G-protein-coupled Receptor (GPCR) Kinase Phosphorylation and β-Arrestin Recruitment Regulate the Constitutive Signaling Activity of the Human Cytomegalovirus US28 GPCR*

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Phosphorylation of G-protein-coupled receptors (GPCRs) by GRKs and subsequent recruitment of β-arrestins to agonist-occupied receptors serves to terminate or attenuate signaling by blocking G-proteins from further interaction with the receptors. Human cytomegalovirus encodes a GPCR termed US28 that is homologous to the human chemokine family of GPCRs but differs from the cellular receptors in that it maintains high constitutive activity in the absence of agonist. Although US28 is constitutively active, mechanisms that regulate this activity are unknown. We provide evidence that US28 is constitutively phosphorylated by GRKs in cells and that in consequence, β-arrestin 2 is localized to the plasma membrane. Deletion of the carboxyl terminal 40 amino acids in US28 generates a receptor that is severely impaired in its ability to become phosphorylated and recruit β-arrestin and accordingly demonstrates increased inositol phosphate signaling. This result indicates that the carboxyl terminus of US28 contains an important signaling regulatory region and mutational analysis deleting carboxyl terminal serines identified serine 323 as a critical residue within this region. In addition, overexpression of wild type GRK5 leads to hyperphosphorylation of US28 that results in a decrease of inositol phosphate accumulation. These results are consistent with the hypothesis that GRK phosphorylation and recruitment of β-arrestin to the US28 viral GPCR attenuates signaling to the traditional Goα-stimulated inositol phosphate pathway. Finally, in contrast to the results with inositol phosphate signaling, we provide evidence that the US28 carboxyl-terminal phosphorylation sites and β-arrestin-interacting domain are required for maximal activation of the p38 mitogen-activated protein kinase. Taken together, these results indicate that US28 interacts with these important regulatory proteins to control multiple aspects of signal transmission. Understanding the regulation of viral GPCRs by GRKs and β-arrestins will provide important new insights into not only aspects of viral pathogenesis but also basic mechanisms of receptor signaling.

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G-protein-coupled receptors (GPCRs) constitute a family of seven-transmembrane domain proteins that transmit signals to the interior of the cell by activating a variety of signaling pathways (1–3). Stimulation of GPCRs with agonist leads to increased GTPase activity of heterotrimeric G-proteins that in turn modulate downstream effectors such as the second messenger-generating adenyl cyclases and phospholipases. Phosphorylation of the receptors by GPCR kinases (GRKs) and recruitment of β-arrestins to the agonist-occupied receptors attenuates signaling by blocking G-proteins from further interaction with the receptors, a process also known as desensitization (4–7).

Many of the herpesviruses encode seven-transmembrane domain proteins that are homologous to the cellular chemokine family of GPCRs (8–13). The function of these virally encoded “receptors” is unknown, although speculation has been that they might enable the host virus to access additional tissue types in vivo and to evade immune surveillance (14–18). This is a particularly attractive hypothesis, since one of the hallmarks of the herpesviruses is their ability to set up a lifelong latent or persistent infection in the host. The genomes of human cytomegalovirus (HCMV), human herpesvirus 6, human herpesvirus 7, and human herpesvirus 8 all contain one or more open reading frames with homology to the chemokine receptors. Interestingly, several of these virally encoded GPCRs exhibit high levels of constitutive activity in the absence of agonist (19–23).

HCMV encodes four GPCR homologues in its genome, US27, US28, UL33, and UL78, whereas mouse cytomegalovirus and rat cytomegalovirus encode genes that correspond to UL33 and UL78 (12, 14, 16, 24). Studies using rodent cytomegaloviruses have indicated that the UL33 counterparts termed M33 and R33 are required for viral dissemination and replication in the salivary gland and are therefore important for virulence in vivo (14, 16). Recent biochemical studies indicate that the HCMV US28 and mouse cytomegalovirus M33 proteins share many common signaling activities, indicating that US28 may also play an important role in viral replication in vivo (25). Expression of HCMV US28 on the surface of leukocytes promotes cell rolling and adhesion in vitro models, whereas US28 expression in smooth muscle cells stimulates cell migration (25, 26).

The abbreviations used are: GPCR, G-protein-coupled receptor; GRK, G-protein-coupled receptor kinase; β2AR, β2 adrenergic receptor; IL8R2, interleukin-8 receptor type 2; RGS, regulator of G-protein signaling; RANTES, regulated on activation normal T cell expressed and secreted; HCMV, human cytomegalovirus; PBS, phosphate-buffered saline; WT, wild type; GFP, green fluorescent protein.
These studies suggest that US28 is important in promoting virus trafficking from the circulation to inflammatory sites and may have significant effects on HCMV-induced diseases such as atherosclerosis. US28 is closely related to the receptors for β-chemokines such as CCR1, and accordingly US28 binds to several chemokines including RANTES, MCP-1, and Fractalkine (13, 24, 27, 28). Although US28 binds to these chemokines, the receptor has high constitutive activity even in the absence of chemokine (19, 23). Some reports indicate that binding of RANTES or MCP-1 can increase US28 activity; however, this activity seems to vary with the cell type as well as the specific signaling pathway being activated (24, 29). Similar to other viral encoded GPCRs, US28 constitutively activates Goα-dependent signaling pathways that stimulate phospholipases, leading to elevation of inositol phosphate levels. US28 signaling also activates mitogen-activated protein kinase pathways as well as the cAMP-response element-binding protein and NF-κB transcription factors, although the mechanisms by which these pathways are regulated by US28 is less clear (19, 23, 29). US28 appears to be functionally similar to its cellular counterparts in that the receptor undergoes internalization via the cellular endocytic machinery; however, unlike the cellular GPCRs that are internalized in response to agonist stimulation, US28 appears to undergo constitutive internalization (30, 31). The data concerning constitutive signaling and constitutive internalization are consistent with the idea that US28 mimics the agonist occupied form of the receptor. In this respect, US28 appears to function quite similarly to cellular GPCRs except that it does so in the absence of agonist.

