THE JOURNAL OF BIOLOGICAL CHEMISTRY Vol. 278, No. 21, Issue of May 23, pp. 19473–19482, 2003

Printed in U.S.A.

The Carboxyl Terminus of Human Cytomegalovirus-encoded 7 Transmembrane Receptor US28 Camouflages Agonism by Mediating Constitutive Endocytosis*

Received for publication, December 26, 2002, and in revised form, March 13, 2003
Published, JBC Papers in Press, March 18, 2003, DOI 10.1074/jbc.M213179200

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US28 is one of four 7 transmembrane (7TM) chemokine receptors encoded by human cytomegalovirus and has been shown to both signal and endocytose in a ligand-independent, constitutively active manner. Here we show that the constitutive activity and constitutive endocytosis properties of US28 are separable entities in this viral chemokine receptor. We generated chimeric and mutant US28 proteins that were altered in either their constitutive endocytic (US28Δ300, US28Δ317, US28-NK$_1$-ctail, and US28-ORF74-ctail) or signaling properties (US28R129A). By using this series of mutants, we show that the cytoplasmic tail domain of US28 *per se* regulates receptor endocytosis, independent of the signaling ability of the core domain of US28. The constitutive endocytic property of the US28 c-tail was transposable to other 7TM receptors, the herpes virus 8-encoded ORF74 and the tachykinin NK$_1$ receptor (ORF74-US28-ctail and NK$_1$-US28-ctail). Deletion of the US28 C terminus resulted in reduced constitutive endocytosis and consequently enhanced signaling capacity of all receptors tested as assessed by inositol phosphate turnover, NF-κB, and cAMP-responsive element-binding protein transcription assays. We further show that the constitutive endocytic property of US28 affects the action of its chemokine ligand fractalkine/CX$_3$CL1 and show that in the absence of the US28 C terminus, fractalkine/CX$_3$CL1 acts as an agonist on US28. This demonstrates for the first time that the endocytic properties of a 7TM receptor can camouflage the agonist properties of a ligand.

Endocytosis of 7 transmembrane (7TM)[1] G protein-coupled receptors is a mechanism that allows control of an appropriate cell surface receptor density in a given physiological setting. Typically, a receptor is activated by an agonist and initiates a signaling cascade before it undergoes endocytosis. However, there are receptors that can signal in a ligand-independent manner, the so-called constitutively active receptors. For some of these, constitutive internalization is an accompanying effect, *e.g.* for mutant parathyroid hormone receptors (PTH1Rc) (1), the constitutively active mutants of the angiotensin II (1A) receptor (2), or the human complement factor C5a receptor (3). Two examples of constitutively endocytosing 7TM receptors have been described in the chemokine system, one of which is the CXCR4 receptor. However, its constitutive internalization rate with ~2.5% of total surface receptor/min in the absence of ligand is rather slow (4). The other receptor is US28, a 7TM receptor encoded by human cytomegalovirus (HCMV) that has been shown to both signal (5, 6) and endocytose in a ligand-independent manner at the rate of ~7% of the cell surface pool/min (7).

Of the four chemokine receptors encoded by this large DNA virus, UL33, UL78, US27, and US28 (8), US28 is so far the most extensively characterized receptor. It binds several CC-chemokines such as MIP-1α/CCL3, MCP-1/CCL2, MIP-1β/CCL4, and RANTES/CCL5 (9) as well as the CX$_3$C-chemokine fractalkine/CX$_3$CL1 with high affinity (10). However, despite its rather broad-spectrum ligand repertoire, US28 signals independently of any ligand in a highly constitutive manner. US28 stimulates the G protein G$_q$/phospholipase C pathway and up-regulates cellular transcription factors including the nuclear factor–κB (NF-κB) and the cAMP-responsive element-binding protein (CREB) (5, 6) in the absence of any ligand. US28 has been implicated to be important for the viral life cycle of HCMV; for example, it has been suggested to enhance cell-cell fusion, demonstrating its potential importance in the cell-to-cell spread of HCMV *in vivo* (10, 11). In addition, US28 has been linked to pathogenic diseases like atherosclerosis and restenosis, following observations that HCMV-infected smooth muscle cells are able to migrate toward a CC-chemokine gradient consisting of the US28-ligands RANTES/CCL5 and MCP-1/CCL2 (12).

In contrast to endogenous chemokine receptors, which are

* This work was supported in part by an EMBO fellowship, an Erwin-Schroedinger-Auslandsstipendium (to M. W.), grants from the Danish Medical Research Council (to T. W. S.), and by funds provided by the State of California for Medical Research and Alcohol and Substance Abuse through the University of California San Francisco (to J. L. W.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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‡‡ Supported by Byk Nederland B. V., Zwanenburg, The Netherlands.

§§ Supported by the Royal Netherlands Academy of Arts and Sciences.

[1] The abbreviations used are: 7TM, 7 transmembrane; CREB, cAMP-responsive element-binding protein; HCMV, human cytomegalovirus; FITC, fluorescein isothiocyanate; DMEM, Dulbecco's modified Eagle's medium; HA, hemagglutinin; RLU, relative light units; ORF, open reading frame; wt, wild type.

TABLE I

C-terminal tails of the US28, NK_1, and ORF74 receptor chimeras beginning with the conserved NPXXY motif

Receptor chimeras were generated by exchanging the cytoplasmic tails 8 residues proximal to the NP*XX*Y motif at the end of TM7. The dotted line indicates the position of the C-terminal "swap"; A, C termini of the wild type US28 receptor; B, ORF74 receptor; C, the tachykinin NK_1-receptor. D, the C termini of the ORF74-US28 chimera; E, the NK_1-US28 chimera; F, the US28-NK_1 chimera; and G, US28-ORF74 chimera.

A.		
US28	wild type	
NPLLYVFVGTKFR	QELHCLLAEFRQRLFSRDVSWYHSMSFSRRSSPSRRETSSDTLSDEVCRVSQIIP	
299		
B.		
ORF74	wild type	
VPLIYSCLGSLFR	QRMYGLFQSLRQSFMSGATT	
335		
C.		
NK1	wild type	
NPIIYCCLNDRFR	LGFKHAFRCCPFISAGDYEGLEMKSTRYLQTQGSVYKVSRLGETTISTVVGAHEEEPEDPKATPSSLDLTSNCSSRSDSKTMTESFSFSSNVS	
335		
D.		
ORF74	US28-c-tail	
VPLIYSCLGSLFR	QELHCLLAEFRQRLFSRDVSWYHSMSFSRRSSPSRRETSSDTLSDEVCRVSQIIP	
335		
E.		
NK1	US28-c-tail	
NPIIYCCLNDRFR	QELHCLLAEFRQRLFSRDVSWYHSMSFSRRSSPSRRETSSDTLSDEVCRVSQIIP	
313		
F.		
US28	NK1-c-tail	
NPLLYVFVGTKFR	LGFKHAFRCCPFISAGDYEGLEMKSTRYLQTQGSVYKVSRLGETTISTVVGAHEEEPEDPKATPSSLDLTSNCSSRSDSKTMTESFSFSSNVS	
299		
G.		
US28	ORF74-c-tail	
NPLLYVFVGTKFR	QRMYGLFQSLRQSFMSGATT	
299		

predominantly located on the cell surface, only a fraction of US28 (at most ~20%) is expressed on the cell surface. The majority of the receptor is found in the membranes of intracellular organelles that include components of the endocytotic pathway, in particular multivesicular late endosomes or multivesicular bodies (7). In fact, US28 has been shown to undergo rapid and constitutive agonist-independent endocytosis, similar to that of activated endogenous chemokine receptors. More specifically, the tonic internalization rate of US28 receptors is comparable with that of activated chemokine receptors (*i.e.* SDF-1 induced internalization in CXCR4 (4)). Interestingly, this seems to be a β-arrestin-independent mechanism, because the trafficking patterns of US28 are unaltered in mouse embryonic fibroblasts devoid of both β-arrestin 1 and β-arrestin 2 proteins (13).

We have used truncated and chimeric US28 receptors to begin to elucidate the domain(s) of US28 necessary for constitutive signaling and endocytosis and have used these mutants to assess the functional consequences of altering receptor signaling and trafficking.

