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Comparison of Agonist-Induced Internalization of the Human EP2 and EP4 Prostaglandin Receptors: Role of the Carboxyl Terminus in EP4 Receptor Sequestration

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ABSTRACT

Prostaglandin E_2 (PGE_2) couples to stimulation of adenylyl cyclase through two distinct G protein-coupled receptors designated EP2 and EP4. Although they have similar affinities for PGE_2, the EP_2 and EP4 receptors have distinct structural characteristics. EP2 is a 358-amino-acid protein with short third intracellular loop and C-terminal domains, whereas EP4 consists of 488 amino acids with a long third intracellular loop and a long cytoplasmic tail. The ability of the HA epitope-tagged receptors to undergo PGE_2-induced internalization was examined by enzyme-linked immunosorbent assay and immunofluorescence microscopy after expression in human embryonic kidney 293 cells. The EP2 receptor did not internalize, whereas the EP4 receptor underwent rapid internalization. Truncation of the EP4 receptor after amino acid 350, which removes 138 residues, abolished internalization. Truncation after amino acid 369 markedly attenuated internalization, whereas truncation after amino acid 383 had little effect. Serine and threonine residues in the region 350 to 383 were mutated to determine their role in internalization. The mutants S370-382A, a full-length receptor containing six serine-to-alanine mutations in the region 370 to 382, and S354-369A, containing four serine mutations and one threonine mutation in the region 350 to 370, both internalized to the same extent as the wild-type. A further mutant, designated S354-382A, containing amino acid substitutions S354A, S359A, S364A, S366G, T369A, S370A, S371A, S374A, S377A, S379A, and S382A, also internalized to the same extent as the wild-type. We conclude that the C terminus of the EP4 receptor is involved in internalization; however, serine and threonine residues do not seem to be involved.

PGE_2 is an important autocrine mediator in the cardiovascular and other systems (Campbell and Halushka, 1996). PGE_2 is a potent vasodilator in the microvasculature, although it may also cause constriction at selected sites. It causes a fall in systemic blood pressure and an increase in blood flow to the heart; it increases blood flow to the kidneys, leading to increased diuresis, natriuresis, and kaluresis, and causes secretion of renin from the renal cortex. PGE_2 exerts its actions through four distinct G protein-coupled receptors, designated EP1, EP2, EP3, and EP4, that are encoded by different genes (for a review, see Negishi et al., 1995). The EP1 receptor couples to phospholipase C, the EP2 and EP4 receptors couple to stimulation of adenylyl cyclase, and the EP3 receptor couples to inhibition of adenylyl cyclase.

EP2 and EP4 receptors are widely distributed; both receptors are found in uterus, spleen, and lung; the EP4 receptor is also present in small intestine, thymus, pancreas, leukocytes, and kidney (Regan et al., 1994; Katsuyama et al., 1995). There are no selective EP receptor antagonists that can be used in studies to functionally distinguish the two receptors. However, the selective EP2 receptor agonist butaprost is not active at the EP4 receptor (Regan et al., 1994; Nishigaki et al., 1995). Some actions of PGE_2 have been assigned to the EP4 receptor through use of butaprost. These include stimulation of matrix metalloproteinase activities in RNK-16 cells (Zeng et al., 1996). In addition, EP4 has been identified as the receptor on T cells responsible for PGE_2-mediated desensitization of the CCR5 receptor, which is a cofactor in HIV infection (Thivierge et al., 1998). Gene knockout of the EP4 receptor shows that it plays a key role in closure of the ductus arteriosus at birth (Nguyen et al., 1997, Segi et al., 1998).

