Surface Expression and Endocytosis of the Human Cytomegalovirus-encoded Chemokine Receptor US28 Is Regulated by Agonist-independent Phosphorylation*

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Human cytomegalovirus encodes the G protein-coupled chemokine receptor homologue US28 that binds several CC chemokines and sequesters extracellular chemokines from the environment of infected cells. Mechanistically, it has been shown that US28 undergoes rapid constitutive receptor endocytosis and recycling. Monoclonal antibodies were raised that allowed the characterization of a ligand-independent phosphorylation and low surface expression of the US28 receptor in transiently transfected HEK293A cells. Phosphoamino acid analysis defined C-terminal serine and threonine residues as phospho-acceptor sites for constitutive receptor phosphorylation. Coexpression of G protein-coupled receptor kinase-2 and US28 enhanced ligand-independent receptor phosphorylation. C-terminal serine to alanine mutagenesis of US28 resulted in a decreased phosphorylation rate that correlated with enhanced surface expression. Maximal surface expression was detected when all C-terminal serines were substituted. Exchange of all C-terminal serines also significantly reduced receptor endocytosis. Thus, constitutive US28 phosphorylation regulates receptor endocytosis and receptor surface display and may thereby provide a pathogenic mechanism for a potential decoy function of the virally encoded receptor.

Latent viruses, particularly the family of herpesviruses, have evolved numerous strategies to elude the innate and adaptive immune response. Such evasive maneuvers are specified by gene functions in the viral genome and include virus-encoded homologues of cellular immune regulators (1). Analysis of the genome of human cytomegalovirus (HCMV) strain AD169 revealed four open reading frames, US27, US28, UL33, and UL78, that encode putative homologues of cellular G protein-coupled receptors (GPCRs). To date, US28 is the only HCMV-encoded GPCR homologue that has been shown to be functional in vitro (2). GPCR homologues have also been identified in other herpesviruses, among them the Kaposi's sarcoma-associated herpesvirus (KSHV or HHV-8) (3).

Upon interaction with extracellular ligands, the chemokines, virally encoded GPCRs, transduce signals into the cell by activating a cascade of cellular processes, which is initiated by the activation of heterotrimeric GTP-binding proteins. The downstream activation of second-messenger pathways has been partially elucidated for Kaposi's sarcoma-associated herpesvirus open reading frame 74 and HCMV-encoded US28 (4, 5). The constitutive activity of virally encoded GPCRs was found to be associated with a variety of human diseases (6). More specifically, Kaposi's sarcoma-associated herpesvirus open reading frame 74 exhibits agonist-independent signaling via activation of phosphoinositide-specific phospholipase C that can abrogate normal growth control mechanisms and lead to oncogenic cellular transformation (7, 4).

The US28-encoded receptor mediates high affinity binding to the CC-chemokines RANTES (CCL5), macrophage inflammatory polypeptide-1α (CCL3), MIP-1β (CCL4), and mono-ocyte chemotactic protein-1 (CCL2) (8) and to the CX3C-chemokine fractalkine (CX3CL1) (9). In view of its pathogenic role, it was demonstrated that expression of US28 facilitated sequestration of extracellular CC chemokines from the environment of HCMV-infected cells (10, 11). Ligand binding to US28 has been shown to mediate intracellular Ca2⁺ flux (8) and leads to activation of the mitogen-activated protein kinase pathways (5). Upon transient expression of US28 in COS-7 cells, US28 constitutively activates phospholipase C and NF-κB via a distinct G protein-mediated pathway, which is partially modulated by fractalkine (12). It was further demonstrated that HCMV infection of primary arterial smooth muscle cells results in significant cellular migration upon RANTES or monocyte chemotactic protein-1 production (13). Chemotaxis can be abrogated by deletion of US28 from the HCMV genome, thus providing a link between HCMV infection and acceleration of atherosclerosis. Surprisingly, epitope-tagged US28 receptors are located predominantly in perinuclear endosomes, possibly as a result of rapid constitutive, agonist-independent endocytosis and recycling of the receptor (14).

To gain more insight into the pathophysiological role of US28 signaling and subcellular distribution, we generated monoclonal antibodies (mAb) specific for the N-terminal extracellular...
lar domain of US28. These monoclonal antibodies specifically recognize US28 in its native form in transiently transfected HEK293A cells as well as in HCMV-infected fibroblasts. The native US28 receptor was largely absent from the cell surface; instead, the receptor was confined to intracellular organelles.