EXPERIMENTAL PROCEDURES

Materials—Substance P was purchased from Peninsula (St. Helens, Merseyside, UK); CX_3CL1 (*i.e.* the chemokine domain corresponding to amino acids 1–69 of fractalkine) was from R & D Systems; and GCP-2/CXCL6 and IL-8/CXCL8 were purchased from PeproTech (London, UK). The radioligands ^{125}I-GCP-2/CXCL6, ^{125}I-CX_3CL1, and ^{125}I-substance P were obtained from Amersham Biosciences. IL-8/CXCL8 was labeled with ^{125}I using the IODO-GEN method (Pierce) according to the manufacturer's protocol. ^{125}I-Labeled IL-8/CXCL8 was separated from free iodine (>99%) using a 25-cm, 10-ml Sephadex G-25 gel filtration column (ICN Pharmaceuticals Inc., Costa Mesa, CA). Iodine incorporation and specific activity were determined via precipitation of the protein by the trichloroacetic acid method (14). The H4A3 LAMP1 antibody developed by J. T. August and J. E. K. Hildreth was obtained from the Developmental Studies Hybridoma Bank developed under the auspices of the NICHD, National Institutes of Health, and maintained by the University of Iowa, Dept. of Biological Sciences, Iowa City.

cDNA Constructs—The DNA coding the US28 chemokine receptor (AD169 strain of HCMV) was kindly provided by Timothy N. C. Wells (Serono Pharmacological Research Institute, Geneva, Switzerland). The R129A mutant of US28 was constructed by site-directed mutagenesis, and the receptor was re-introduced into the multiple cloning site of the expression vector. The truncated versions of the US28 receptor, US28Δ300 and US28Δ317, were constructed by inserting a STOP codon following residues Gln-300 or Val-317 of the wild type US28 receptor, respectively (see Fig. 2*A*), and thereafter introducing an *Eco*RI site for fusion into the multiple cloning site of the expression vector. The receptor chimeras ORF74-US28-ctail and NK_1-US28-ctail were created as follows. The ORF74 receptor carrying the US28-ctail (Table I, D) was constructed by oligonucleotide-directed mutagenesis, with oligonucleotides consisting of a 10–15-bp overlap from both the C-terminal truncated part of the ORF74 receptor (truncation at residue Arg-335) and the C terminus (residues Gln-300 to Pro-354) of the US28 receptor. Likewise, the NK_1 receptor bearing the C-terminal domains of US28 (Table I, E) was constructed by fusing the C terminus of the wild type US28 receptor (residues Gln-300 to Pro-354) to the NK_1 receptor truncated at residue Arg-313. The receptor chimeras US28-NK_1-ctail (Table I, F) and US28-ORF74-ctail (Table I, G) were created by fusing the C-terminal truncated part of the wild type US28 receptor (truncation at residue Arg-299) to either the C terminus of the human NK_1 receptor (residues Leu-314 to Ser-407) or the C terminus of the ORF74 receptor (residues Gln-336 to Thr-355). All constructs were tagged by inserting the 9-amino acid sequence (YPYDVPDYA) recognized by the anti-HA 11 mouse monoclonal antibody immediately after the N-terminal initiate methionine of the respective receptor DNAs. Addition of the N-terminal HA tag did not impair binding of the respective ligands to any of the

tested receptors. All constructs were cloned into the eukaryotic expression vector pTEJ8 (15) and verified by DNA sequencing.

Transfections and Cells—COS-7 cells were grown at 10% CO_2 and 37 °C in Dulbecco's modified Eagle's medium 1885 supplemented with 10% fetal calf serum, 2 mM glutamine, and 100 units/ml penicillin G/streptomycin. HEK293 cells were grown at 5% CO_2 and 37 °C in Dulbecco's Ham's F-12 medium supplemented with 10% fetal calf serum, 2 mM glutamine, and 100 units/ml penicillin G/streptomycin. Cells were transiently transfected with the respective amounts of cDNA using the calcium phosphate precipitation method (16) or with LipofectAMINE 2000 (Invitrogen) in the luciferase-reporter assay.

Phosphatidylinositol Assay—Inositol phosphate turnover in COS-7 cells was performed as described previously (6). In brief, COS-7 cells (2.5×10^5 cells/well) were incubated in 12-well plates (Biotechline, Denmark) for 24 h with 5 μCi of *myo*-[^{3}H]inositol, followed by a washing step in 20 mM HEPES buffer, pH 7.4, supplemented with 140 mM NaCl, 5 mM KCl, 1 mM $MgSO_4$, 1 mM $CaCl_2$, 10 mM glucose, and 0.05% (w/v) bovine serum albumin. Subsequently, cells were incubated in 0.5 ml of buffer supplemented with 10 mM LiCl at 37 °C for 60 min in the presence of various concentrations of chemokines. The incubation was stopped by aspiration of the binding buffer and addition of 1 ml of ice-cold 10 mM formic acid. After incubation on ice for 60 min, the generated [^{3}H]inositol phosphates were purified using Dowex AG 1X8 anion-exchange columns (Bio-Rad) and counted by liquid scintillation.

Reporter Gene Assays—COS-7 and HEK293 cells were transfected with reporter plasmids as described (6). In short, cells (35,000 cells/well) were seeded out in 96-well plates and transiently transfected with either a "reporter/cDNA mixture" consisting of pFA2-CREB and pFR-Luc reporter plasmids (PathDetect CREB Trans-Reporting System, Stratagene) or pNF-κB-Luc (PathDetect Cis-Reporting System, Stratagene) and the indicated amounts of receptor DNA. Following transfection, cells were treated with the respective compounds in an assay volume of 100 μl for 24 h. When treated with chemokines, cells were maintained in low serum (2.5%) throughout the experiments. The assay was terminated with the addition of luciferase assay reagent (LucLite, Packard Instrument Co.), and luminescence was measured in a TopCounter (Top Count NXT™, Packard Instrument Co.) for 1 s. Luminescence values are given as relative light units (RLU). All reporter-gene experiments were conducted in parallel in HEK293 and COS-7 cells and showed comparable results; however, the chemokine stimulation assays in HEK293 cells yielded a better signal-to-noise ratio.

Radioligand Binding Experiments—Whole cell radioligand binding assays were performed on transiently transfected COS-7 cells. 24 h post-transfection, cells were transferred to 24-well culture plates, and the number of cells seeded per well was such as to obtain 5–10% specific binding of the added radioactive ligand. Homologous competition binding was performed for 3 h at 4 °C in 0.5 ml of binding buffer containing 12 pM of the respective radioligands ^{125}I-GCP-2/CXCL6 or ^{125}I-CX_3CL1 plus unlabeled ligand in the concentrations indicated. The binding buffer consisted of 50 mM HEPES buffer, pH 7.4, supplemented with 1 mM $CaCl_2$, 5 mM $MgCl_2$, and 0.5% bovine serum albumin. Following incubation cells were washed quickly four times in 4 °C binding buffer supplemented with 0.5 M NaCl to reduce unspecific binding. Nonspecific binding was determined in the presence of 0.1 μM of the corresponding unlabeled ligand. ^{125}I-Substance P binding was conducted in 50 mM Tris-HCl buffer, pH 7.4, supplemented with 150 mM NaCl, 5 mM $MnCl_2$, 0.1% (w/v) bovine serum albumin, and 40 μg/ml bacitracin. Following binding, cells were washed twice in ice-cold buffer. Nonspecific binding was determined in the presence of 0.1 mM of unlabeled ligand. IC_{50} values were determined by nonlinear regression using GraphPad Prism 2.1 (GraphPad Software, San Diego), and B_{max} values were calculated from the homologous competition binding curves using the equation $B_{max} = B_0 \times (IC_{50}/[L])$, where B_0 indicates the specific bound radioligand, and [L] indicates the concentration of free radioligand. Saturation assays were performed for 3 h at 4 °C in 0.25 ml of binding buffer containing increasing amounts of ^{125}I-CX_3CL1 radioligand, and nonspecific binding was determined in the presence of 0.1 μM of the unlabeled ligand.

Radioligand Endocytosis Assays—Transiently transfected COS-7 cells were plated in 24-well culture plates and allowed to grow for 2 days. Cells were washed once with ice-cold phosphate-buffered saline and incubated with ^{125}I-CX_3CL1 (0.3 nM) or ^{125}I-IL-8/CXCL8 (1.5 nM) for 3 h at 4 °C in 50 mM HEPES buffer, pH 7.4, supplemented with 1 mM $CaCl_2$, 5 mM $MgCl_2$, and 0.5% bovine serum albumin. Subsequently, cells were washed twice with cold binding buffer supplemented with 0.5 M NaCl, followed by one wash with prewarmed DMEM at 37 °C to remove free ligand. Cells were then incubated in DMEM at 37 °C to allow receptor internalization. Internalization was stopped by aspirating the medium and returning the samples on ice. For each time point, one set of cells was immediately lysed in 0.2 M NaOH, whereas a second set of cells was subjected to an acidic wash to remove surface-bound chemokine. Briefly, cells were rinsed once in ice-cold DMEM (pH 2.0), followed by two 3-min washes in the same medium, and finally lysed in 0.2 M NaOH. Samples were counted in a gamma-counter. Control binding experiments were conducted and showed that the acidic wash fully removed surface-bound chemokine while leaving the receptor surface intact.