Desensitization is a key regulatory mechanism required to control the magnitude and duration of GPCR signaling (1). The important factors in controlling desensitization are the GRKs and β-arrestins. Currently there are seven known GRKs (GRK1-GRK7) and four arrestins (β-arrestin 1, β-arrestin 2, rod arrestin, and cone arrestin). The specificity of GRKs and arrestin family members for different receptors is just beginning to be ascertained, but it is clear that the limited number of GRK and arrestin proteins are responsible for regulating the much larger number of receptor proteins (32). Cellular GPCRs have differing numbers of GRK phosphorylation sites, and this appears to be important in the strength as well as duration of β-arrestin binding to receptor (33, 34). The strength of β-arrestin binding will likely prove to be important in determining the relative sensitivity of different receptors to desensitization. This paradigm of GRK phosphorylation followed by β-arrestin recruitment leading to desensitization has been experimentally defined in biochemical experiments and in cultured cells and has recently been confirmed by studies using knockout and transgenic mice (35–37). Deletion of GRK phosphorylation sites results in receptors that have stronger signaling responses to agonist stimulation in transfection studies (38–40). Recent experiments in GRK and arrestin knockout mice demonstrate hypersensitive responses to several agonists (36, 37).

US28 appears to mimic agonist-occupied receptors in that it uses traditional GPCR pathways to transmit signals and also undergo internalization via classic endocytic pathways. It is unknown if traditional regulatory proteins such as GRKs and β-arrestins modulate US28 signaling. It has recently been demonstrated that US28 is constitutively phosphorylated in cells; however, it remains unknown if this phosphorylation leads to β-arrestin binding or directly affects signaling to downstream effectors such as inositol phosphate (31). Accordingly, in this study we examine the effects of GRK phosphorylation and β-arrestin binding on several aspects of US28 signaling. The data indicate that US28 interacts with GRK and β-arrestin proteins and that these interactions have multiple effects on US28-directed signal transmission.

### EXPERIMENTAL PROCEDURES

**Cell Culture and Transfection**—All cell lines were obtained from the American Type Culture Collection (ATCC). HEK-293 cells were maintained in minimal essential medium supplemented with 10% fetal bovine serum and penicillin/streptomycin (Invitrogen). Cells were routinely subcultured 1:5 two times weekly. COS-7 cells were maintained in Dulbecco’s modified Eagle’s medium supplemented as above. Cells were routinely subcultured 1:10 three times weekly. HEK-293 cells were transfected in 100-mm dishes with 5 μg of total plasmid DNA using a modified calcium phosphate method. COS-7 cells were transfected in 100-mm dishes with 5 μg of total plasmid DNA and 20 μl of LipofectAMINE according to the manufacturer’s instructions. Cells were routinely harvested 48 h post-transfection.

**Plasmids**—The mammalian expression vector for US28 was derived from the HCMV strain VH/E. The US28 cDNA is cloned into the pcDNA3 mammalian expression vector and contains an amino-terminal FLAG tag and prolactin signal peptide followed by the US28 open reading frame. The US28(1–314), US28(1–322), US28(1–324), and US28(1–332) mutants were constructed by PCR incorporating a stop codon and XhoI restriction enzyme site into the antisense primer. All seven GRK cDNAs were confirmed using an automated ABI DNA sequencer (Howard Hughes Nucleic Acid Facility, Duke University). β-Arrestin 2 tagged with GFP was a kind gift of Marc Caron and Larry Barak (Duke University). pcDNA3-based expression plasmids for the βAR and IL8R2 and pRK5-based expression plasmids for GRK2 and GRK5 have been described previously (41, 42). The pRK5-based expression constructs containing the kinase-inactivating K220R (GRK2) and K215R (GRK5) have also been described (43). The p38α expression plasmid was a kind gift of Roger J. Davis.

**Inositol Phosphate Accumulation**—24 h post-transfection, cells were transferred to 12-well tissue culture plates and allowed to adhere. Cells were incubated for 18 h in complete medium containing 3 μC/m of [3H]inositol (PerkinElmer Life Sciences), washed once with ice-cold PBS, and whole cell extracts were prepared by lysing cells in 1 ml of lysis buffer (20 mM HEPES, 0.5% Triton X-100, 0.1 M formic acid, 1 M ammonium formate; and eluates were counted in a liquid scintillation counter.

