recruitment of clathrin coats

Palmitoylation at C-terminal, membrane-proximal cysteine residues was suggested to induce the formation of a fourth intracellular loop and might thereby enhance GRK and β-arrestin recruitment [25]. Consequently, more efficient receptor endocytosis might result. Palmitoylation of US28 was determined by transient transfection and labeling of HEK293 cells with [^{3}H]palmitic acid. Cells were lysed, and US28 was immunoprecipitated with monoclonal antibody Tub-45 (Fig. 4A). Although the level of [^{3}H]palmitic acid incorporation was less compared to CCR5 transfected control cells, a specific band for US28 was obtained. We note that a portion of the receptor did not enter the separating gel very well, which is due to protein aggregation. To study the functional consequences of US28 palmitoylation, a membrane-proximal cysteine at position 304 (C304A), which provides a likely acceptor site for fatty acylation, was mutated to alanine. Agonist-induced internalization was not changed compared to wt US28 (Fig. 4B). A double mutant, C304/347A where both C-terminal cysteines were replaced by alanine, exhibited an identical internalization rate as US28 wt receptor (Fig. 4C).

Tyrosine-based or dileucine-based motifs have been established as specific signals that direct rapid internalization of transmembrane receptors. It has been shown that these signals bind to the AP-2 complex, followed by the local concentration of transmembrane proteins within clathrin-coated pits [13]. We targeted a C-terminal dileucine motif, LL305/306, for mutation to alanine residues and measured the capacity of the double-mutant (LL305/306AA) to undergo agonist-induced internalization (Fig. 4D). Although the endocytosis rate at the later 60 min time point was indistinguishable between US28 wt and the LL305/306AA mutant, a statistically significant reduction of 34% was seen at the 10 min time point. This result suggests that leucines at positions 305 and 306 are critical for rapid internalization. Mutation of three potential tyrosine based internalisation motifs, delta Y130-138, Y208, and Y321, did not alter agonist-induced internalization or surface expression (Supplementary information IV).

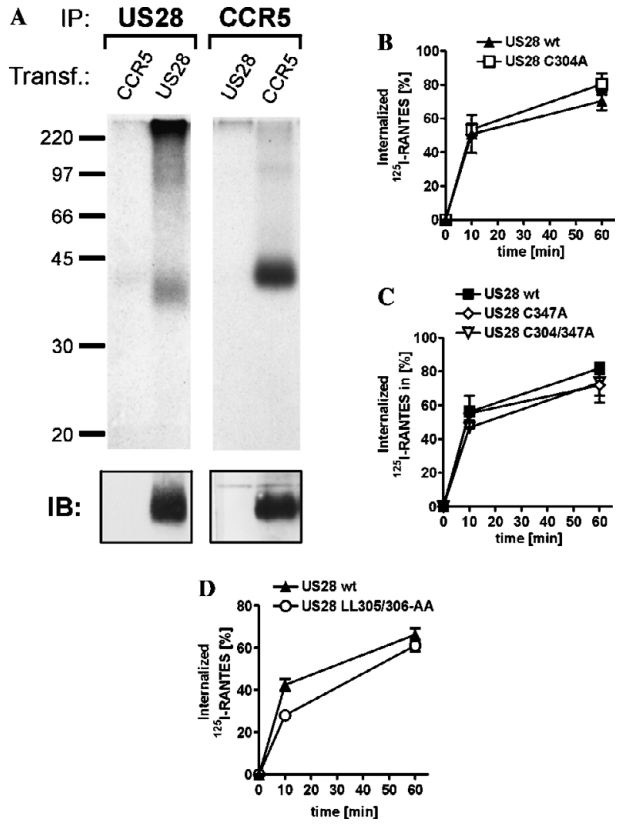

Fig. 4. US28 is palmitoylated and rapid endocytosis of US28 is controlled by a dileucine motif in the C-terminus. (A) HEK293 cells were transiently transfected with US28 or CCR5. Forty-eight hours later, cells were metabolically labeled with [^{3}H]palmitate and lysed. US28 and CCR5 were immunoprecipitated from cell lysates using mAb Tub-45 and R22/7, respectively. Immunoprecipitates were resolved by SDS–PAGE under non-reducing conditions. To assess total expression rates of the receptors in detergent lysates, aliquots were analyzed in parallel for CCR5 and US28 expression by immunoblotting (IB). (B) HEK293 cells were transiently transfected with US28 wt or US28 C304A. Internalization of ^{125}I-labeled RANTES was analyzed as described before (Fig. 3). All data points represent means ± SD of three independent experiments with duplicates in each experiment. (C) A second cysteine mutant, US28 C347A, and the double mutant US28 C304/347A were tested in a standard internalization assay. All data points represent means ± SD of at least three independent experiments performed in duplicate. (D) HEK293 cells were transfected with US28 wt or US28 LL305/306AA, followed by a standard internalization assay, as detailed in Fig. 3. Mutation of the US28 C-terminal dileucine motif reduces the rate of rapid endocytosis, but not late endocytosis.