Immunofluorescence—Cells expressing the receptor constructs 2 days after transfection were used for immunofluorescence. 24 h post-transfection, cells were plated to coverslips and allowed to grow for 24 h. Living cells were fed monoclonal anti-HA 11 antibody (Covance, Berkeley, CA) for 30 min to label receptors. For co-localization studies with transferrin, cells were starved in serum-free medium for 60 min and fed Texas Red-conjugated transferrin (Molecular Probes) for 30 min. Subsequently, cells were fixed with 3% paraformaldehyde in phosphate-buffered saline for 20 min at room temperature and permeabilized with 0.1% Triton X, essentially as described (17). For co-localization studies with LAMP1, receptors were labeled with affinity-purified rabbit anti-mouse antibody to convert the HA-11 IgG1-labeled receptors into rabbit-IgGs and subsequently visualized with a secondary goat anti-rabbit FITC (Jackson ImmunoResearch) antibody. In parallel, cells were stained with the LAMP1 H4A3 IgG1 antibody from Developmental Studies Hybridoma Bank and visualized with a CY5-conjugated secondary donkey anti-mouse antibody (Molecular Probes). Controls (omission and cross-staining with either first or secondary antibodies) were made in order to verify that the staining was specific to both receptor and LAMP1, respectively. Following staining, cells were mounted in Vectashield Mounting Medium (Vector Laboratories) and analyzed using a Zeiss fluorescence microscope equipped with a Coolsnap CCD camera and a Zeiss LSM510 META Axioplan 2 confocal microscope. We emphasize that all the antibody feeding experiments were conducted in parallel in both HEK293 and COS-7 cells, but we could not detect any substantial differences between these two cell lines.

RESULTS

An Inactive Signaling Mutant of US28 Is Constitutively Endocytosed—We first asked whether the constitutive endocytosis of US28 was a consequence of its high constitutive signaling activity (5, 6). To examine this, we generated a US28 mutant that was no longer constitutively active. The conserved DRY motif located at the boundary of transmembrane helix 3 and the second intracellular loop of most 7TM receptors plays a pivotal role in G protein activation (18, 19). We created a mutant US28 receptor where the arginine in this DRY motif was substituted with an alanine (US28 R129A). As expected, US28 R129A no longer showed constitutive signaling properties when tested in inositol phosphate turnover assays (Fig. 1*O*, *black bar*). To directly examine constitutive endocytosis, we conducted antibody feeding experiments, because this experimental setup allows us to assess distributions of receptors that were accessible to the HA antibody during a 30-min incubation period (for details see "Experimental Procedures"). Furthermore, this approach guarantees that the labeled receptors were in fact intact and reached the cell surface and were not merely stuck intracellularly or retained in the endoplasmatic reticulum due to improper protein folding. The cellular distribution of US28 R129A (Fig. 1, *A–G*) was indistinguishable from that of the wild type US28 receptor (*H–N*). Both US28 wild type and US28 R129A receptors were localized in early endosomes and lysosomes (*G* and *N*, respectively) as assessed by co-localization studies with the early endosomal marker transferrin (*A–C* and *H–J, red*) and the lysosomal marker lysosomal-associated membrane protein 1 (LAMP1) (*D–F* and *K–M, blue*). In addition, US28-containing vesicles that lacked these markers were also observed (*green*). These data are in excellent agreement with the elaborate study of Fraile-Ramos *et al.* (7), where localization of US28 was assessed in HeLa cells. The same co-localization profiles were found in COS-7 cells (data not shown). We further verified that both US28wt and USR129A receptors were expressed on the cell surface in comparable