**[32P]Orthophosphate Labeling and Receptor Immunoprecipitation**—48 h post-transfection, cells were starved in phosphate-free Dulbecco’s modified Eagle’s medium supplemented with 2% diazylated fetal bovine serum and 20 mM HEPES for 3 h. Reactions were stopped by aspirating medium, adding 1 ml of 0.4 M perchloric acid, and cooling undisturbed at 4 °C for 5 min. 800 μl of supernatant was neutralized with 400 μl of 0.2 M KOH, 0.8 M KHCO3, and subjected to centrifugation. 1 ml of supernatant was diluted with 3 ml of distilled H2O, and applied to freshly prepared Dowex columns (AG1-X8, Bio-Rad). Columns were washed two times with distilled H2O; total inositol phosphates were eluted with 4.0 ml of 0.1 M formic acid, 1 mM ammonium formate, and eluates were counted in a liquid scintillation counter.

**[32P]Orthophosphate Labeling and Receptor Immunoprecipitation**—48 h post-transfection, cells were starved in phosphate-free Dulbecco’s modified Eagle’s medium supplemented with 2% diazylated fetal bovine serum and 20 mM HEPES, 0.5% Nonidet P-40, 250 mM NaCl, 10% glycerol, 2 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, 2.5 μg/ml aprotinin, 25 μg/ml leupeptin, 100 μM sodium orthovanadate, and 50 mM sodium fluoride for 20 min at 4 °C. The supernatant was clarified by centrifugation and stored at 80 °C until use. FLAG-tagged receptors were immunopurified from the supernatant with 20 μl of anti-FLAG M2 agarose beads and washed once with PBS for 30 min. 800 μl of supernatant was neutralized with 400 μl of 0.2 M KOH, 0.8 M KHCO3, and subjected to centrifugation. 1 ml of supernatant was diluted with 3 ml of distilled H2O and applied to freshly prepared Dowex columns (AG1-X8, Bio-Rad). Columns were washed two times with distilled H2O; total inositol phosphates were eluted with 4.0 ml of 0.1 M formic acid, 1 mM ammonium formate, and eluates were counted in a liquid scintillation counter.

**Immunoblotting and Detection of Phosphorylated p38α**—48 h post-transfection, cells were harvested directly in 3× Laemml sample buffer and briefly sonicated, and the cell extracts were subjected to SDS-PAGE. Resolved proteins were transferred to nitrocellulose filters (Schleicher and Schuell), and nonspecific reactivity was blocked with Tris-buffered saline containing 0.1% Tween 20 (TBST) and 5% nonfat dried milk. Anti-serum directed against the phosphorylated form of p38 was purchased from Cell Signaling Technology, Beverly, MA. Reactive proteins were detected using the appropriate secondary antibodies in the enhanced chemiluminescence system (ECL, Amersham Biosciences).

**Fluorescence-activated Cell Sorting Analysis of Cell Surface US28 Expression and Confocal Microscopy**—Transfected cells were trans-
The HCMV US28 viral GPCR maintains constitutive signaling and is phosphorylated in the absence of agonist. A, HEK-293 cells were transfected with empty vector (MOCK) or FLAG-US28, and identical populations of cells were either left untreated or stimulated with 10 nM RANTES. Inositol phosphates were isolated using anion exchange chromatography, and the results represent the mean induction ± S.E. of three independent experiments performed in duplicate (left panel). HEK-293 cells were transfected with empty vector (Mock), FLAG-US28, FLAG-IL8R2, or FLAG-β2AR, labeled with 250 μCi/ml [32P]orthophosphate, and either left untreated or stimulated for 5 min with the appropriate ligand (10 nM RANTES, 10 nM GROα, or 10 μM ISO, respectively). Receptors were immunoprecipitated with anti-FLAG affinity gel, and phosphorylated receptors were visualized by autoradiography (right panel). The autoradiograph shown is representative of two or three independent experiments.

RESULTS

HCMV US28 Is Constitutively Phosphorylated in the Absence of Agonist—Stimulation of cellular GPCRs with agonist leads to a rapid and transient increase in signaling activity. The transient nature of this increase in signaling activity is due to the fact that activated receptors are strong targets for phosphorylation by GRK proteins and subsequent interaction with β-arrestin proteins. The interaction of GPCRs with these regulatory proteins leads to the dissociation of receptor from its cognate G-protein and effectively terminates signal transduction. Although the HCMV-encoded US28 GPCR has been shown to maintain strong constitutive signaling capacity in the absence of agonist, it remains unknown whether phosphorylation and β-arrestin binding regulate this signaling. To determine whether US28 signaling is regulated by phosphorylation and β-arrestin binding, we first analyzed its constitutive signaling activity. Since US28 appears to be predominantly coupled to Goq proteins, we analyzed the ability of US28 to induce accumulation of inositol phosphates, a classical readout for Goq signaling. US28 induces a 9.3-fold increase in the level of inositol phosphates when compared with mock-transfected HEK-293 cells (Fig. 1A, left panel). Stimulation with the CC chemokine RANTES has only a weak effect on the ability of US28 to induce inositol phosphate accumulation in HEK-293 cells. To analyze US28 phosphorylation, HEK-293 cells expressing US28, IL8R2, or β2AR were labeled with [32P]orthophosphate and stimulated with the appropriate ligand, and the receptors were immunoprecipitated. As expected, both the IL8R and the β2AR exhibited weak receptor phosphorylation that is strongly stimulated by agonist (Fig. 1A, right panel). Interestingly, US28 is strongly phosphorylated in the absence of exogenous ligand, and this phosphorylation appears to be unaffected by the β-chemokine RANTES. We also analyzed signaling and phosphorylation in COS-7 cells.