Although they have similar affinities for PGE_2, and function similarly to activate adenylyl cyclase, the EP_2 and EP4 receptors have distinct structural characteristics. EP2 is a 358-amino-acid protein with relatively short third intracellular loop and C-terminal domains (Regan et al., 1994). On

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ABBREVIATIONS: PGE_2, prostaglandin E_2; ELISA, enzyme-linked immunosorbent assay; HA, hemagglutinin; PCR, polymerase chain reaction; HEK, human embryonic kidney; DMEM, Dulbecco's modified Eagle's medium; EBNA, Epstein-Barr virus nuclear antigen; IBMX, 3-isobutyl-1-methylxanthine; PFA, paraformaldehyde; HBSS, Hank's balanced salt solution; BLOTTO, bovine lacto transfer optimizer.

the other hand, EP4 consists of 488 amino acids, and has a long third intracellular loop and a long cytoplasmic tail (An et al., 1993). The EP2 receptor does not undergo agonist-induced, short-term desensitization, whereas EP4 desensitizes rapidly (Nishigaki et al., 1996; Bastepe and Ashby, 1997; Bastepe and Ashby, 1999), within minutes. Both the EP2 and EP4 receptor undergo down-regulation upon long term (>2 h) exposure to agonist. Hence, although boundaries between the roles of the EP2 and EP4 receptors remain to be clarified, agonist-induced short-term desensitization is a significant regulatory element in EP4-mediated signaling, but not in EP2-mediated signaling, so that EP2 may be involved in mediating sustained actions of PGE_2, whereas EP4 mediates rapidly waning events.

In previous studies, we have shown that the C-terminal domain of EP4 is essential for agonist-induced desensitization (Bastepe and Ashby, 1997). By deletion mutagenesis we identified a 14-amino-acid stretch containing six serine residues that is involved in short-term EP4 desensitization (Bastepe and Ashby, 1999). We mutated the six serine residues at positions 370, 371, 374, 377, 379, and 382 to alanine to generate a mutant, designated hEP_4-S370-382A, that retained the entire C-terminal domain of wild-type EP_4. Mutant hEP_4-S370-382A showed no desensitization, indicating that at least one of the six serines located between positions 370 and 382 may be necessary.

In the present work, we have extended our studies to examine structural features involved in internalization of the EP4 receptor, and have shown that the EP2 receptor does not undergo internalization. We assayed internalization on epitope-tagged forms of the receptors by an ELISA and by confocal immunofluorescence microscopy. We examined agonist-induced internalization of the native EP4 receptor and of a number of truncated forms as well as mutant forms.

Our results indicate that the C terminus of the EP4 receptor is involved in sequestration in common with other G protein-coupled receptors; however, serine or threonine residues in the C terminus do not seem to be involved.

Materials and Methods

Construction of Epitope-Tagged EP4 Truncations and Mutants. The cDNAs for the EP4 receptor, truncated forms of the receptor and the mutant receptor S370-382A were prepared as described previously (Bastepe and Ashby, 1999). The EP2 and EP4 receptor constructs were epitope-tagged by inserting the nine-amino-acid epitope (YPYDVPDYA) of hemagglutinin between the N-terminal methionine and the second amino acid of each by use of PCR. The 5′ end of the PCR product carried a *Hin*dIII site. The forward primer for the EP4 receptor was 5′-ATG **AAG CTT** ATC ATG TAC CCA TAC GAC GTC CCA GAC TAC GCT TCC ACT CCC GGG GTC AAT-3′, where the *Hin*dIII site is indicated in bold and the HA-tag is underlined, and the reverse primer was 5′-GGG ATG GAG CAG ATG A GC-3′. The forward primer for the EP2 receptor was 5′ CTA **AAG CTT** ATG TAC CCA TAC GAC GTC CCA GAC TAC GCT GGC AAT GCC TCC AAT GAC TCC-3′, where the *Hin*dIII site is indicated in bold and the HA-tag is underlined, and the reverse primer was 5′-TGT AGG CCT AAG GAT GGC AAA GAC-3′. The PCR product included a *Sac*II site located at nucleotide 696 of the coding region allowing us to epitope tag constructs already created with truncations and mutations in the C-terminal domain that begin at the same *Sac*II site and stretch into the 3′end of the molecule. Each construct was epitope-tagged by cutting with *Hin*dIII and *Sac*II and removing the 5′ end of the receptor, which does not contain any of the truncations or mutations. The HA epitope-tagged PCR product was ligated into the *Hin*dIII/*Sac*II site. All constructs were prepared in pUC18 (Life Technologies-BRL, Grand Island, NY) and subcloned into the mammalian expression vector pCEP4 (Invitrogen, Carlsbad, CA) for transfection.