In this study, we show that US28 is constitutively phosphorylated through a G protein-coupled receptor kinase (GRK)-mediated phosphorylation mechanism. We further identify amino acid residues within the US28 C terminus that are essential for the high basal receptor phosphorylation. Our data provide experimental evidence that such unique receptor phosphorylation is a prerequisite for the subcellular localization and for the constitutive agonist-independent endocytosis of the receptor.

EXPERIMENTAL PROCEDURES

Cell Lines—HEK293A cells were cultured in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal calf serum, penicillin (100 units/ml), streptomycin (100 μg/ml), and 2 mM glutamine at 37 °C in humidified air with 5% CO2. Human embryonic lung fibroblasts were maintained in culture medium containing 5% fetal calf serum and 10 mM Heps.

Plasmid Constructs and Site-directed Mutagenesis—The US28 sequence was amplified by PCR using a cDNA derived from the HCMV genome, strain AD169 (GenBank™ accession number X17403) as template. The PCR product flanked by a 5′ XbaI and a 3′ HindIII restriction site was ligated into the mammalian expression vector pcDNA3.1(−) (Invitrogen). PCR was employed to generate US28 mutants with serine to alanine mutations at Ser-315, Ser-319, Ser-323, Ser-325, Ser-327, Ser-330, Ser-331, Ser-333, Ser-335, Ser-339, Ser-343, and Ser-350 and threonine to alanine mutations at Thr-337 and Thr-341 by using degenerated primers.

Plasmids containing FLAG-CCR5 and bovine GRK2 and GRK2/K220R have been described previously (15, 16). The NF-κB reporter plasmid 6NF-xBtkluc.neo was described elsewhere (17), and pRL-TK plasmid was obtained from Promega. A glutathione S-transferase-US28 fusion protein (511 N-terminal amino acids) in pGEX-2T1 (Amerham Biosciences) was expressed in Esherichia coli and purified according to standard procedures. All cDNA constructs were confirmed by sequencing.

Generation of Monoclonal Antibodies—The mAbs Tub-45 (IgG2b) and Tub-6 (IgG2b) used in this study were raised by immunizing C57BL/6 mice with purified glutathione S-transferase-US28 fusion protein in PBS and incomplete Freund’s adjuvant in three two-weekly intervals followed by three final boosts. Pools were formed according to standard procedures (18). Hybridoma supernatants were screened by direct enzyme-linked immunosorbent assay using glutathione S-transferase-US28 fusion protein or irrelevant glutathione S-transferase as the antigen. MAbs that reacted with PBS, 5% paraformaldehyde, PBS (w/v) for 20 min at room temperature. For intracellular staining, cells were permeabilized after fixation with PBS, 0.1% Triton X-100 for 15 min at room temperature. Cells were blocked by incubation with PBS, 3% rat serum, and permeabilized cells were incubated with the receptor-specific mAb for 60 min at room temperature. A 60-min incubation of a biotin-conjugated rat anti-mouse Ig antibody (Jackson ImmunoResearch) was followed by a final incubation with streptavidin-conjugated Alexa Fluor® 568 (Molecular Probes) for 30 min at room temperature. Slides were mounted in Moviol (Calbiochem) and analyzed by fluorescence microscopy on a Leica DM IRBE (Leica). Images were collected using a digital camera (AxioCam™, Zeiss).

For analysis of receptor expression by flow cytometry, cells were incubated with US28-specific mAb Tub-45 or anti-FLAG mAb (CCCR5-FLAG) M2 on ice for 30 min followed by incubation in 5% paraformaldehyde, PBS (w/v) for 20 min at room temperature. For intracellular staining, cells were permeabilized after fixation with PBS, 0.1% Triton X-100 for 15 min at room temperature. Cells were blocked by incubation with PBS, 5% rat serum, and permeabilized cells were incubated with the receptor-specific mAb for 60 min at room temperature. A 60-min incubation of a biotin-conjugated rat anti-mouse Ig antibody (Jackson ImmunoResearch) was followed by a final incubation with streptavidin-conjugated Alexa Fluor® 568 (Molecular Probes) for 30 min at room temperature. Slides were mounted in Moviol (Calbiochem) and analyzed by fluorescence microscopy on a Leica DM IRBE (Leica). Images were collected using a digital camera (AxioCam™, Zeiss).