ferred into six-well dishes 24 h after transfection. 48 h after transfection, medium was aspirated, and cells were incubated with anti-FLAG primary antibody diluted 1:500 in PBS on ice for 2 h, washed once in PBS, and incubated with fluorescein isothiocyanate-conjugated anti-mouse antibody diluted 1:250 in PBS on ice for 2 h, washed once in PBS, and incubated with fluorescein isothiocyanate-conjugated antiamouse antibody diluted 1:250 in PBS on ice for 1 h. Cells were fixed in phosphate-buffered saline containing 4% formaldehyde, and the presence of cell-surface receptors was determined by flow cytometry (Duke University Flow Cytometry Facility). For confocal microscopic localization of β-arrestin, HEK-293 cells were transfected with the appropriate combination of β-arrestin 2-GFP, US28, and GRK plasmids. 24 h after transfection, cells were transferred into collagen-coated glass bottom dishes. 48 h after transfection, confocal microscopy was performed on a Zeiss LSM510 laser-scanning microscope using a × 100/1.4 numerical aperture oil immersion lens.
GRK and β-Arrestin Regulation of US28 Signaling

Along with GRK2 or GRK5 and labeled with 250

transfected with empty vector (MOCK). The amino acid substitutions that lead to the inactivation contains an inactive amino-terminal RGS domain and a central catalytic domain. The amino acid substitutions that lead to the inactivation are indicated. B, COS-7 cells were transiently transfected with empty vector (MOCK), FLAG-US28, or FLAG-US28 along with GRK2 or GRK5 and labeled with 250 μCi/ml [32P]orthophosphate. Receptors were immunoprecipitated with anti-FLAG affinity gel, and phosphorylated receptors were visualized by autoradiography. The autoradiograph shown is representative of at least five independent experiments.

The constitutive phosphorylation of US28 is strongly enhanced by GRK expression. A, structure of the GRK2 and GRK5 proteins. The GRKs are multifunctional proteins that have several structural domains in addition to the catalytic kinase domain. GRK2 contains an active amino-terminal RGS domain, a central catalytic domain, and a carboxyl-terminal pleckstrin homology domain. GRK5 contains an inactive amino-terminal RGS domain and a central catalytic domain. The amino acid substitutions that lead to the inactivation of the catalytic domains are indicated. B, COS-7 cells were transiently transfected with empty vector (MOCK), FLAG-US28, or FLAG-US28 along with GRK2 or GRK5 and labeled with 250 μCi/ml [32P]orthophosphate. Receptors were immunoprecipitated with anti-FLAG affinity gel, and phosphorylated receptors were visualized by autoradiography. The autoradiograph shown is representative of at least five independent experiments.

We hypothesized that if GRKs were capable of phosphorylating US28, overexpression of GRK2 or GRK5 would lead to an increase in observable US28 phosphorylation. US28 was expressed in COS-7 cells either alone or co-transfected with either GRK2 or GRK5, cells were metabolically labeled with [32P]orthophosphate, and the level of US28 phosphorylation was analyzed in US28 immunoprecipitates (Fig. 2B). GRK2 is a very potent stimulator of US28 phosphorylation, whereas GRK5 is less potent but still strongly increases US28 phosphorylation. These experiments indicate that GRKs are capable of phosphorylating US28 and suggest that at least some of the basal US28 phosphorylation observed in cells is due to the activity of endogenous GRKs.

β-Arrestin 2 is Constitutively Recruited to US28 in the Absence of Agonist—Agonist-induced phosphorylation of cellular GPCRs typically results in the rapid recruitment of β-arrestins to the membrane-associated receptor complex. Although US28 appears to be localized to several cellular organelles, previous reports have indicated that ~20% of US28 is localized to the plasma membrane (30). To determine whether β-arrestins are also recruited to US28, we analyzed the subcellular localization of a GFP-tagged β-arrestin protein using confocal microscopy (Fig. 3). β-Arrestin 2-GFP is distributed uniformly in the cytoplasm of mock-transfected cells that do not contain US28 (Fig. 3, left panel). Expression of US28 induces translocation of β-arrestin 2-GFP to the plasma membrane (Fig. 3, middle panel). This translocation can be visualized as punctate localization of β-arrestin 2 at the cell periphery and is indicated by arrows. Expression of GRK2 leads to hyperphosphorylation of US28 and more robust recruitment of β-arrestin (Fig. 3, right panel). These results, taken together with the phosphorylation studies, indicate that although US28 is a constitutively active receptor, cellular regulators of GPCR activity such as GRKs and β-arrestins retain the ability to interact with this viral encoded GPCR.