Site-Directed Mutagenesis. Construction of a mutant containing the amino-acid substitutions S354A, S359A, S364A, S366G, T369A (designated S354-369A) in the full-length receptor was achieved by oligonucleotide-directed mutagenesis according to the method of Higuchi et al. (1988). Two overlapping fragments of EP4 were amplified by PCR using Turbopfu DNA polymerase (Stratagene, La Jolla, CA) using the flanking primers P1 = 5′-CCA CCG CGG CCG CCT CGG TTG CCT CC-3′ and P4 = 5′-GGA GGG CCC TAT TTA TTC ATA TAC ATT TTT CTG ATA AGT TCA G- 3′, and the following primers, which incorporate the five mutations. P2 = 5′-GGC GGG GCC CGC AGG GAG CGC GCC GGA CAG CAC TGC GCA GAC GGT CAA AGG GCA TCT TCT −3′, and P3 = 5′- AGA AGA TGC CCT TTG ACC GTC TGC GCA GTG CTG TCC GGC GCG CTC CCT GCG GGC CCC GCC - 3′. PCR was carried out for 25 cycles with 1 min at 95°C, annealing for 1 min at 62°C, and elongating for 1 min at 72°C. One of the PCR products extends from the unique *Sac*II site to nucleotide 1116 (underlined in P1) and the other one from nucleotide 1095 to the *Apa*I site (underlined in P4) adjacent to the termination codon. Small aliquots of the overlapping PCR products were combined, denatured, reannealed, and subjected to additional 25 cycles of PCR using primers P1 and P4. After purification and restriction enzyme digestion with *Sac*II and *Apa*I, the resultant PCR product was substituted into the corresponding region of HA tagged-EP4 receptor in pUC 18 and the full-length construct subcloned into pCEP4 for transfection.

Construction of a full-length mutant containing the amino acid substitutions S354A, S359A, S364A, S366G, T369A, S370A, S371A, S374A, S377A, S379A, and S382A, was achieved by a similar strategy using the mutant S370-382A as template for the initial round of PCR using primers P2 and P3. This construct was designated S354-382A.

PCR of the Human Prostaglandin EP2 Receptor. We obtained the cDNA for the human EP2 prostaglandin receptor by RT-PCR of vascular smooth muscle total RNA. The full-length EP2 PCR product was prepared with primers containing a *Hin*dIII site located on the forward primer and a *Bam*HI site located on the reverse primer and subcloned into the vector puC18. The cDNA was sequenced and shown to be identical with the sequence of the EP2 receptor reported by Oakley (1995). The EP2 cDNA was HA epitope tagged and cloned into the expression vector pCEP4.

Expression in 293-EBNA Cells. EP4 receptors and constructs were transfected into 293-EBNA cells obtained from Invitrogen. This line is a derivative of HEK 293 cells that stably expresses EBNA1. 293-EBNA cells were grown in DMEM supplemented with 10% fetal bovine serum in the presence of 250 μg/ml G418 to maintain expression of the EBNA1 plasmid. The cells were stably transfected with EP4 or EP2 construct cDNAs in pCEP4 by use of Lipofectamine (Life Technologies) according to the manufacturer's instructions with 200 μg/ml hygromycin B for selection, and clonal cells isolated by serial dilution. Positive clones were identified by the HA-tagged ELISA assay and by measuring PGE_2-induced cAMP formation, and maintained in medium containing 10% fetal bovine serum, 250 μg/ml G-418 sulfate and 200 μg/ml hygromycin B.