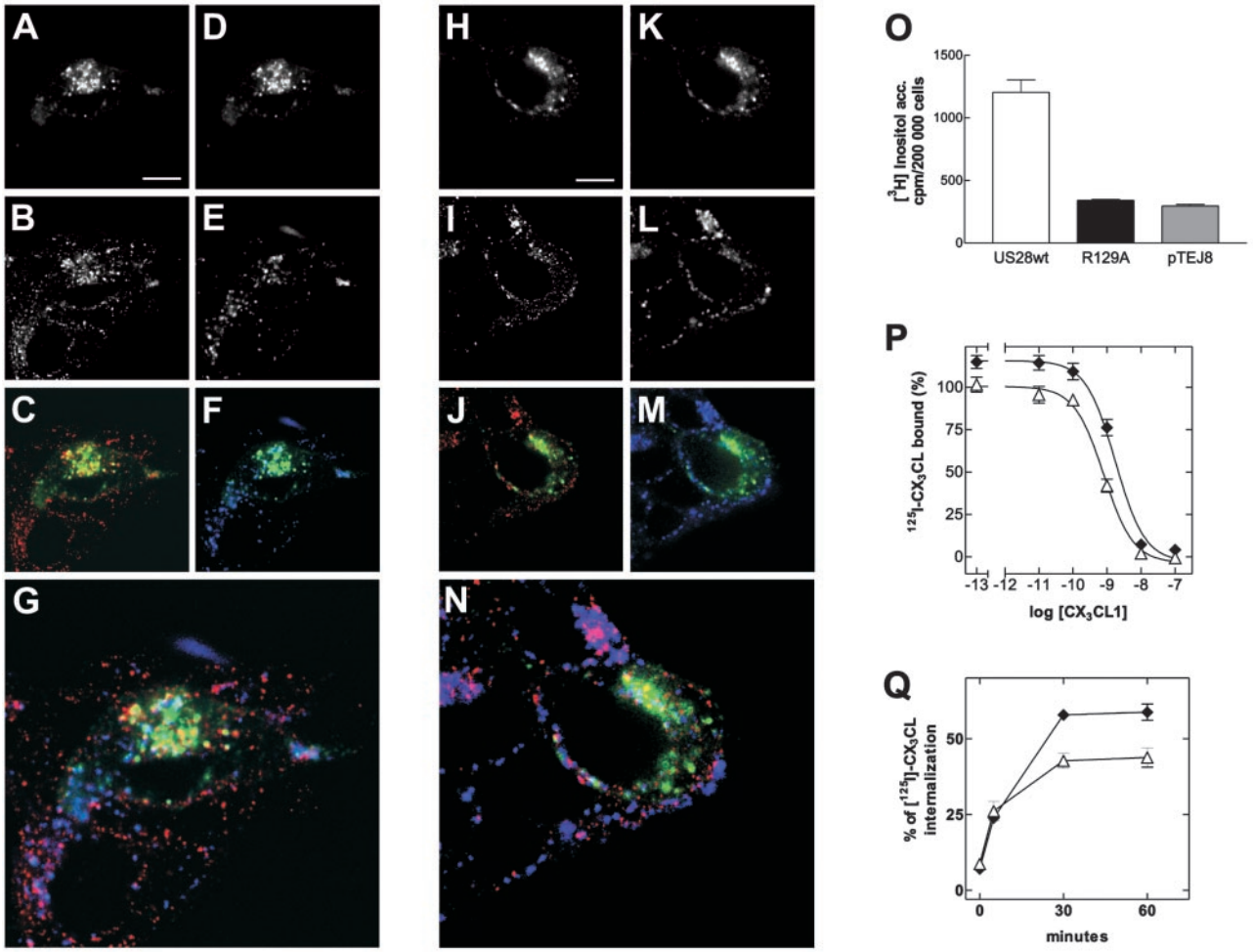

FIG. 1. **Internalization and signaling properties of the US28 mutant R129A.** *A–N*, HEK293 cells were transiently transfected with wild type US28 (*A–G*) or US28 R129A (*H–N*) cDNAs, and an antibody feeding experiment was conducted as described under "Experimental Procedures." US28wt (*A*) and US28 R129A (*H*) co-localize with the early recycling endosomal marker transferrin (*B* and *I*), as shown in the overlays (*C* and *J*), respectively. Receptor, *green*; transferrin, *red*; and overlay, *yellow*. US28wt (*D*) and US28 R129A (*K*) co-localize with the lysosomal marker LAMP1 (*E* and *L*), as shown in the overlays *F* and *M*, respectively. Receptor, *green*; LAMP1, *blue*; overlay, *light blue*. *G* and *N,* triple overlay of US28wt (*G*, *green*) or US28 R129A (*N*, *green*) with transferrin (*red*) and LAMP1 (*blue*). *Scale bars*, 10 μm. *O,* constitutive inositol phosphate turnover was measured in COS-7 cells transiently transfected with 40 μg of US28 wt (*white bar*) or US28R129A (*black bar*) DNAs. 40 μg of vector DNA (*gray bar*) served as a control. Shown are means ± S.E. of 3 independent experiments carried out in triplicate. *P*, surface expression of the wild type US28 and the US28 R129A receptor determined by whole cell binding of the chemokine ligand ^{125}I-CX$_3$CL1/fractalkine. Whole cell homologous competition binding was performed on COS-7 cells transiently transfected with 40 μg of either US28 wild type (△) or US28 R129A (♦) receptor DNAs. One day post-transfection, cells were labeled with ^{125}I- CX$_3$CL1/fractalkine and incubated with increasing amounts of cold chemokine ligand as indicated. Data are mean ± S.E. from 2 experiments performed in duplicate. *Q,* COS-7 cells were transiently transfected with either US28 wild type (△) or US28 R129A (♦) receptor cDNAs, and receptor internalization was measured with ^{125}I-CX$_3$CL1 for 60 min at 37 °C. Data are means ± S.E. from 3 experiments performed in triplicate.

amounts by conducting whole cell ^{125}I-CX$_3$CL1 radioligand binding experiments. Because these experiments are performed at 4 °C, no endocytosis of receptors occurs, and therefore this assay represents a "snapshot" of the steady state level of receptors on the cell surface. Fig. 1*P* shows a homologous displacement curve for both US28wt (△) and US28 R129A (♦), and Table II shows that the B_{max} levels of US28R129A-expressing cells were comparable with those of US28wt receptor-expressing cells. In addition, we tested the constitutive endocytosis rate of US28wt and US28 R129A by radioligand internalization assays with the chemokine ligand ^{125}I-CX$_3$CL1. When compared with the wild type receptor US28 (Fig. 1*Q*, △), US28 R129A (Fig. 1*Q*, ♦) was constitutively internalized at 37 °C at a slightly higher rate than the wild type US28 receptor. Summarized, these data show that constitutive activity of US28 is not necessary for its constitutive endocytosis.

Effect of Full or Partial Truncation of the C Terminus on the Trafficking and Signaling, Properties of US28—We next designed a set of experiments to determine what domain(s) in US28 were mediating its constitutive endocytosis. A very recent study (20) showed that the C terminus of US28 is constitutively phosphorylated and that the deletion of all phosphorylation sites resulted in increased surface expression of US28. Moreover, palmitoylation of the C terminus of various 7TM receptors (for review see Ref. 21) and for one chemokine recep-

TABLE II
Steady state surface expression of US28 constructs

The table shows K_D ± S.E. for the indicated number of experiments (n), followed by B_{max} ± S.E.

Receptor	K_D	B_{max}
	nM	*fmol/10^5 cells*
US28wt	0.23 ± 0.06 (3)	13.2 ± 1.6
US28R129A	1.5 ± 0.3 (2)	21.6 ± 4.2
US28Δ300	0.79 ± 0.01 (3)	128.4 ± 4.1
US28Δ317	0.79 ± 0.15 (2)	67.9 ± 4.3
US28-NK$_1$-ctail	0.83 ± 0.12 (2)	86.1 ± 1.1
US28-ORF74-ctail	0.66 ± 0.07 (2)	66.1 ± 5.6

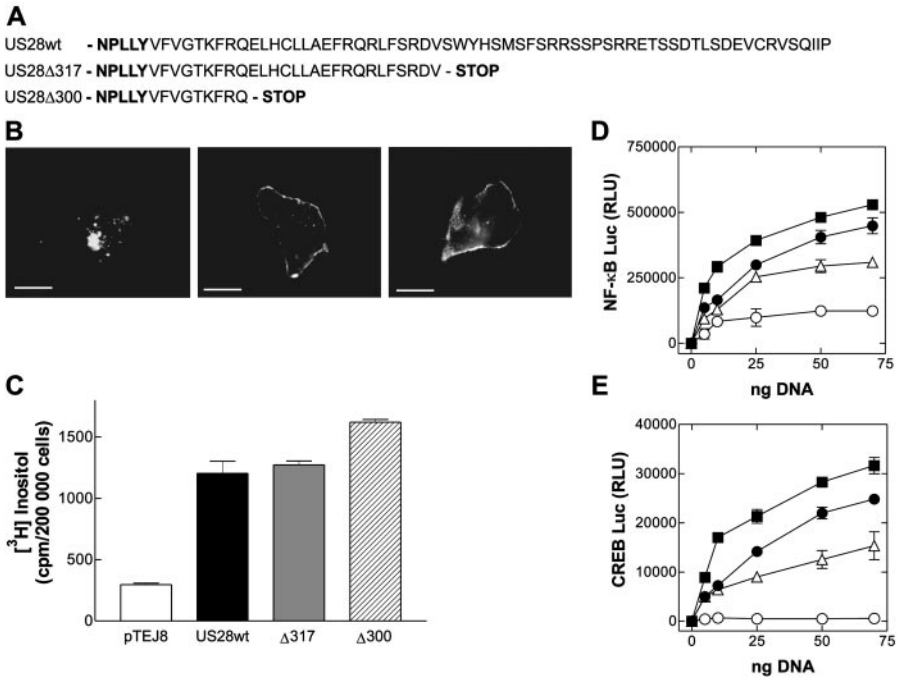

FIG. 2. **Localization and signaling of wild type and C-terminally truncated versions of US28.** *A*, amino acid sequences for C termini of US28wt and truncated sites for US28Δ300 and US28Δ317. *B*, HEK293 cells were transiently transfected with either wild type US28 receptor (*left panel*), US28Δ317 (*middle panel*), or US28Δ300 (*right panel*). 48 h post-transfection, cells were incubated with antibodies directed against the N-terminal HA tags of the respective receptors, fixed, and permeabilized, and receptors were visualized with fluorescent antibodies (see "Experimental Procedures"). *Scale bars*, 10 μm. *C*, constitutive inositol phosphate turnover was measured in COS-7 cells transiently transfected with 40 μg of US28wt (*black bar*), US28Δ317 (*gray bar*), or US28Δ300 (*hatched bar*) DNAs. 40 μg of vector DNA (*white bar*) served as a control. Shown are means ± S.E. of 3 independent experiments carried out in triplicate. *D*, induction of NF-κB-driven luciferase expression was determined in transiently transfected COS-7 cells (35,000 cells/well). Cells were co-transfected with 50 ng of NF-κB-luciferase vector and increasing amounts of either pTEJ8 vector DNA (○) as a control, US28wt (△), US28Δ300 (●), or US28Δ317 (■) DNAs. *E*, activation of CREB-luciferase was determined in COS-7 cells (35,000 cells/well) co-transfected with a CREB/luciferase/vector mixture (for details see "Experimental Procedures") and with increasing amounts of either vector DNA (○) as a control or US28wt (△), US28Δ300 (●), or US28Δ317 (■) DNAs. For both reporter assays, representative experiments out of at least four independent experiments performed in quadruplicate are shown. RLU was as measured in a Packard TopCounter (1 s/well).

tor, the CCR5 receptor, has been reported to be essential for (i) the expression of a functional receptor on the cell surface and for (ii) the endocytic properties of these receptors (22–24). However, to date no study has been reported that palmitoylation of the C terminus plays a role in the function of US28 or any other viral chemokine receptor. We first tested whether truncation of the last 36 residues of the US28 C terminus (US28Δ317), thereby leaving its putative palmitoylation site intact, would alter the endocytic properties of US28. US28Δ317 protein was expressed predominantly on the surface of HEK293 cells (Fig. 2*B, middle panel*) as opposed to US28 wild type receptor, which was predominantly located intracellularly (Fig. 2*B, left panel*). Full truncation of the last 54 residues of the US28 C terminus (US28Δ300) resulted in the same phenotype as US28Δ317, with the majority of US28Δ300 protein expressed on the cell surface (Fig. 2*C, right panel*).