For analysis of receptor expression by flow cytometry, cells were incubated with US28-specific mAb Tub-45 or anti-FLAG mAb M2 followed by incubation with fluorescein-conjugated goat anti-mouse Ig Abs (Jackson ImmunoResearch). Flow cytometric analysis was carried out using FACSCalibur™ (BD Biosciences). Hybridomas were screened by direct enzyme-linked immunosorbent assay using glutathione S-transferase-US28 fusion protein or irrelevant glutathione S-transferase as the antigen. MAbs that reacted with PBS, 5% paraformaldehyde, PBS (w/v) for 20 min at room temperature.
cell-associated activity). To remove surface-bound radioligand, the remaining wells were washed twice for 3 min in RPMI 1640 without bicarbonate, 0.2% bovine serum albumin, 10 mM MES, pH 2.7 (acid-resistant activity). After one wash with BM, cells were harvested as described above, and all lysates were transferred to tubes for γ-counting. The proportion of internalized radioligand was calculated by dividing the acid-resistant activity by the total cell-associated activity.

Statistics—Results are expressed as the mean ± S.D. Statistical significance was assessed by the Student’s t test.

RESULTS

Localization and Expression Kinetics of the Native US28 Receptor—It has been shown that epitope-tagged versions of the US28 receptor localize predominantly in intracellular organelles but only at marginal amounts at the plasma membrane, although this receptor efficiently binds its ligands and conveys signals to downstream effector molecules at a high rate (14). Because such unusual behavior of an epitope-tagged GPCR might not necessarily reflect the behavior of wild type (wt) receptor, we employed newly developed US28-specific mAbs to analyze native US28 expression in transient transfectants as well as in HCMV-infected fibroblasts. In flow cytometry analysis, US28 expression in transiently transfected HEK293A cells was detectable in permeabilized cells, whereas surface expression in non-permeabilized cells was marginal (Fig. 1A). To exclude a cell type-specific peculiarity, we also tested transiently transfected HeLa cells and COS cells, obtaining identical results compared with HEK293A cells (data not shown). US28 gene transcription and US28 functional activity has been described in HCMV-infected primary fibroblasts. Using the US28-specific mAb Tub-45 in immunoprecipitation of 35S-labeled cells, we were able to resolve the kinetics of US28 protein expression in HCMV-infected human fetal lung fibroblasts. More specifically, US28 could be detected by immunoprecipitation as early as 48 h after infection, with a peak expression at 72–96 h (Fig. 1B). This result supported our notion that the US28-specific mAbs Tub-45 and Tub-6 were able to detect native US28 receptor in transfected cell lines as well as in HCMV-infected primary cells.

US28 Receptor Exhibits High Basal Phosphorylation Levels—Because GPCR function and intracellular distribution are often regulated by receptor phosphorylation (19, 20), we analyzed such US28 receptor modification in transiently transfected HEK293A cells. Cells were labeled with 32P, and stimulated with various chemokines. Receptors were immunoprecipitated from cell lysates with the anti-US28 mAb Tub-45, which recognizes a US28-specific band of 36 kDa in SDS-PAGE (Fig 2A). We observed a high basal level of phosphorylation of US28, which could not be enhanced upon stimulation with the cognate ligands RANTES, MIP-1α, or the common PKC inducer PMA. It has been reported that the chemokine fractalkine acts as an inverse agonist on US28-mediated phospholipase C activation, resulting in down-modulation of phospholipase C-dependent second messenger molecules (12). Fractalkine, when added to transiently transfected HEK293A cells, resulted in partial inhibition of US28 basal phosphorylation (Fig. 2B). Compared with the physiological receptor for RANTES and MIP-1α (i.e. CCR5), which exhibited a ligand-dependent phosphorylation and a low basal phosphorylation (Fig. 2A), such constitutive ligand-independent phosphorylation is rather unique among chemokine receptors.