Determination of cAMP Formation. To determine whether selected clones produced cAMP in response to PGE_2, clonal cells grown in six-well, 35-mm culture plates were labeled overnight with 2 μCi/ml of [^{3}H]adenine (25 Ci/mmol). Labeling medium was removed, and cells incubated for 10 min with fresh medium containing 2 mM IBMX. Cells were treated with 100 nM PGE_2 for 15 min in the presence of 2 mM IBMX. Reactions were stopped by the replacement of the medium with a stopping solution containing 0.2 M HCl, 0.2% SDS and 2000 cpm of [^{14}C]cAMP used as recovery standard. The

amount of [^{3}H]cAMP was determined as a percentage of the total labeled adenine nucleotide pool according to Salomon (1979).

Measurement of Internalization. Internalization of receptors was determined by measuring loss of cell-surface immunoreactivity of HA-epitope-tagged receptors using an ELISA assay and by immunofluorescence microscopy.

ELISA Assay. 293-EBNA cells stably expressing the HA-epitope-tagged EP4 or EP2 receptor were grown in 12 plates. On the day of assay, the medium was replaced by DMEM buffered with 50 mM HEPES, pH 7.4. Cells were treated with PGE_2 or vehicle for various times at 37° and incubations terminated by placing the plate in on ice for 5 min. Cells were fixed with 2% PFA for 10 min, washed, blocked, and incubated with anti-HA antibody [monoclonal HA 11 from Babco (Richmond, CA)] for 30 min. After washing, cells were incubated with horseradish peroxidase-conjugated secondary antibody for 30 min then washed and exposed to substrate *O*-phenylenediamine (Pierce, Rockford, IL) and hydrogen peroxide. Aliquots were transferred to 96-well plates, the reaction stopped by adding sulfuric acid, and read at 450 nm on a microtitre plate reader. Results were expressed relative to the value obtained with untreated cells as percent surface HA immunoreactivity.

Immunofluorescence Confocal Microscopy. 293-EBNA cells stably expressing HA-tagged receptors were grown in 35-mm dishes on coverslips. Cells were washed with PBS and treated with PGE_2 for various times and at various concentrations. Cells were fixed and prepared by the following protocols to examine disappearance of receptors from the surface and appearance of receptors inside the cell using immunofluorescence confocal microscopy.

Detection of Surface Receptors. Cells were incubated with HA specific antibody (monoclonal HA 11 from Babco) at a 1:500 dilution for 30 min at 4°C in DMEM with 1% BSA and then washed twice with HBSS. The cells were warmed to 37°C and treated with or without 100 nM PGE_2 for the indicated times in 0.5% BSA, 20 mM HEPES, pH 7.4 and then fixed with 2% PFA at room temperature for 10 min. The cells were washed three times with HBSS and blocked for 30 min at 37°C with PBS containing 5% nonfat milk (blotto). Goat anti-mouse fluorescein isothiocyanate-conjugated secondary antibody (Molecular Probes, Eugene, OR) was added at a dilution of 1:150 in BLOTTO for 30 min at 37°C. The cells were washed three times with HBSS. The coverslips were mounted on slides using slow-fade mounting medium (Molecular Probes).