Next we examined whether the US28Δ300 and US28Δ317 receptor proteins were still functionally coupled to G proteins. As we have demonstrated previously, US28 is a constitutively active receptor that signals via a G_q/phospholipase C-coupled pathway and up-regulates transcription factors such as NF-κB and cAMP-responsive element-binding protein (CREB) (5, 6). Fig. 2*C* shows that the partial or full deletion of the C terminus of US28, US28Δ317 (*gray bar*), or US28Δ300 (*hatched bar*), respectively, did not impair constitutively induced inositol phosphate turnover in COS-7 cells when compared with wild type US28 (*black bar*). In fact, the fully truncated US28Δ300 (*hatched bar*) protein showed a slightly enhanced signaling profile compared with the other receptors. When tested for their ability to up-regulate reporter genes like NF-κB or CREB, both US28Δ317 (■) and US28Δ300 (●) were able to constitutively induce NF-κB-luciferase (Fig. 2*D*) and CREB-luciferase (Fig. 2*E*) activity above control levels (Fig. 2, *D* and *E*, ○) in a gene dose-dependent manner.

These findings show that the C terminus of US28 is involved in the endocytotic trafficking of the receptor, but it is not necessary for the constitutive activity of this viral protein. Together with the data from Fig. 1, these results suggest that the constitutive activity and endocytosis of US28 are separable entities.

US28 C Terminus Conveys Constitutive Endocytosis Properties to Other 7TM Receptors—Although the signaling properties of US28 were not impaired by deletion of its C terminus, the endocytic properties of US28 were significantly altered, *i.e.* the truncated receptors were predominantly expressed on the cell surface. To assess whether the US28 C terminus contains signals for constitutive endocytosis of 7TM receptors in general, we exchanged the C termini of both a virally encoded, constitutively active receptor, the herpes virus 8-encoded 7TM receptor ORF74 (25), and a nonvirally encoded nonconstitutively active 7TM receptor (the human tachykinin NK_1 receptor) with the last 55 C-terminal amino acids of US28 (see Table I, D and E, respectively). Like US28, ORF74 constitutively signals via the G_q/phospholipase C pathway and induces NF-κB transcription (26, 27). In terms of cellular localization, however, ORF74 differs from US28 in that it is predominantly expressed on the cell surface of transfected cells (26) (Fig. 3*A, green panel*). When expressed in HEK293 cells, a significant portion of the receptor chimera ORF74-US28-ctail (Table I, D) was now found intracellularly (Fig. 3*B*) as assessed by antibody

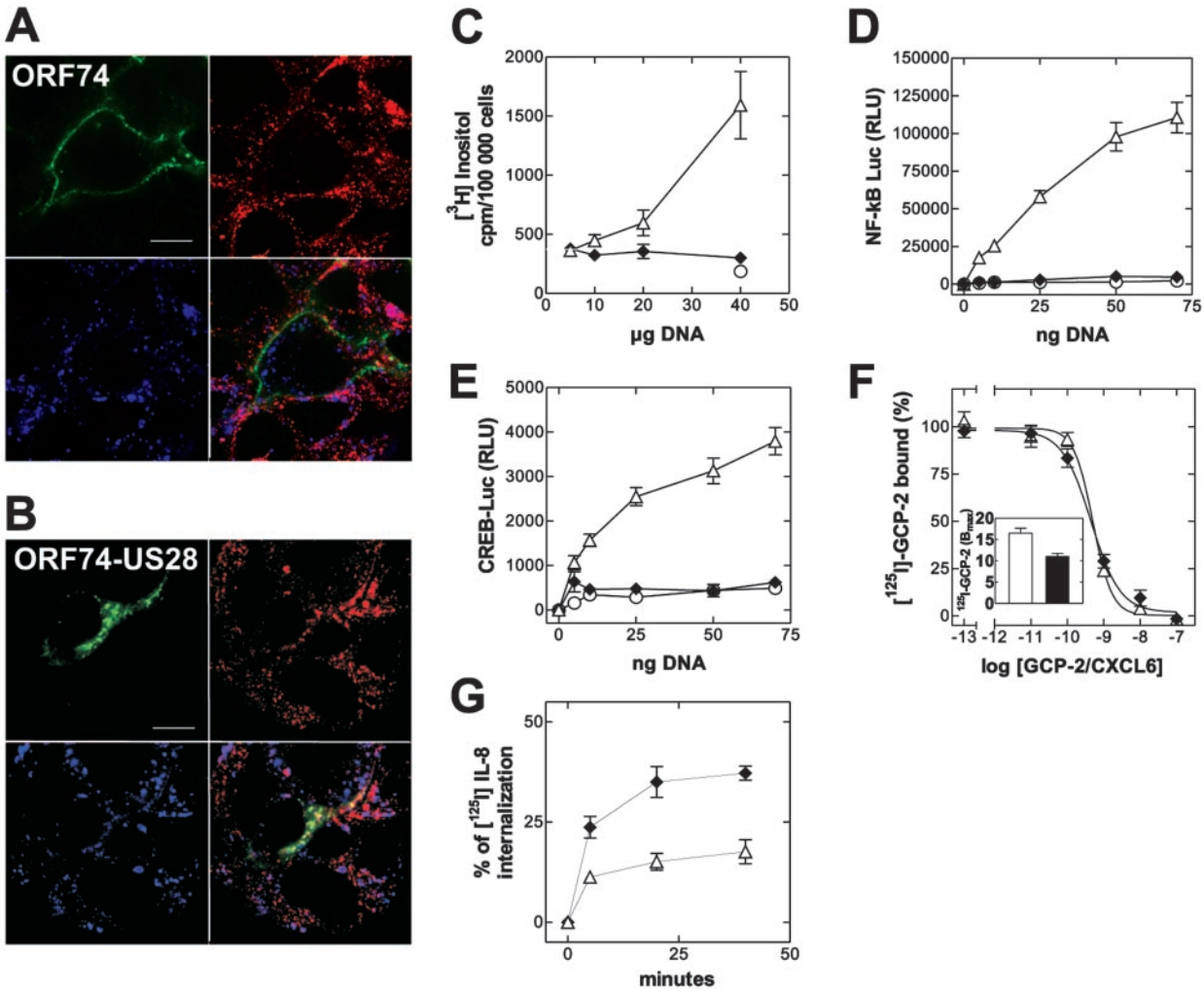

FIG. 3. **ORF74 wild type receptor and ORF74-US28-ctail chimeric receptor signaling and sorting properties.** HEK293 cells were transiently transfected with either ORF74 wild type receptor (*A*) or ORF74-US28-ctail (*B*) receptor DNA. Cells were incubated with an HA antibody for 30 min, and co-localization (*right lower panel* in *A* and *B*) with the early endosomal markers transferrin (*red*) and the lysosomal marker LAMP1 (*blue*) was assessed as described under "Experimental Procedures." *Scale bars*, 10 μm. *C*, gene dose-dependent inositol phosphate turnover measured in COS-7 cells transiently transfected with increasing concentrations of ORF74 wild type receptor (△) or ORF74-US28-ctail receptor (◆) DNAs. 40 μg of vector DNA (○) served as a control. Shown are means ± S.E. of 3 independent experiments carried out in triplicate. *D*, induction of NF-κB-driven luciferase expression in transiently transfected COS-7 cells. Cells were co-transfected with NF-κB-luciferase vector and increasing amounts of either pTEJ8 vector DNA (○), ORF74 wild type receptor (△), or the ORF74-US28ctail receptor (◆) DNAs. *E*, activation of CREB-luciferase in COS-7 cells. Cells were co-transfected with a CREB/luciferase/vector mixture and with increasing amounts of either vector DNA (○) as a control or ORF74 wild type receptor (△) or the ORF74-US28ctail receptor (◆) DNAs. For both reporter assays, representative experiments out of at least four independent experiments performed in quadruplicate are shown. *F,* surface expression of the wild type ORF74 and the ORF74-US28-ctail receptor determined by whole cell binding of the CXC-chemokine ligand ^{125}I-GCP-2/CXCL6. Whole cell homologous competition binding was performed on COS-7 cells transiently transfected with 40 μg of either ORF74 wild type (△) or ORF74-US28-ctail (◆) receptor DNAs. One day post-transfection, cells were labeled with ^{125}I-GCP-2/CXCL6 and incubated with increasing amounts of cold chemokine ligand as indicated. *Inset*, B_{max} values for ^{125}I-GCP-2/CXCL6 binding on ORF74 wild type (*open bar*) and ORF74-US28-ctail (*black bar*) are given in fmol/10^5 cells. Data are means ± S.E. from 3 experiments performed in duplicate. *G,* endocytosis of ORF74wt and ORF74-US28-ctail proteins with the neutral chemokine ligand ^{125}I-IL-8/CXCL8. COS-7 cells were transiently transfected with either US28 wild type (△) or US28-ORF74-ctail (◆) constructs, and receptor internalization was measured with 1.5 nM ^{125}I-IL-8/CXCL8 for 60 min at 37 °C. Data are mean ± S.E. from 2 experiments performed in triplicate.

feeding experiments and co-localization studies with the early recycling endosomal marker transferrin (Fig. 3*B, red panel*) and the lysosomal marker LAMP1 (Fig. 3*B, blue panel*).