Basal Phosphorylation of US28 Is Modulated by Second Messenger-dependent Kinases—In general, phosphorylation of GPCRs is mediated by ligand-induced GRKs or, alternatively, by second messenger dependent kinases. To probe potential mechanisms of US28 phosphorylation, we analyzed the amino acid sequence at the C terminus for potential consensus sites, accessible to serine/threonine kinases. Serines at positions 327 and 333 are potential candidates for PKC, whereas serine-331 and threonine-337 are located at potential phosphorylation sites of cAMP/cGMP-dependent kinases. Consensus sequences for casein kinase 2 were identified for threonine at positions 337 and 341 and serine 333. To assess the contribution of various kinases to US28 phosphorylation, we made use of pharmacological inhibitors. Treatment with the broad spectrum protein kinase inhibitor staurosporine or bisindolylmaleimide, a PKC specific inhibitor, or the casein kinase 2-specific inhibitor 5,6-dichloro-1-β-d-ribofuranosylbenzimidazol (DRB) reduced US28 phosphorylation to 63, 59, or 68% of basal levels, respectively (Fig. 3A). In contrast, genistein treatment, an inhibitor of epidermal growth factor receptor kinase and other protein tyrosine kinases, had no inhibitory effect. We conclude that PKC and casein kinase 2 contribute partially to US28 receptor phosphorylation under agonist-independent conditions. None of the inhibitors tested was able to abrogate US28 phosphorylation completely, suggesting that alternative phosphorylation pathways must contribute to the constitutive phosphorylation of US28. The modulatory effect of the protein tyrosine kinase inhibitor, AG490, on basal US28 phosphorylation provides a potential link to the role of the Jak family tyrosine kinases (Fig. 3A).
Overexpression of GRK2 Enhances US28 Phosphorylation—
Among GRKs, the ubiquitously expressed GRK2 has a key role in regulating chemokine receptor phosphorylation (21). GRK-dependent phosphorylation of GPCRs could occur directly as phosphorylation of ligand-occupied receptor or indirectly as a result of GRK activation via PKC (19). Because basal US28 phosphorylation was partially PKC-dependent, we explored the ability of GRK2 to phosphorylate US28. Overexpression of GRK2 in HEK293A cells together with US28 resulted in a 2–3-fold increase in US28 phosphorylation. A dominant negative mutant of GRK2, GRK2/K220R, which lacks kinase activity, served as a negative control (Fig. 3B). Stimulation with RANTES did not significantly alter the phosphorylation status.

In contrast, CCR5, when coexpressed with GRK2, showed a 5–6-fold enhanced phosphorylation upon stimulation with RANTES.

Phosphoamino Acid Analysis and Identification of Phosphorylation Sites of US28—
The C-terminal domain of US28 contains 12 serine and 2 threonine residues that constitute potential phosphorylation sites for different kinases (Fig. 4A). To analyze which amino acids contribute to receptor phosphorylation in living cells, a two-dimensional phosphoamino acid analysis of US28 transfectants (Fig. 4B) was performed. We observed a predominance of serine phosphorylation, whereas threonine phosphorylation was also detectable but not on tyrosine residues. The C-terminal domain of US28 contains 12 serine and 2 threonine residues that constitute potential phosphorylation sites for different kinases (Fig. 4A). To analyze which amino acids contribute to receptor phosphorylation in living cells, a two-dimensional phosphoamino acid analysis of US28 transfectants (Fig. 4B) was performed. We observed a predominance of serine phosphorylation, whereas threonine phosphorylation was also detectable but not on tyrosine residues. To localize the phosphorylated amino acid positions in more detail, we substituted the C-terminal serine residues 3–5 (Ser-323, -325, -327), 6–8 (Ser-330, -331, -333), or 9–12 (Ser-338, -339, -343, -350) resulted in an impaired US28 phosphorylation (Fig. 4C). Because this alteration was strongest for the S9–12A mutant, amino acids positions 338–350 most likely contain the majority of phosphorylation sites. Mutagenesis of serines 1 and 2 (Ser-315 and -319) did not alter US28 phosphorylation at all, indi-
Labeled cells were stimulated for 10 min with RANTES (10 nM) or Ala mutant constructs were transfected into HEK293A cells. Cells were assessed by running phosphoamino acid standards. T

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Tub-45 followed by phosphoamino acid analysis. Positions of phosphoamino acids were determined using phosphoamino acid standards. Data represent means ± S.D. of three to five independent experiments, each in duplicate.