Detection of Internalized Receptors. Cells were incubated with HA-specific antibody (monoclonal HA 16B12 from Babco, Richmond, CA) at a 1:500 dilution for 30 min at 4°C in DMEM with 1% BSA. The cells were washed twice with HBSS. The cells were warmed to 37°C and treated with or without 100 nM PGE_2 for the indicated times in 0.5% BSA, 20 mM HEPES, pH 7.4 and then fixed with 2% PFA at room temperature for 10 min. To mask surface receptors, unconjugated goat antimouse IgG (Jackson Immunoresearch, West Grove, PA) was incubated with the cells for 60 min at 37°C at a dilution of 1:25. The cells were then washed six times with HBSS and permeabilized with 0.05% Triton X-100 in PBS for 10 min at room temperature. After blocking for 30 min with BLOTTO containing 0.05% Triton X-100, the secondary fluorescein isothiocyanate-conjugated antibody was added (1:150) in BLOTTO for 30 min at 37°C. Cells were washed six times with PBS containing 0.05% Triton X-100 and the last wash left for 30 min at 37°C. The cells were fixed with 2% PFA at room temperature for 10 min, washed three times with HBSS, and mounted on slides with coverslips using Slow-fade mounting medium.

Confocal Microscopy. Fluorescence was examined using a Leica TCS-NT confocal microscope. Single optical sections across cells are presented. Only a few cells are shown in each case; the cells are representative of the whole field. The photomultiplier gain and magnification were kept constant for each set of photographs to permit comparison among them.

Results

The C-terminal tails of the prostaglandin EP2 and EP4 receptors are compared in Fig. 1. Hydropathy analysis indicates that the C terminus of EP2 begins around residue 318, comprising 40 amino acids that include 10 serine and threonine residues. The C terminus of EP4 begins around residue 350, comprising 148 amino acids that include 36 serine and threonine residues. Fig. 1 also illustrates the position of sites where truncation mutations were made in EP4 and the positions of serine and threonine residues that were mutated.

Comparison of EP2 and EP4 Receptor Internalization by ELISA. HA-tagged EP2 and EP4 prostaglandin receptors were expressed separately in 293-EBNA cells. Addition of the HA-epitope tag to either receptor did not affect its ability to generate cAMP and the EC_{50} values of the receptors were unchanged (data not shown). Typically, we observe values of between 2 and 3 nM for the EC_{50} values of both EP2 and EP4 receptors. To examine internalization, the cells were challenged with 1 μM PGE_2 for times ranging from 0 to 60 min and the amount of remaining surface HA antigen was determined by an ELISA at each time. As shown in Fig. 2, the EP4 receptor underwent rapid internalization to the extent of 40% with a half-time of just a few minutes. By contrast, the EP2 receptor was not internalized to any extent even after 60-min exposure to agonist. We also examined the dose-response relationship for internalization of the EP4 receptor after 30 min exposure to PGE_2. The EC_{50} for internalization of EP4 was 5 nM (data not shown).

Comparison of EP2 and EP4 Receptor Internalization by Immunofluorescence Confocal Microscopy. In a parallel series of studies, we measured internalization of the transfected HA-tagged EP2 and EP4 receptor by confocal microscopy. We used different procedures to examine 1) re-

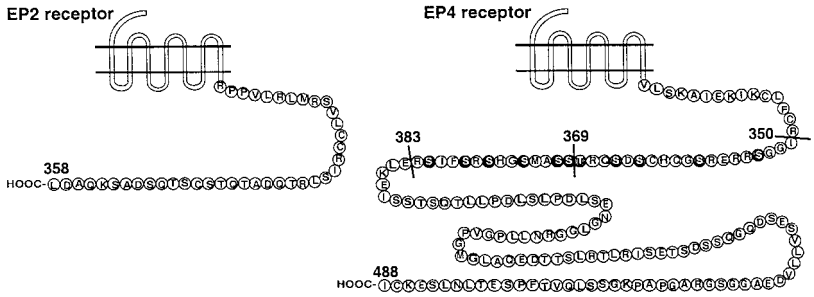

Fig. 1. Schematic representation of the amino acid sequence of the hEP2 and hEP4 carboxyl terminus. Lines on the EP4 C terminus indicate the truncation positions at amino acids 383, 369, and 350. Dark circles indicate the positions of alanine substitutions of the full-length receptor S370-382A. Gray circles indicate positions of alanine or glycine substitution of the full-length mutant receptor S354-369A. Mutant receptor S354-382A combined all of these substitutions.

ceptors only on the surface and 2) receptors that had been internalized.