Discussion

In this study we made the puzzling observation that the HCMV encoded chemokine receptor homologue US28 internalizes in a β-arrestin-independent manner, yet we note a US28-dependent relocalization of GFP-tagged β-arrestin 2 into vesicular structures. Colocalization of US28 with the β_2-adaptin subunit was seen in some vesicular structures, suggesting that US28 internalizes in a clathrin-coated vesicle (CCV)-dependent manner. Although arrestin also colocalized with the AP-2 adaptor complex subunit, those signals were clearly distinct from those obtained from the US28-β_2-adaptin staining.

The signaling deficient [20], but internalization competent, US28 R129A mutant failed to induce β-arrestin 2 GFP redistribution, indicating that signaling but not internalization is required for this process. Other examples for β-arrestin-independent internalization, with an apparently differential receptor-induced sorting of β-arrestins and GPCR, include the 5-HT$_{2A}$ serotonin receptor [14].

An explanation for the relocalization of receptors and β-arrestin molecules into distinct vesicular components might include mechanisms of heterologous cross-desensitization [26,27]. Mechanistically, the fact that β-arrestin and US28 reside on separate endocytic vesicles might reflect a situation where an unknown receptor in close proximity to the "priming" US28 receptor receives a cross-activation that might result in its β-arrestin-dependent internalization as well. In a second step, both receptors, US28 and a second, interacting receptor, are then endocytosed on separate endocytic vesicles. US28-induced heterologous cross-desensitization might be associated with the CXCR4 receptor, which is down-modulated upon expression of the US28 molecule, but not by coexpression of the human RANTES receptors CCR1 and CCR5 [28].

Since the arrestin adaptor was apparently dispensable to link the US28-cargo to CCV [10], we focused on endocytic motifs which could allow US28 to bind directly to the AP-2 complex. Among the motifs tested, only the dileucine motif at aa position 305/306 at the C-terminus influences rapid endocytosis, but not late endocytosis. Another necessary component of the receptor endocytosis pathway is the large GTPase dynamin [13]. Coexpression of a dominant negative mutant, dynamin K44A, and US28 significantly diminished receptor endocytosis, resulting in enhanced US28 surface display.

In general, dynamin also contributes to the raft or caveolae-dependent pathway of receptor endocytosis [13,29]. Caveolae-associated signaling and endocytosis has been shown for several GPCRs including the cellular homologue CCR5 [22]. Pharmacological inhibitors that interfere with the incorporation of cholesterol into lipid raft membranes induced a decrease either in early or a reduction in both early and maximal receptor uptake. MCD has been found previously to influence transferrin receptor uptake through the clathrin-mediated pathway. In contrast, filipin treatment showed no effect on transferrin internalization when tested in HEp-2 cells [24]. However, compared to the inhibition of the CCV-pathway, mediated by hyperosmolaric sucrose, the raft-dependent pathway was less efficient.

In support of a potential role of rafts in US28 endocytosis, we found a substantial enrichment of US28 in

the detergent-resistant membrane fraction. Proteins associated with them display different degrees of detergent resistance. In this context, in this report we show that US28 is palmitoylated. Palmitoylation is a dynamic process in the course of receptor activation and was suggested to provide a targeting signal to caveolae [30,31]. Caveolae might define a subdomain of the lipid rafts whose expression is associated with the marker protein caveolin 1 [32]. The failure to obtain colocalization of US28 and caveolin 1 in confocal microscopy does not exclude a raft-dependent internalization. Recent findings indicate that endocytosis through a lipid raft pathway does not necessarily require caveolin 1, as shown for CD36 [33].

Based on the employment of pharmacological inhibitors, we suggest that the preferred route of US28 uptake in HEK293 cells is via the clathrin-coated vesicle pathway. A lipid raft or caveolae pathway might be recruited as well, but only at lower abundance. Recruitment of considerable portions of US28 to membrane rafts has several implications for the function of this virally encoded receptor. The initiation of signaling scaffolds in raft domains has been well established for a variety of receptors of the immune system [34]. Furthermore, raft localization may be required for the proposed co-receptor activity of US28 for HIV-entry [35]. Recent studies have suggested that lipid rafts serve as the site for HIV entry based on interactions of gp120 with lipid raft components, such as CD4 and cellular chemokine receptors [36]. In conclusion, our data provide evidence that the US28 receptor has evolved unusual flexibility in the recruitment of cellular mechanisms, necessary for its rapid endocytosis. The simultaneous employment of different endocytosis pathways most likely relates to the capacity to serve as a decoy for inflammatory chemokines.

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Appendix. Supplementary material

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