Fig. 5. US28-mediated NF-κB activation is preserved in phosphorylation-deficient mutants US28S1–12A and US28S12A. HEK293A cells were transiently transfected with 6NF-κBtkluc, pRL-TK, and either US28, US28S1–12A, or US28S12A, CXCR5, or pcDNA3.1 (−) (empty). 24 h after transfection, cells were stimulated with tumor necrosis factor-α (10 ng/ml) or fractalkine (100 nM) or remained unstimulated as indicated. Cells were harvested 6 h later, and the NF-κB-driven luciferase expression was determined (relative luciferase units (RLU)). Results are expressed as luciferase expression compared with control transfectants (empty). Data represent means ± S.D. of three to five independent experiments, each in duplicate.

Fig. 4. Mutagenesis of C-terminal Ser/Thr residues abrogates US28 phosphorylation. A, schematic representation of US28 C-terminal domain and receptor mutants. All 12 serine and threonine residues (bold letters) were mutagenized to alanine in various combinations. B, HEK293A cells were transiently transfected with US28 32P-labeled, and US28 receptor was immunoprecipitated with Tub-45 followed by phosphoamino acid analysis. Positions of phosphoserine (S), phosphothreonine (T), and phosphotyrosine (Y) were assessed by running phosphoamino acid standards. C, US28 Ser/Thr → Ala mutant constructs were transfected into HEK293A cells. Cells were 32P-labeled, and US28 receptor was immunoprecipitated with Tub-45 from cell lysates and subjected to SDS-PAGE. Phosphorylation levels of mutants were quantitated as described (Fig. 2). Data represent the means of three independent replicates ± S.D. Reduction of basal phosphorylation was calculated in relation to wild type US28 receptor.

cating that these two residues display no phosphoacceptor sites. This conclusion is further confirmed by the observation that the S3–12A mutant, which preserved these first two serines, was phosphorylated at comparable levels to mutant S1–12A, lacking all C-terminal serines. As expected from the phosphoamino acid analysis, remaining phosphorylation of mutants S3–12A and S1–12A might be due to phosphorylation at threonine residues. An additional substitution of the threonine residues at positions 337 and 341 (ST/A mutant) did not lead to a further reduction of US28 phosphorylation. We conclude that C-terminal serines (Ser-323–Ser-350) contribute to basal US28 phosphorylation. Upon stimulation with the cognate ligand RANTES, no alteration in the phosphorylation status of any mutant receptor was observed.

US28-induced NF-κB Activity Is Not Affected by Deletion of C-terminal Phosphorylation Sites—It has been demonstrated (12) that US28 constitutively activates phospholipase C and the transcription factor NF-κB in COS-7 cells, transiently expressing US28. This effect was partially inhibited by the CX3C chemokine fractalkine. To investigate whether US28-induced NF-κB activity is dependent on receptor phosphorylation, we performed a reporter-gene assay. Plasmids encoding 6NF-κBtkluc and either US28 wt or US28 (S1–12A; ST/A) mutants were cotransfected into HEK293A cells. We observed constitutive activation of NF-κB upon expression of US28 wt, and essentially no differences to the US28 mutants (S1–12A; ST/A) were seen (Fig. 5). We conclude that a decreased phosphorylation of US28 did not lead to an impaired signaling via activation of NF-κB. Furthermore, US28-dependent NF-κB activity of both the US28 wt (Fig. 5) and mutant receptors (data not shown) was not significantly modulated upon stimulation with RANTES or fractalkine.

Constitutive US28 Phosphorylation Regulates Cell Surface Expression and Endocytosis—Recently, it has been reported that epitope-tagged constructs or green fluorescent protein fusion proteins of US28 were expressed poorly at the cell surface and accumulated in intracellular organelles of transiently transfected HeLa cells (14). Reduction or even loss of surface expression has been observed for many GPCRs upon ligand stimulation. Such cellular redistribution is the result of ligand induced endocytosis, which is facilitated in many cases by GRK2-mediated receptor phosphorylation (20, 19). Because GRK2 was capable of phosphorylating US28, we asked if the high basal US28 phosphorylation level was responsible for receptor endocytosis and low receptor display at the plasma membrane.