Fig. 3 shows the time course of disappearance of the wild-type receptor from the surface and the time course of appearance in transfected 293-EBNA cells after exposure to 100 nM PGE_2. Disappearance of surface receptor is complemented by an increase in intracellular fluorescence with labeled receptor appearing in punctate regions inside the cell, which presumably represent early endosomes. The process shows a rapid time course similar to that observed by ELISA.

Fig. 4 shows comparable studies on the EP2 receptor, which shows no tendency to internalize as measured by either disappearance of surface receptor or appearance of internalized receptor, which confirms the result obtained by ELISA.

Examination of the Role of the C Terminus of EP4 in Internalization. To examine the role of the C terminus and to examine structural determinants involved in internalization of the EP4 receptor we created a number of truncation mutations that are illustrated in Fig. 1.

The wild-type receptor is designated t488 to indicate that it was truncated at the native stop codon to remove the 3′-untranslated tail. Other forms of the receptor were truncated at amino acid 383, 369, or 350 and are designated t383, t369, and t350. The truncations were characterized in previous studies from this laboratory (Bastepe and Ashby, 1999) and shown to bind PGE_2 with equal affinity (K_d of 4 nM) to the wild-type receptor and to couple to stimulation of adenylyl cyclase. Addition of the HA epitope tag to the N terminus did not alter these properties (data not shown). We studied the effect of truncation or mutation on internalization of the EP4 after stable transfection of the various constructs into 293-EBNA cells.

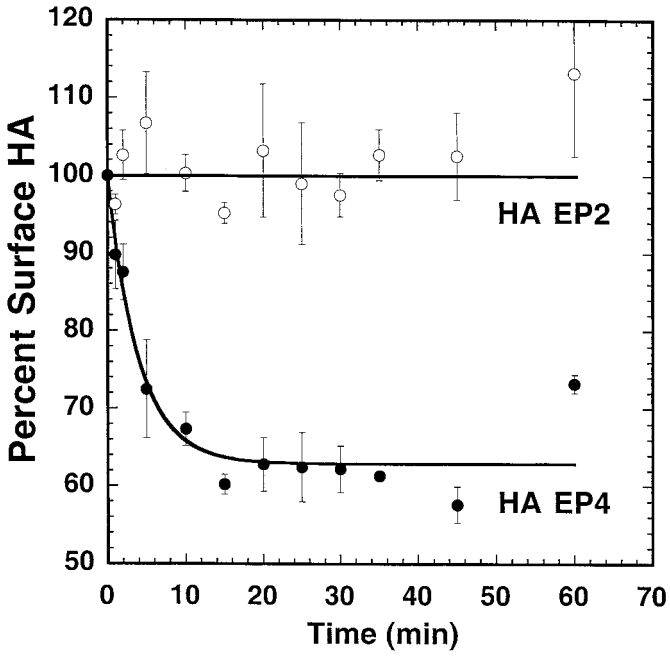

Fig. 2. Time course analysis of EP2 and EP4 receptor internalization induced by incubation with 1 μM PGE_2. 293-EBNA cells were stably transfected with HA epitope-tagged EP2 (○) or EP4 (●) receptors and the percentage of receptors remaining at the cell surface at various times after agonist stimulation was measured by ELISA analysis, as described under *Materials and Methods*. The results represent the mean ± S.E. of three independent experiments, each done in triplicate.

We examined the rate and extent of internalization of the EP4 receptor truncations and mutants after exposure to 100 nM PGE_2 at 37°C. Internalization of the HA epitope-tagged, wild-type EP4 receptor (HA-t488) was measured by ELISA. Fig. 5 shows that HA-t488 was rapidly internalized with a 40% loss of cell surface receptor occurring rapidly with a half-time of 1 to 2 min. Truncation of the receptor after amino acid 383, to remove 105 amino acids from the C-tail (HA-t383) had little effect on the extent of rapidity of surface receptor loss. By contrast, internalization of the truncated receptor HA-t369 was attenuated and that of HA-t350 almost abolished, suggesting that the C-terminal tail is involved in internalization and that amino acids in the region 350 to 382 are involved in the process.