Hence, we propose that the C terminus of US28 can serve as a transposable constitutive endocytic module. Furthermore, the ability of the US28 c-tail to stimulate endocytosis of ORF74 did not appear to depend on its constitutive signaling activity. Truncation of the C terminus of ORF74 has been shown to abolish its constitutive and ligand-induced signaling activity (26). In fact, when we substituted the ORF74 c-tail with that of US28 and tested this chimera in inositol phosphate turnover (Fig. 3*C*), NF-κB (Fig. 3*D*), or CREB-luciferase (Fig. 3*E*) activation assays, the ORF74-US28-ctail receptor (◆) was inactive as opposed to the wild type ORF74 receptor (△), which showed gene dose-dependent activation in all three signaling assays. Although the ORF74 wild type receptor activity could be further stimulated by the addition of the chemokine agonist GRO-α/CXCL1 (28), treatment of cells expressing the ORF74-US28-ctail receptor with agonist concentrations up to 1 μM did not elicit a response above base line in any of the assays employed (data not shown). We verified that this was not due to a lack of binding capacity of the ORF74-US28-ctail chimera to chemokines and/or profound differences in steady state receptor surface expression. The chemokine GCP-2/CXCL6 (Fig. 3*F*) bound ORF74-US28-ctail (◆) with affinities comparable with those of wild type ORF74 receptor (△). The B_{max} values (see *inset* in Fig.

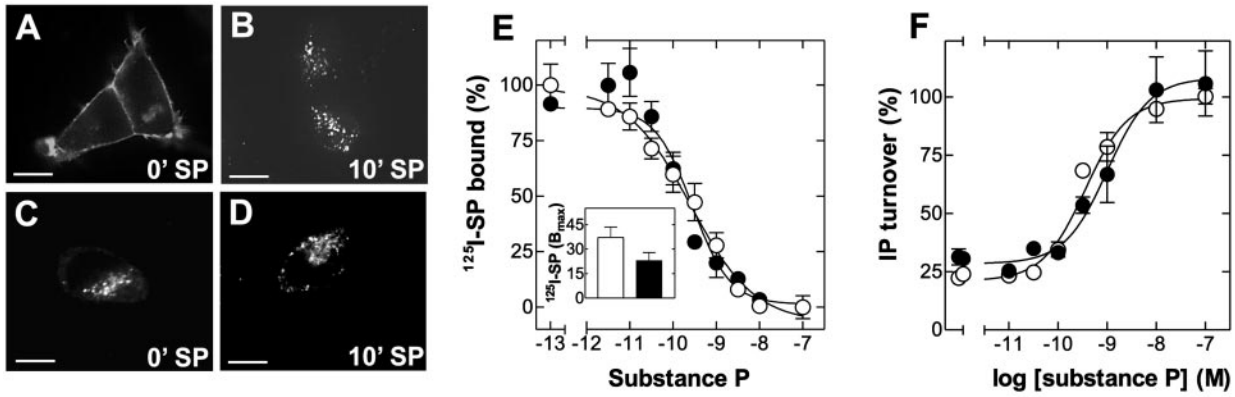

FIG. 4. **The cellular distribution and signaling activity of the tachykinin NK_1 wild type and NK_1-US28-ctail receptors.** HEK293 cells were transiently transfected with wild type NK_1 receptor (*A* and *B*) or NK_1-US28ctail receptor DNAs (*C* and *D*). 2 days following transfection, cells were incubated with an HA antibody for 30 min and either fixed (*A* and *C*) or treated with 1 μM substance P for 10 min (*B* and *D*) and fixed, permeabilized, and stained with a FITC-conjugated secondary antibody. *Scale bars,* 10 μm. *E*, surface expression of the wild type NK_1 and the NK_1-US28 receptor determined by whole cell binding of ^{125}I-substance P (*SP*). Whole cell homologous competition binding was performed on COS-7 cells transiently transfected with 40 μg of either NK_1 wild type (○) or NK_1-US28 (●) receptor DNAs. One day post-transfection, cells were labeled with ^{125}I-substance P and incubated with increasing amounts of cold ligand as indicated. *Inset*, B_{max} values for ^{125}I-substance P binding on NK_1 wild type (*open bar*) and NK_1-US28 (*black bar*) are given in fmol/10^5 cells. Shown is a representative experiment performed in duplicate. *F*, inositol phosphate turnover in response to increasing concentrations of substance P in COS-7 cells transiently transfected with 40 μg of NK_1wt (○) or NK_1-US28-ctail (●) receptors. 100% corresponds to the maximally obtained activity in the wild type receptor. Data are mean ± S.E. from 3 experiments performed in triplicate.

3*F*) and IC_{50} values of GCP-2/CXCL6 were 16.5 ± 1.95 fmol/10^5 cells and 0.50 ± 0.08 nM for the wild type and 11.0 ± 1.74 fmol/10^5 cells and 0.45 ± 0.08 nM for the ORF74-US28-ctail receptor. Hence, the steady state surface receptor levels of these two receptors differed by less than 50%, a difference that we do not believe can account for the signaling mute phenotype of the ORF74-US28-ctail chimera. To assess more quantitatively the endocytosis rates of the wild type ORF74 receptor and the ORF74-US28-ctail chimera, we conducted radioligand internalization assays with the neutral chemokine ligand ^{125}I-IL-8/CXCL8 (Fig. 3*G*). When compared with the wild type ORF74 (Fig. 3*G*, △) receptor, the ORF74-US28-ctail (Fig. 3*G*, ♦) chimera was internalized at 37 °C at a significantly higher rate than the wild type ORF74 receptor. These data show that the US28 C terminus is able to transfer its constitutive internalization properties to another viral chemokine receptor.

We further validated the ability of the US28-ctail to promote constitutive endocytosis using a nonviral and nonconstitutively active 7TM receptor. We chose the tachykinin NK_1 receptor, because it is known to utilize similar signaling pathways, *i.e.* the G_q/phospholipase C pathway and NF-κB transcription in a nonconstitutive, ligand-dependent manner. In addition, the trafficking properties of the NK_1 receptor have been extensively studied, and the involvement of its C terminus in interacting with proteins from the endocytotic machinery is well documented (29–31). The wild type NK_1 receptor is typically found on the surface of transfected cells (Fig. 4*A*), whereas upon stimulation with the agonist substance P, receptors rapidly internalize (Fig. 4*B*) (30). The replacement of the NK_1-ctail with the last 55 residues of the US28 receptor (NK_1-US28-ctail, see also Table I, E) generated a receptor that was found intracellularly both in the absence (Fig. 4*C*) or presence (Fig. 4*D*) of substance P. Fig. 4*E* shows that both the wild type NK_1 (○) and the NK_1-US28-ctail chimera (●) bind the radioligand ^{125}I-substance P with comparable affinities, with an IC_{50} of 0.33 ± 0.07 nM (n = 5) for the NK_1-wild type receptor and 0.23 ± 0.10 nM (n = 10) for the NK_1-US28-ctail receptors. Steady state surface receptor levels were 37.12 ± 6.4 nM (n = 5) for the NK_1 wild type receptor (*inset* in Fig. 4*E, white bar*) and 22.95 ± 4.9 nM (n = 10) for the NK_1-US28-ctail chimera (*inset* in Fig. 4*E, black bar*).