US28 expression in transiently transfected HEK293A cells was detected in intracellular compartments, with the majority of the receptor localized in perinuclear structures, but not at the plasma membrane, as analyzed by immunocytochemistry (Fig. 6A). In contrast, the US28 mutant S1–12A, which lacks all C-terminal serine phosphorylation sites, showed a characteristic cell surface distribution, comparable with that observed for CXCR5 (Fig. 6A). To specifically address the influence of different C-terminal phosphorylation sites of US28 on plasma membrane localization, we tested all of the serine substitution mutants for cell surface expression. Flow cytometry analysis revealed that complete abrogation of serine phosphorylation (US28S1–12A, US28S12A) resulted in a maximal surface expression of US28 (Fig. 6B). By inhibition of de novo protein synthesis with cycloheximide, we could largely exclude that enhanced

2 T. Mokros and U. E. Hopken, unpublished observations.
Constitutive Phosphorylation of US28

It has been shown that the HCMV-encoded receptor US28 is constitutively active and undergoes rapid agonist-independent endocytosis. The mechanisms controlling such constitutive receptor down-regulation have not been characterized yet. Because agonist-dependent phosphorylation leads to rapid desensitization and internalization of GPCRs, we investigated whether constitutive signaling and internalization of US28 (12, 14) might be linked to enhanced basal phosphorylation levels. Considerable basal phosphorylation levels have been described for another chemokine receptor, CXCR4 (22), and for some constitutively active GPCR mutants (19). As expected, phosphorylation of these receptors was further enhanced upon stimulation with their cognate ligands.

In this study, we demonstrate that the virally encoded chemokine receptor US28 is constitutively phosphorylated. In contrast to the before-mentioned GPCRs, high basal phosphorylation of US28 in transiently transfected HEK293A cells was not altered upon stimulation with PMA or the cognate ligands RANTES and MIP-1α (Fig. 2A). Employing phosphoamino acid acid and mutational analysis, we identified phosphoserin located between Ser-323 and Ser-350 as the major phospho-acceptor sites on US28 (Fig. 4).

Surface phosphorylation might have an additive effect on surface expression of US28. US28 undergoes rapid constitutive endocytosis and recycling, as demonstrated by radiolabeled antibody uptake and internalization of radiolabeled specific ligand (14). To determine if high basal phosphorylation of US28 affects constitutive endocytosis, uptake of 125I-labeled RANTES was compared between US28 wt and US28 mutant transfectants. Radiolabeled ligand bound to US28 wt receptor underwent rapid endocytosis, and receptor endocytosis was maximal after 60 min when ~80% of the 125I-labeled RANTES initially bound to the cell surface was internalized. In contrast, mutation of phosphoacceptor sites in the C terminus of US28 (ST/A mutant) resulted in a significantly reduced 125I-labeled RANTES uptake already after 10 min (Fig. 7), suggesting that the endocytotic capacity of US28 is largely dependent on a constitutive receptor phosphorylation. To confirm that US28 wt and ST/A mutant receptor were expressed at comparable levels, we performed fluorescence-activated cell sorter analysis with both receptor variants (data not shown). Because constitutive endocytosis is significantly down-regulated in the ST/A mutant, we conclude that rapid endocytosis and low surface expression of the native US28 receptor might be due to its high basal phosphorylation.

**DISCUSSION**

It has been shown that the HCMV-encoded receptor US28 is constitutively active and undergoes rapid agonist-independent endocytosis. The mechanisms controlling such constitutive receptor down-regulation have not been characterized yet. Because agonist-dependent phosphorylation leads to rapid desensitization and internalization of GPCRs, we investigated whether constitutive signaling and internalization of US28 (12, 14) might be linked to enhanced basal phosphorylation levels. Considerable basal phosphorylation levels have been described for another chemokine receptor, CXCR4 (22), and for some constitutively active GPCR mutants (19). As expected, phosphorylation of these receptors was further enhanced upon stimulation with their cognate ligands.

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Second messenger-dependent kinases like PKC and PKA, activated by Gαi-coupled and Gαs-coupled receptors, respectively, can participate in the phosphorylation of GPCRs (19). Once activated, second messenger-dependent kinases obtain the potential to phosphorylate not only ligand-occupied, but also free receptors, provided that phosphorylation consensus sequences exist. US28 was recently reported to constitutively activate phospholipase C via Gα11 molecules in COS-7 cells (12). Thus, a phospholipase C-dependent activation of PKC could be hypothesized for the constitutive (ligand-independent) phosphorylation of US28. The PKC inhibitors staurosporine and bisindolylmaleimide used in our study partially inhibited consti-
Constitutive Phosphorylation of US28