Construction of US28 Deletion Mutants and Localization of US28 Phosphorylation Sites—A large number of seven membrane-spanning GPCRs contain Ser/Thr phosphorylation sites in the carboxyl-terminal tail. The carboxyl terminal tail of US28 is 58 amino acids long, of which 14 are potential sites of Ser/Thr phosphorylation. To analyze the effects of carboxyl-terminal Ser/Thr residues on US28 phosphorylation and signaling, we constructed four mutants, US28(1–314), US28(1–322), US28(1–324), and US28(1–332) (Fig. 4). The 1–314 mutant is deleted for all of the carboxyl-terminal Ser/Thr residues, whereas the other mutants contain two (1–322), three (1–324), and seven (1–332) carboxyl-terminal Ser/Thr residues.

To determine the phosphorylation status of mutant receptors, we expressed US28 (WT) as well as US28(1–322) and US28(1–332) in COS-7 cells and immunoprecipitated receptors from [32P]orthophosphate-labeled cells. As expected from earlier experiments, US28 (WT) is highly phosphorylated when expressed in COS-7 cells (Fig. 5). The majority of US28 phosphorylation is located between amino acids 333 and 354, since US28(1–332) is largely defective in its ability to undergo phosphorylation. US28(1–314) exhibits a phosphorylation status slightly weaker than that of US28(1–332), indicating that although the majority of US28 phosphorylation is between amino acids 333 and 354, residues between amino acids 315 and 332 are also weakly phosphorylated. Cell surface expres-
potential sites of serine and threonine phosphorylation are marked by arrows. respectively, from the carboxyl terminus of US28. The locations of US28-(1–314). These mutants delete 22, 30, 32, and 40 amino acids, and phosphorylated receptors were visualized by autoradiography. The images shown are representative of 3–5 independent experiments.

Since phosphorylation of the US28 carboxyl terminal mutant was severely impaired, we sought to determine whether receptor that demonstrated an intermediate level of phosphorylation and retained the ability to interact with β-arrestin 2 and US28-(1–324) mutant that exhibited low level phosphorylation and is unable to interact with β-arrestin induced levels of inositol phosphate accumulation 8.7-fold over control cells. This activity exhibited by US28-(1–314) is roughly 50% greater than that of US28 (WT) and supports the hypothesis that this mutant is unable to undergo regulation by GRK and β-arrestin proteins.

GRK and β-Arrestin Regulation of US28 Signaling

**Fig. 3.** β-Arrestin 2 is constitutively recruited to phosphorylated US28. HEK-293 cells were transiently transfected with empty vector and β-arrestin 2-GFP (MOCK, left panel), FLAG-US28, and β-arrestin 2-GFP (US28, middle panel) or FLAG-US28, GRK2, and β-arrestin 2-GFP (US28 + GRK2, right panel). Subcellular localization of β-arrestin 2-GFP was localized using a Zeiss Axiomat confocal microscope. The confocal images shown are representative of 3–5 independent experiments.

**Fig. 4.** Structure of the US28 carboxyl-terminal domain. The carboxyl-terminal domain of US28 contains 12 serines and 2 threonines that could be potential phosphorylation sites. Four mutants were generated for this study: US28(1–332), US28(1–324), US28(1–322), and US28(1–314). These mutants delete 22, 30, 32, and 40 amino acids, respectively, from the carboxyl terminus of US28. The locations of potential sites of serine and threonine phosphorylation are marked by arrows.

**Fig. 5.** Localization of US28 phosphorylation sites to the carboxyl-terminal domain. COS-7 cells were transiently transfected with empty vector (MOCK), FLAG-US28 (WT), FLAG-US28(1–314), and FLAG-US28(1–332) and labeled with 250 μCi/ml [32P]orthophosphate. Receptors were immunoprecipitated with anti-FLAG affinity gel, and phosphorylated receptors were visualized by autoradiography. The autoradiograph shown is representative of at least four independent experiments.

This finding is similar to those obtained for carboxyl-terminal mutants of other receptors and prompted us to analyze the signaling properties of the carboxyl-terminal US28 mutants.

The US28(1–314) Mutant Exhibits Increased Signaling to the Inositol Phosphate Pathway—The duration and magnitude of signaling mediated by agonist activated GPCRs is regulated largely by phosphorylation of activated receptors by GRKs and subsequent interaction with β-arrestins. Our data indicate that US28 is constitutively phosphorylated and bound by β-arrestin 2. We therefore hypothesized that the US28(1–314) mutant that is unable to be phosphorylated or recruit β-arrestin might have significantly altered signaling properties. Wild type and mutant US28 proteins were expressed in COS-7 cells and analyzed for their abilities to induce accumulation of inositol phosphates (Fig. 7). US28 (WT) induced inositol phosphate accumulation 5.7-fold over control cells. The US28(1–332) mutant that demonstrated an intermediate level of phosphorylation and retained the ability to interact with β-arrestin 2 induced inositol phosphate accumulation slightly weaker than US28 (WT). Interestingly, the US28(1–314) mutant that exhibits low level phosphorylation and is unable to interact with β-arrestin induced levels of inositol phosphate accumulation 8.7-fold over control cells. This activity exhibited by US28(1–314) is roughly 50% greater than that of US28 (WT) and supports the hypothesis that this mutant is unable to undergo regulation by GRK and β-arrestin proteins.