However, the swap of the US28 C terminus onto the NK_1 receptor did not confer constitutive signaling to this receptor,

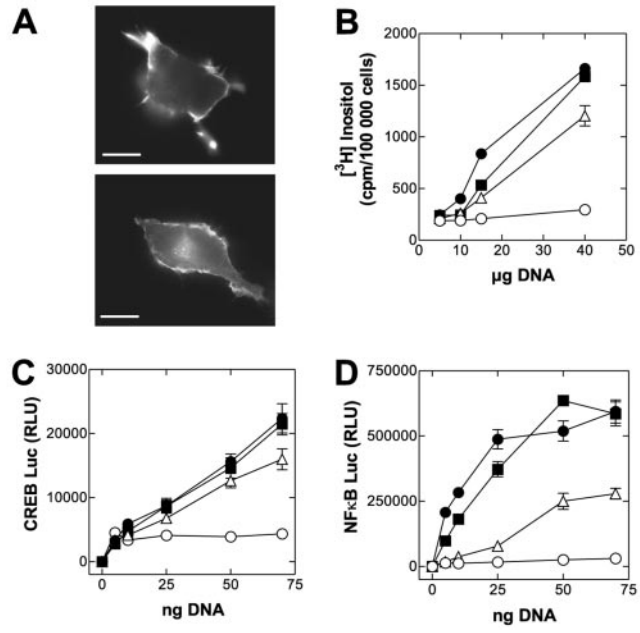

FIG. 5. **Sorting and signaling of the US28-ORF74-ctail and US28-NK_1-ctail receptor chimeras.** HEK293 cells were transiently transfected with either US28-ORF74-ctail receptor (*A*) or US28-NK_1-ctail (*B*) receptor DNAs. An antibody feeding experiment was performed with HA antibody for 30 min, and cells were fixed, permeabilized, and stained with a FITC-conjugated secondary antibody and imaged by fluorescence microscopy. *Scale bars*, 10 μm. *C,* constitutive inositol phosphate turnover in COS-7 cells transiently transfected with increasing concentrations of wild type US28 receptor (△), US28-ORF74-ctail receptor (●), or US28-NK_1-ctail receptor (■) DNAs. Cells transfected with vector pTEJ8 DNA (○) served as a control. *D*, CREB-luciferase activity measured in COS-7 cells co-transfected with a CREB/luciferase/vector mixture (for details see "Experimental Procedures") and the indicated amounts of US28 wild type (△), US28-ORF74-ctail (●), or US28-NK_1-ctail (■) receptor DNAs. Cells co-transfected with the respective reporter DNAs and increasing amounts of pTEJ8 vector DNA (○) served as a control. *E,* induction of NF-κB-driven luciferase expression in COS-7 cells co-transfected with 50 ng of NF-κB-luciferase DNA and increasing concentrations of US28 wild type receptor (△), US28-ORF74-ctail receptor (●), or US28-NK_1-ctail receptor (■) DNAs. US28. For all reporter assays, representative experiments out of at least four independent experiments performed in quadruplicate are shown.

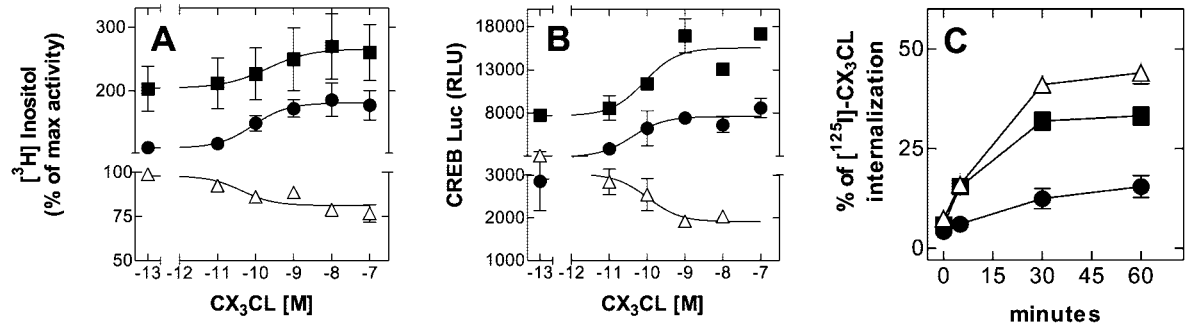

FIG. 6. **CX_3CL1/fractalkine is an agonist on US28Δ300 and US28-NK_1-ctail proteins.** *A*, effect of increasing concentrations of the chemokine domain of fractalkine/CX_3CL1 on the basal inositol phosphate turnover in COS-7 cells transiently transfected with 40 μg of wild type US28 (△), US28Δ300 receptor (●), or US28-NK_1-ctail (■) receptor DNAs. 100% corresponds to the maximally obtained basal activity with wild type US28 receptor, and 0% corresponds to background activity obtained with 40 μg of the empty expression vector pTEJ8. Results show the mean ± S.E. of 3 different experiments carried out in duplicate. *B*, HEK293 cells were transiently transfected with 50 ng of wild type US28 (△), US28Δ300 receptor (●), or US28-NK_1-ctail (■) receptor DNAs and 50 ng of CREB-luciferase cDNA. Cells were exposed to increasing concentrations of fractalkine/CX_3CL1 immediately following transfection. Shown are representative experiments out of at least four independent experiments performed in quadruplicate. *C*, endocytosis of US28wt, US28Δ300, and US28-NK_1-c-tail proteins in the presence of CX_3CL1/fractalkine. COS-7 cells were transiently transfected with either US28 wild type (△), US28Δ300 (●), or US28-NK_1-ctail constructs (■), and receptor internalization was measured with ^{125}I-CX_3CL1 for 60 min at 37 °C. Data are mean ± S.E. from 3 experiments performed in triplicate.

because both the wild type NK_1 receptor (○) and the NK_1-US28-ctail receptor (●) required stimulation with substance P to induce inositol phosphate accumulation (Fig. 4*F*) or NF-κB-luciferase transcription (data not shown).

To summarize, these studies demonstrate that the C terminus of US28 is capable of constitutively trafficking other 7TM receptors into the cytosol, but it does not transpose constitutive signaling properties.

Substitution of the US28 C Terminus with Other 7TM C-tails—Fig. 2 shows that US28Δ300, the complete C-terminal truncated version of US28, is still a highly constitutively active receptor in all signaling assays employed (see Fig. 2, *C–E*). However, the lack of the C terminus abolishes constitutive endocytosis of this receptor (Fig. 2*B*, *right panel*). Substitution of the US28 C terminus with (i) the rather long and serine/threonine-rich C terminus of the NK_1 receptor (Table I, F) or (ii) the short C terminus of the viral ORF74 receptor (Table I, G) onto the US28Δ300 protein did not promote constitutive endocytosis of US28 (Fig. 5, *A* and *B*). However, both US28-NK_1-ctail (■) and US28-ORF74-ctail (●) receptors signaled in a constitutive manner like wild type US28 (△). Both chimeric receptors were highly constitutively active when measured in gene dose-dependent inositol phosphate turnover (Fig. 5*C*), CREB-luciferase (Fig. 5*D*), or NF-κB transcription (Fig. 5*E*) assays. In fact, the US28-NK_1-ctail and US28-ORF74-ctail proteins showed consistently higher constitutive activity than did the wild type US28 receptor in all signaling assays employed.

Thus, the substitution of the US28 C terminus with C termini from two other 7TM receptors rather enhanced the constitutive signaling activity of US28. In addition, both the long and nonviral NK_1 receptor c-tail as well as the short, viral ORF74 receptor c-tail did not restore the constitutive endocytic activity seen in the wild type receptor. Taken together, these results suggest that the c-tail of US28 is both sufficient (even in the absence of constitutive signaling activity) and necessary to induce constitutive endocytosis of US28. These data also demonstrate that the constitutive activity of US28 *per se* was not sufficient to facilitate endocytosis of US28 receptors (i) lacking the US28 c-tail or (ii) containing a different C terminus.

CX_3CL1/Fractalkine Is an Agonist on US28 C-terminally Deficient Receptors—US28 is known to bind a broad spectrum of CC-chemokines (9, 10, 32, 33), but none of the CC-chemokine ligands tested to date affect the constitutive signaling properties of US28 (6). However, US28 has been optimized to recognize specifically the membrane-associated CX_3C chemokine, fractalkine/CX_3CL1, with high affinity (10), and as we have shown previously (5, 6), fractalkine/CX_3CL1 is a partial inverse agonist on US28 as assessed by inositol phosphate turnover (Fig. 6*A*, △), CREB transcription activation (Fig. 6*B*, △), and NF-κB transcription (6). To our surprise, we found that fractalkine/CX_3CL1 could increase constitutive inositol phosphate accumulation in both US28Δ300 receptor (Fig. 6*A*, ●) and US28-NK_1-ctail receptor (Fig. 6*A*, ■) expressing COS-7 cells. The same phenomenon was observed in HEK293 cells (and COS-7 cells, data not shown) co-transfected with the CREB-luciferase reporter and either the US28Δ300 receptor (Fig. 6*B*, ●) or the US28-NK_1-ctail receptor (Fig. 6*B*, ■) as well as in NF-κB-transcription assays (data not shown). The extent of fractalkine/CX_3CL1 stimulation above the base-line receptor activity in both US28Δ300 and US28-NK_1-ctail-expressing cells was almost reciprocal to that of its inverse agonist activity in cells expressing the wild type US28 receptor.