Fig. 6 compares single optical sections of 293-EBNA cells expressing HA-tagged wild-type receptor, HA-t350 receptor, and HA-t369 receptor treated with and without PGE_2. Wild-type receptor showed marked PGE_2-induced internalization. The truncated receptor t350 did not internalize in response to PGE_2 even after 60-min exposure to the agonist. Similarly, HA-tagged t369 (HA-369) did not internalize after 60-min treatment with PGE_2.

Examination of the Role of C-Terminal Serine and Threonine Residues in Internalization of EP4. For many G protein-coupled receptors, the presence of serine or threonine phosphorylation sites is important for desensitization and internalization. In previous studies, we examined the mutant S370-382A, in which six serine residues between positions 370 and 382 were mutated to alanine in the full-length receptor. We showed that mutation of the six serines abolishes rapid agonist-induced desensitization. However, examination of the HA-tagged mutant S370-382A (HA-S370-382A) by ELISA analysis after exposure to PGE_2 showed that it displayed a similar extent and rate of internalization as the wild-type receptor, indicating that the six serine residues in this region play no role in internalization (Fig. 7). The result was confirmed by examination of cells expressing HA-S370-382A by confocal immunofluorescence microscopy (Fig. 8). Challenge with 100 nM PGE_2 for 30 min resulted in marked disappearance of HA-S370-382A from the surface of the cell and a pronounced increase in intracellular fluorescence in vesicles. Intermediate time points showed that internalization took place rapidly with a time course (not shown) similar to that observed in the ELISA assay.

We also examined the effect of modifying serine and threonine residues in the region 350 to 382. We prepared a mutant, designated S354-369A, containing the amino acid substitutions S354A, S359A, S364A, S366G, and T369A in the full-length receptor. ELISA analysis after exposure to PGE_2 showed that it displayed a similar extent and rate of internalization as the wild-type receptor, indicating that the one threonine and four serine residues in this region play no role in internalization (Fig. 7). The result was confirmed by examination of cells expressing HA-S354-369A by confocal immunofluorescence microscopy (not shown).

In addition, we examined a mutant, designated S354-382A, containing amino acid substitutions S354A, S359A, S364A, S366G, T369A, S370A, S371A, S374A, S377A, S379A, and S382A. This mutant again was a full-length receptor, combining the mutations in S354-369A with the mutations in S370-382A. Mutant S354-382A, containing a total of 10 serine mutations and one threonine mutation in

the C terminus, internalized to the same extent as the wild-type (Fig. 7).

Discussion

In the current work we have shown that epitope-tagged prostaglandin EP4 receptor undergoes rapid internalization in response to PGE_2, whereas the epitope-tagged prostaglandin EP2 receptor does not. Internalization of epitope-tagged forms of the receptors was measured by ELISA analysis, which showed that EP2 is not internalized after 1-h exposure to PGE_2, whereas EP4 internalizes rapidly with a $t_{1/2}$ of 2 min, to the extent of 40% loss of surface EP4 receptor. The results were closely paralleled in time-course experiments showing disappearance of cell surface EP4 receptor and appearance of internalized receptor by immunofluorescence confocal microscopy.

The role of the carboxyl tail in desensitization and internalization has been demonstrated for a number of G protein coupled receptors using studies of truncated receptors and site-directed mutagenesis. In the case of the EP4 receptor, we have previously shown that the C terminus plays a role in rapid agonist-induced desensitization of adenylyl cyclase activity. To examine the role of the C terminus in internalization of EP4, we prepared an epitope-tagged form of the receptor truncated at amino acid 350 (HA-t350), close to the proximal end of the C terminus. Studies by both ELISA analysis and immunofluorescence confocal microscopy demonstrated that mutant t350 underwent minimal internalization. Further deletion mutants were studied. Truncation of the receptor at residue 369 also attenuated internalization, whereas truncation at residue 383 had little effect.