These data indicate that residues between amino acids 315 and 332 contain important determinants for regulation of US28 signaling to inositol phosphates, so we constructed several additional mutants that systematically delete the serine residues between amino acids 315 and 332 (Fig. 4). US28(1–322) and US28(1–324) delineate the important residues required for this regulation, since US28(1–322) induces accumulation of inositol phosphates 8.3-fold over control cells, whereas the US28(1–324) mutant induced accumulation of inositol phosphates only 3.6-fold over control cells (Fig. 7). These experiments indicate that serine 323 is important for attenuation and regulation of US28 signaling, since the US28(1–322) mutant exhibits the increased signaling phenotype similar to US28(1–314), whereas the US28(1–324) mutant exhibits signaling equivalent to or less than that of US28 (WT).

**Effect of GRKs on US28(1–314) Signaling to the Inositol Phosphate Pathway—**Previous studies have indicated that...
overexpression of wild type or kinase-inactive GRK2 blocks signaling from US28 (19). Based on these studies, it is unclear whether GRK phosphorylation plays a significant role in US28 signaling, since both wild type and kinase-inactive GRK2 blocked US28 signaling to the same degree. We analyzed the ability of US28 (WT) and US28-(1–314) to induce inositol phosphate accumulation in the presence of either GRK2 or GRK5 (Table I). In agreement with previous studies, US28 (WT) signaling is strongly inhibited by either wild type GRK2 (52–62% inhibition) or kinase-inactive GRK2 (40–65% inhibition). This effect is dose-dependent, since increasing amounts of GRK more strongly inhibited US28 signaling. We next analyzed the US28-(1–314) mutant that is only minimally phosphorylated in cells to determine whether GRK2 overexpression would affect signaling of this mutant. Since the effect of GRK2 is probably attributable to the RGS domain, we expected wild type and kinase-inactive GRK2 to potently inhibit signaling of US28-(1–314). US28-(1–314) signaling is inhibited similar to US28 (WT) by either wild type GRK2 (47–61% inhibition) or kinase-inactive GRK2 (40–60% inhibition). These data support our hypothesis that the effects of overexpressed GRK2 in this system are largely independent of its activity as a GPCR kinase.

GRK5, on the other hand, is unable to function as a G protein-sequestering protein, since it contains an inactive RGS domain in its amino terminus (Fig. 2) and is therefore a useful tool to analyze the specific effects of GRK kinase activity on US28 signaling. Overexpression of wild type GRK5 potently inhibited US28 (WT) signaling (34–66% inhibition), but kinase inactive GRK5 had little effect and actually slightly enhanced signaling at a low concentration of transfected DNA (9% increase). These results indicate that GRK phosphorylation of US28 is capable of regulating US28 signaling and that the generation of the minimally phosphorylated US28-(1–314) mutant is a useful reagent to study US28 signaling in the absence of regulation by phosphorylation.

The US28-(1–314) Mutant Exhibits Decreased Signaling to the p38α MAP Kinase—We next sought to determine whether activation of the MAP kinase p38α pathway is induced by US28 and to examine the effect of deletion of the carboxyl terminus on this process. Using chemical inhibitors of p38, it has been reported that US28-mediated activation of the p38 pathway is partially involved in stimulation of the cAMP-response element-binding protein transcription factor (23). These experiments do not specifically assess p38 activation, so it remains unknown whether US28 can induce phosphorylation and hence activation of p38. We transfected increasing amounts of the US28 expression plasmid (0.03–1.0 μg) along with p38α and used a phosphospecific antibody to explore the ability of US28 to activate p38α (Fig. 8A). US28 potently induces phosphorylation of p38α, indicating that this viral GPCR constitutively initiates a signaling event that leads to activation of the p38 pathway. Interestingly, although US28-(1–314) is capable of activating p38α, the magnitude of this activation is decreased in comparison with US28 (WT). This decreased ability of US28-(1–314) to activate p38 is particularly evident using 1 μg of transfected DNA, and quantitation of phospho-p38 levels indicates that US28-(1–314) exhibits a 34% defect compared with US28 (WT) in its ability to activate this signaling pathway (Fig. 8B). The analysis of p38 signaling is in sharp contrast to inositol phosphate signaling, since the US28-(1–314) mutant demonstrates increased ability to induce inositol phosphate accumulation. These data indicate that US28 activation of
COS-7 cells were transiently transfected with FLAG-US28 (WT) or FLAG-US28-(1–314) along with increasing amounts of wild type and kinase-inactive GRKs. 48 h after transfection, inositol phosphates were allowed to accumulate for 3 h in the presence of 20 mM LiCl, and total inositol phosphates were isolated using anion exchange chromatography. The results are presented in table form and represent the effect of GRK proteins relative to US28 (WT) or US28-(1–314) alone. The numbers reported are the percent change in signaling ± S.E. of three or four independent experiments performed in duplicate.