Agonist Behavior of CX_3CL1/Fractalkine Is Determined by the Endocytic Properties of US28—The finding that CX_3CL1/fractalkine is a partial agonist on the C-terminally deficient US28 proteins led us to speculate that fractalkine might in fact be an agonist on US28, but its agonism is masked by the constitutive endocytosis of the wild type receptor. If this were the case, we would predict that blocking the endocytosis of receptors and consequently increasing the number of receptors on the cell surface at a given time would reveal the agonist properties of CX_3CL1/fractalkine. To examine this possibility, we investigated the endocytic properties of the c-tail-deficient proteins US28Δ300 (Fig. 6*C*, ●) and US28-NK_1-ctail (Fig. 6*C*, ■) by measuring the internalization rates of these receptors with ^{125}I-CX_3CL/fractalkine. Both US28Δ300 and US28-NK_1-ctail showed markedly reduced internalization rates when compared with wild type US28 (Fig. 6*C*,△), thus resulting in an increased receptor number on the cell surface at a given time as assessed by whole cell saturation radioligand binding (for B_{max} and K_D values see Table II).

DISCUSSION

The cytoplasmic tail of the majority of 7TM receptors typically determines their trafficking fate once they have been activated by a given ligand and initiated a cellular signaling cascade. Here we show that the cytoplasmic tail of the HCMV-encoded chemokine receptor US28 regulates receptor endocytosis in a ligand-independent, constitutively active manner and that this feature is transposable to other 7TM receptors. We

show that constitutive activity and constitutive endocytosis are separable entities in this viral chemokine receptor, and more specifically, omittance of the US28 C terminus results in enhanced signaling capacity of the receptor. Moreover, we demonstrate that the endocytic properties of a 7TM receptor can camouflage the agonist properties of a ligand.

To test whether the constitutive endocytic activity of the cytoplasmic tail of US28 was dependent on the constitutive signaling activity of US28, we engineered the signaling-mute US28 R129A receptor (Fig. 1). In both antibody feeding (Fig. 1, *A–N*) and chemokine internalization assays (Fig. 1*Q*), US28 R129A was constitutively internalized similar to the wild type receptor. To summarize, this was evidence that constitutive endocytosis was totally independent of the signaling capacity of the receptor and suggested that the US28-ctail *per se* is an endocytic signal. In order to ask whether this constitutive endocytic property of the US28 c-tail was transposable to other 7TM receptors, we chose two receptors that are predominantly surface expressed as follows: the virally encoded, constitutively active ORF74 receptor from herpes virus 8 (25) and the nonvirally, nonconstitutively active human tachykinin NK_1 receptor. As Figs. 3 and 4 show, the endocytic functionality of the US28 c-tail was transposable to both the ORF74 and NK_1 receptors. The signaling properties of the NK_1-US28-ctail chimera were essentially undistinguishable from the wild type NK_1 receptor (Fig. 4*F*); however, the ORF74-US28-ctail receptor was totally mute in all signaling assays employed (Fig. 3, *C–E*). We speculated that this was due to the fact that the far C-terminal region of ORF74 is crucial for transducing signal information to downstream effector proteins (26) and by substitution of this "signaling domain" with the US28-ctail abolished the constitutive signaling activity of ORF74.

A very recent study (20) showed that the C terminus of US28 is constitutively phosphorylated and that the deletion of all phosphorylation sites resulted in increased surface expression of US28. Both of our c-tail truncated mutants of US28 are devoid of all but one (US28Δ317 still contains serine 315) characterized phosphorylation sites in this study. However, the rather drastic protein modifications in US28Δ317 and US28Δ300 did not impair the signaling capacities of these receptors (Fig. 2, *C* and *D*). On the contrary, cells expressing US28 constructs either (i) lacking the US28 C terminus, *i.e.* US28Δ300 and US28Δ317, or (ii) carrying C-terminal substitutions from other 7TM receptors, *i.e.* the US28-ORF74-ctail and US28-NK_1-ctail chimera, showed enhanced levels of constitutive activity in inositol turnover, NF-κB, and CREB transcriptional activation when compared with the wild type receptor (Figs. 2 and 5). This apparent "gain of function" might simply reflect the fact that these receptors lack residues important for their sorting to late endosomes and multivesicular bodies (7), and thus concentrate more on the cell surface and in closer vicinity to their cognate G protein(s) (see Table II). This in turn would result in more efficient signaling at a given time, hence the increased level of activity.

Taken together, our experiments clearly demonstrate that the C terminus of US28 contains sufficient information for the constitutive internalization of 7TM receptors, independent of the signaling capacity of the receptor.

To date, only one chemokine ligand modulating the constitutive signaling activity of US28 has been identified, the membrane-associated fractalkine/CX_3CL1 (5, 6). Fractalkine/CX_3CL1 is a partial inverse agonist on wild type US28 receptors as assessed by inositol phosphate turnover, CREB transcription activation (Fig. 6), and NF-κB transcription (6) and has been shown to induce some down-modulation of the wild type US28 receptor (25% of receptor pool in 30 min) (7). Strikingly, when testing fractalkine/CX_3CL1 on the US28-c-tail-deficient receptors, we observed a dose-dependent increase in signaling activity in both the US28Δ300 and the US28-NK_1-ctail receptors (Fig. 6). Neither US28Δ300 nor US28-NK_1-ctail receptors are constitutively endocytosed, and in the presence of CX_3CL1, the endocytosis rates of these two receptors was significantly reduced compared with the wild type US28 receptor (Fig. 6*C*). We hence speculated that fractalkine might in fact be an agonist on US28; however, because the wild type US28 receptor is rapidly internalizing, the agonist signaling properties of fractalkine are simply undetectable. US28 receptors devoid of their constitutively internalizing C termini reveal the true agonistic strength of fractalkine, because US28 is now concentrated predominantly on the surface of the cell. It is tempting to speculate that the concentration of US28 receptor on the surface of the cell reflects a more *in vivo* like setting. Evidence for this is found in the striking study of Streblow *et al.* (12), in which US28 was able to mediate the migration of smooth muscle cells in response to the chemokines RANTES/CCL5 and MCP-1/CCL2, thus suggesting US28 to be one of the possible causative agents in the pathogenesis of atherosclerosis and restenosis. In this study, immunofluorescence analysis of smooth muscle cells infected with an adenovirus carrying a FLAG-tagged US28 receptor indicated that US28 was present on the plasma membrane of these cells in a polarized fashion, suggesting that US28 was concentrated at the leading edge of smooth muscle cells when exposed to a chemokine gradient. Hence, it might be feasible that in certain cellular environments combined with certain extracellular stimuli, US28 is indeed expressed to a greater extent on the plasma membrane than generally observed in the laboratory used heterologous expression systems.

From a virological point of view, US28 is likely to be located intracellularly in cell types that provide the best environment for the replication and spreading of the virus, thereby acting as an immediate early gene to up-regulate cellular transcription factors like CREB or NF-κB as suggested previously (6, 34, 35). However, other cell types might provide different sorting and/or interaction protein partners for US28, resulting in a more pronounced surface expression of the receptor, which then in turn becomes more susceptive to extracellular stimuli, such as fractalkine. In these cell types, fractalkine might in fact now act as an agonist, eventually leading to aberrant cell growth and pathological phenotypes (12), an unpleasant side effect of HCMV infections.

From a drug developmental point of view, the striking finding that constitutive receptor endocytosis can mask the true agonistic properties of a ligand might have important implications in the generation of more effective therapeutic agents. A variety of constitutively activating mutations for 7TM receptors has been implicated to be causative agents in the development of diseases. For example, mutations in the thyrotropin and luteotropin receptors cause thyrotoxicosis (36) and premature puberty (37), respectively. One might envision that the treatment of such constitutively signaling receptors with an "apparent" inverse agonist, which in pathophysiological cellular environments is truly a "camouflaged agonist," might in fact lead to the opposite rather than the desired therapeutic effect.

Acknowledgments—We thank Trine Lind, Lisbet Elbak, and Jamie Fong for excellent technical assistance. We are grateful to Selena Bartlett for critically reading this manuscript.

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