tutive US28 phosphorylation, but they were not able to abrogate it completely (Fig. 3A). Thus, US28 mediated constitutive activation of phospholipase C, and subsequently PKC is not sufficient for a constitutive US28 phosphorylation in HEK293A cells. Together with our finding that inhibition of casein kinase 2 additionally decreased US28 phosphorylation, we propose that various second messenger-dependent kinases may participate in the regulation of agonist-independent US28 phosphorylation. This conclusion is supported by the observation that inhibition of tyrosine kinases with AG490 resulted in a 31% decrease in US28 phosphorylation (Fig. 3A). In the absence of tyrosine phosphorylation of US28, the influence of tyrosine kinases on US28 phosphorylation might be indirect.

In addition to constitutive signaling through the phospholipase C pathway, ligand-independent activation of the transcription factor NF-κB as well as cyclic AMP response element-binding protein (CREB) have been described (12, 23). We investigated the potential influence of constitutive receptor phosphorylation in regulating the activation of NF-κB-mediated transcription. US28 mutants (S1–12A, ST/A) and US28 wt were equally effective in activating NF-κB (Fig. 5), suggesting that constitutive NF-κB activity is largely independent from receptor phosphorylation. We further assessed the effects of the chemokine fractalkine on the constitutive activation of NF-κB, since a role of fractalkine as a partial inverse agonist has been suggested recently (12). However, we failed to observe a regulatory effect of fractalkine on NF-κB activity in our experimental system (Fig. 5). Because all former studies have been performed in COS-7 cells instead of HEK293A cells, NF-κB activation can be regulated by multiple pathways, which might differ depending on cell type,GPCRs, and heterotrimeric G-protein subunit composition. Such differences might also account for fractalkine-independent constitutive NF-κB activation in US28-transfected HEK293 cells. In contrast to second messenger-dependent kinases, GRKs have been shown to preferentially bind and phosphorylate agonist-occupied receptors (24). When coexpressed, the ubiquitously occurring GRK2 isoform significantly increased US28 phosphorylation. When compared with other GPCRs, GRK2 enhanced phosphorylation of US28 was not altered upon RANTES stimulation (Fig. 3B). It has been shown that PKCs can modulate GRK2 and PKC activity (25, 26), thus providing a protein kinase cascade that may represent an alternative mechanism for the observed partial PKC dependence of the constitutive US28 phosphorylation. Our results indicate that US28 is a substrate for GRK2 and that both ligand-occupied and free US28 receptor represent targets for GRK2 phosphorylation.

Epitope-tagged and fusion constructs of US28 exhibit low surface expression and localize predominantly to intracellular vesicles (14). Such modifications might not necessarily reflect the behavior of a native receptor; therefore, we made use of our novel mAb, which specifically recognizes the native US28 receptor in transiently transfected HEK293A cells as well as in HCMV-infected human fibroblasts (Fig. 1). Immunostaining showed a preferential intracellular localization of US28 in transiently transfected HEK293A cells. US28 is known to undergo rapid constitutive internalization and recycling (14). In general, internalization involves the GRK-dependent phosphorylation of activated GPCRs on cytoplasmic serine and threonine residues followed by the recruitment of β-arrestin and, subsequently, the internalization via clathrin-coated pits. Therefore, we asked if the constitutive phosphorylation of US28 is crucial for its rapid endocytosis and low surface expression. Flow cytometry analysis of US28 mutants revealed a reciprocal relationship between receptor surface display and number of phospho-acceptor sites. More specifically, maximal surface expression was detected for mutants with all C-terminal serines deleted (US28S1–12A and US28ST/A) (Fig. 6). The same mutants revealed a significant down-regulation of receptor endocytosis, as demonstrated by a reduced internalization of [125I]-labeled RANTES (Fig. 7). However, the effect observed was only incomplete, suggesting that endocytosis and subcellular redistribution of US28 might not be regulated exclusively by receptor phosphorylation. Various cellular factors can contribute additionally to the internalization of GPCRs, among them clathrin, AP-2, and dynamin (19). Amino acid motifs of the receptor C terminus might further enhance receptor internalization, as has been reported for CCR5 (27, 28) and CXCR4 (22). In view of the role of US28 in HCMV immune escape via chemokine sequestration, this study provides experimental evidence that constitutive receptor internalization and recycling are dependent on US28 phosphorylation.

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