| Amount of GRK | US28 (WT) % | US28-(1–314) % |
|---------------|-------------|----------------|
| 0.5 μg of GRK2 (wild type) | (-) 52 ± 11 | (-) 47 ± 9 |
| 2.5 μg of GRK2 (wild type) | (-) 62 ± 3 | (-) 61 ± 3 |
| 0.5 μg of GRK2 (kinase-inactive) | (-) 40 ± 8 | (-) 40 ± 6 |
| 2.5 μg of GRK2 (kinase-inactive) | (-) 65 ± 2 | (-) 60 ± 4 |
| 0.5 μg of GRK5 (wild type) | (-) 34 ± 6 | ← |
| 2.5 μg of GRK5 (wild type) | (-) 66 ± 3 | ← |
| 0.5 μg of GRK5 (kinase-inactive) | (+) 19 ± 16 | (+) 19 ± 11 |
| 2.5 μg of GRK5 (kinase-inactive) | (-) 2 ± 11 | (-) 3 ± 7 |

![Diagram](http://www.jbc.org/)

**Table 1: Inhibition of US28 signaling by GRK overexpression**

**Discussion**

We present evidence that the virally encoded GPCR US28 is constitutively phosphorylated and recruits β-arrestin in the absence of agonist. The data indicate that the GRKs are capable of inducing strong phosphorylation of Ser/Thr residues in the carboxyl terminal tail of US28, subsequently leading to β-arrestin recruitment. Deletion of the phosphorylation sites in the US28 carboxyl terminal tail results in a mutant, US28-(1–314), that demonstrates weak overall phosphorylation, fails to recruit β-arrestin, and accordingly exhibits enhanced signaling capacity to the second messenger inositol phosphate. Additional mutational analysis identifies serine 323 as a critical residue involved in US28 signaling. Moreover, the US28-(1–314) mutant exhibits decreased signaling to the MAP kinase p38, suggesting that the US28-directed p38 pathway is regulated differently than the inositol phosphate pathway. This regulation of signal transduction by GRK and β-arrestin proteins is typical of a variety of cellular GPCRs and can now be extended to include the viral family of GPCRs (1, 39).

The data presented here are largely in agreement with a very recent study that examines the effects of carboxyl-terminal deletion mutants on cell surface expression of US28 (47). This study demonstrates that the US28 carboxyl terminus contains determinants for constitutive internalization, thereby affecting the magnitude of signaling to inositol phosphates. However, the mutants we generated and studied in this report exhibit cell surface expression patterns very similar to wild type US28 and indicate that the altered signaling we observe is due specifically to the fact that the US28-(1–314) mutant is unable to engage GRK and β-arrestin proteins.

Our data indicate that in cells expressing constitutively active US28, the receptor is constantly undergoing phosphorylation and regulation by the cellular machinery. This is somewhat surprising, since the constitutive nature of US28 might suggest that these viral receptors have evolved a mechanism to avoid being regulated by cellular proteins such as GRKs and arrestins. One speculation is that the viral proteins need to be regulated to prevent chronic hyperstimulation of signaling pathways that are not beneficial for viral replication and infection. Mammalian cells have evolved numerous mechanisms to prevent aberrant chronic activation of signaling pathways. By engaging cell regulatory machinery, US28 may be able to signal only to moderate levels, thus potentially avoiding recognition by apoptotic or other cell defense machinery.

US28 appears to maintain an active conformation, so that immediately upon synthesis and trafficking to the plasma membrane, US28 is capable of engaging G-proteins and activating signal transduction pathways. Since the active conformation of US28 is also a substrate for phosphorylation, the receptor becomes GRK-phosphorylated and bound by β-arrestin. The β-arrestin-bound receptor complex is therefore sterically inhibited from interaction with G-proteins, and traditional signaling through these G-proteins is terminated. In direct support of this model are the data obtained with overexpression of GRKs. As the level of GRK proteins in the cell is raised (in this case by gene transfection experiments), the ratio of phosphorylated to unphosphorylated receptor increases, and the magnitude of signaling activity decreases. This may also be important with regard to the total constitutive activity of these two signaling pathways is controlled by distinct mechanisms. Whereas the GRKs and β-arrestins are critical negative regulators of the traditional Goα-stimulated inositol phosphate pathway, it appears that the GRKs and/or β-arrestins play positive roles in activation of the p38 pathway.

**Fig. 8.** US28-(1–314) mutant exhibits decreased signaling to p38α MAP kinase. COS-7 cells were transiently transfected with increasing amounts (0.03–1.0 μg) of FLAG-tagged US28 (WT) and US28-(1–314) proteins. A, 48 h after transfection, cell extracts were analyzed by immunoblot using a phosphospecific p38 antibody. B, the results are presented graphically and represent the mean ± S.E. of four or five independent experiments. *, p < 0.05, comparing cells expressing 1 μg of US28-(1–314) with cells expressing 1 μg of US28 (WT).

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served with these receptors in different cell types. The magnitude of US28 signaling in cells in vivo may be directly correlated with the relative amount of GRKs expressed, so that cells expressing low levels of GRK may have high levels of constitutive inositol phosphate signaling and vice versa. Interestingly, data obtained with the HHV8-encoded ORF74 protein indicate that this viral receptor constitutively induces inositol phosphate production in COS-7 and HEK-293 cells but only induces inositol phosphate production in an agonist-dependent manner in lung endothelial cells (21, 22, 48). It will be interesting to determine whether GRK phosphorylation and β-arrestin binding also regulate the ORF74 viral GPCR and to analyze the relative effects of GRKs on US28 signaling in additional cell types.

It is interesting that the GRKs have evolved multiple mechanisms to shunt off GPCR signaling through traditional G-protein signaling pathways. The presence of multiple mechanisms of GRK regulation is especially evident when studying the constitutive viral receptor US28 in comparison with the carboxyl terminal mutants that are impaired in phosphorylation. The discovery of the RGS homology domain in the amino terminus of GRK2 first indicated that the mechanisms of GRK-mediated inhibition of GPCR signaling might extend beyond the ability to phosphorylate the receptor (44, 45). We and others have shown that kinase-inactive GRK2 (K220R) inhibits US28 signaling to the same degree as wild type GRK2 (19). This kinase-independent mechanism can probably be attributed to the RGS homology domain in the amino terminus of GRK2, which potently binds Goα proteins, preventing receptors from interacting with and activating the G-protein. The RGS homology domain in GRK2 only inhibits signaling involving Goα proteins, since it does not interact to any measurable degree with other G-proteins. This effect of GRK2 is therefore specific to Goα signaling. GRK5-mediated inhibition of US28 signaling utilizes the more traditional mode of signaling inhibition by GRKs. Whereas wild type GRK5 potently inhibits US28 signaling, the kinase-inactive mutant of GRK5 (K215R) is unable to inhibit US28 signaling. In this case, the mechanism of inhibition by GRK5 can be attributed to its ability to phosphorylate US28. This traditional mechanism of inhibition is more widespread and applies to GPCR signaling in general, since it is not specific for Goα-mediated signaling pathways. US28 has been reported to also signal through Goα and Goα16 proteins, so the different GRKs could certainly have different effects on US28 signaling depending on the specific pathway and G-protein being used.

Accumulating evidence indicates that in addition to their roles in the termination of G-protein signaling, the GRKs and β-arrestins also function as adapter proteins to couple GPCRs to additional types of signaling pathways (49, 50). GRKs have been shown to interact with or phosphorylate several nonreceptor proteins, including tubulin, synuclein, and GIT-1 (51–53). The β-arrestins appear to be important adapter proteins involved in coupling receptors to a variety of signaling pathways including most notably the Src tyrosine kinases and several MAP kinases (54). GRKs and β-arrestins have also been shown to be important in interacting with members of the endocytic machinery and are important in promoting receptor internalization (55).

We have shown in this study that the viral US28 GPCR is engaged and regulated by the GRKs and β-arrestins. Moreover, deletion of the carboxyl terminal phosphorylation and β-arrestin binding sites generates a US28 protein with enhanced inositol phosphate signaling capacity due to an inability to be regulated by GRKs and β-arrestins. Interestingly, this same mutant demonstrates a defect in its ability to induce high levels of the p38 MAP kinase in comparison with wild type US28. This result is consistent with the hypothesis that US28 uses a β-arrestin scaffold to induce maximal levels of the p38 MAP kinase similar to that observed with extracellular signal-regulated kinase and c-Jun NH2-terminal kinase activation by cellular GPCRs such as the angiotensin AT1 receptor (56, 57).

Several cellular chemokines including RANTES, MCP-1, and fractalkine interact with US28 (24, 27, 28). We have shown that RANTES modestly increases US28-induced signaling to inositol phosphates but has no detectable effect on US28 phosphorylation. US28 has demonstrable capacity to internalize these chemokines, so it will be interesting to see how the binding of chemokines in addition to RANTES affects receptor phosphorylation. In particular, fractalkine partially inhibits US28 signaling, and it would be interesting if this chemokine induced additional phosphorylation leading to decreased signaling capacity.

The phosphorylation and regulation of US28 by GRKs is unlikely to be unique to US28 within the viral GPCR subfamily. The other herpesviral GPCRs also contain a significant number of serine and threonine residues in their carboxyl terminal domains. The HHV8-encoded ORF74 protein has six potential sites in the carboxyl-terminal region, whereas the HHV6-encoded U12 protein contains nine sites and the HCMV-encoded UL33 contains 20 sites. Since these other viral proteins signal in a similar manner to US28, it seems reasonable to speculate that the other viral GPCRs might be regulated by cellular GRKs and β-arrestins. Particularly interesting is the ORF74 protein that has demonstrable oncogenic activities in vitro, interacts with c-Src, and activates several MAP kinase signaling pathways (58, 59). Recent evidence linking GRKs and β-arrestins to c-Src and MAP kinase signaling pathways suggests that these cell-regulatory proteins may play an important role in ORF74 signaling.

Taken together, these findings indicate that phosphorylation of US28 and regulation of signaling by GRK and β-arrestin proteins play an important role in US28-mediated signal transduction. We have identified a US28 mutant that demonstrates altered signaling through inositol phosphate and p38 MAP kinase pathways due to deletion of the key determinants required for engagement of cellular GRK and β-arrestin proteins and have defined several mechanisms used by GRKs to regulate US28 signaling. Future studies on the role of GRK- and β-arrestin-mediated regulation should provide considerable new insight not only into the mechanism(s) by which US28 activates cellular signaling pathways but also into how cellular proteins such as GRKs and β-arrestins function in mediating GPCR-mediated signal transduction in general.

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G-protein-coupled Receptor (GPCR) Kinase Phosphorylation and β-Arrestin Recruitment Regulate the Constitutive Signaling Activity of the Human Cytomegalovirus US28 GPCR

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