The results indicate that the region of the C terminus between residues 350 and 383 may be important in internalization. We have previously shown that the same region seems to be involved in agonist-induced desensitization of adenylyl cyclase. Within this 33-amino-acid stretch are 10 serines and one threonine, which are potential sites for phosphorylation. In previous studies (Bastepe and Ashby, 1999), we mutated the six serines in the region 370 to 382 to alanine to generate the mutant S370-382A, a full-length form of the receptor with serine-to-alanine mutations at positions 370,

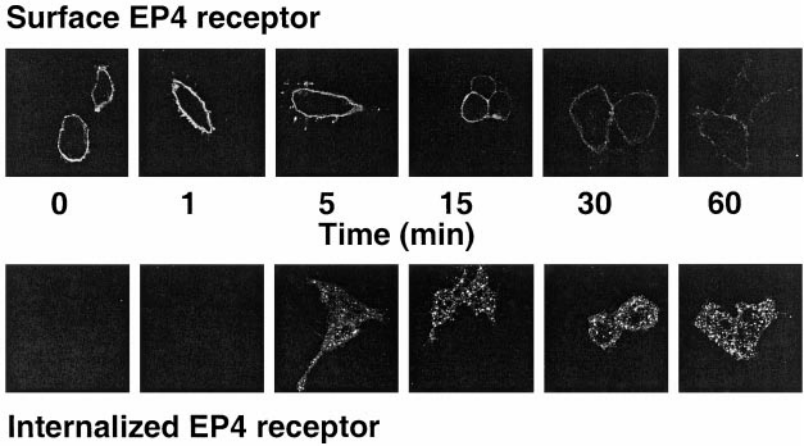

Fig. 3. Immunofluorescence analysis of EP4 (HA-t488) distribution in 293-EBNA cells. Stably transfected HA epitope-tagged receptors were prelabeled at 4°C with the anti-HA monoclonal antibody and then treated with 100 nM PGE_2 for various times. Cells were fixed and processed for detection of surface or intracellular immunofluorescence as described under *Materials and Methods*. Top, time course of disappearance of wild-type EP4 receptor from the surface. Bottom, time course of appearance of epitope-tagged receptor inside the cell. Single optical sections obtained by confocal microscopy are shown. Results are representative of three individual experiments.

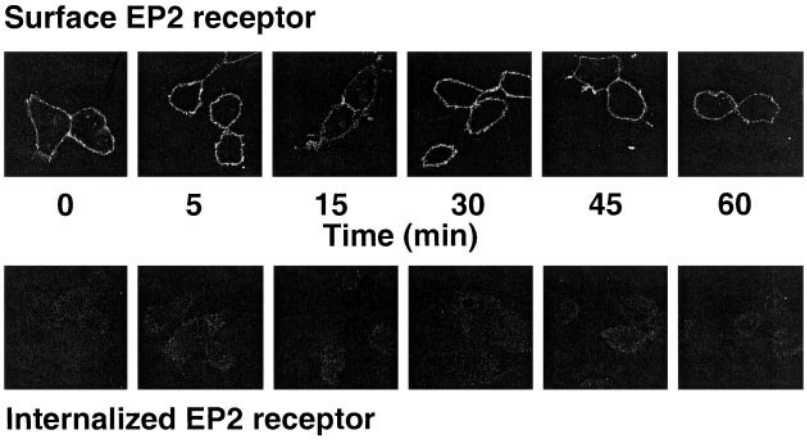

Fig. 4. Immunofluorescence analysis of EP2 distribution in 293-EBNA cells. Stably transfected HA epitope-tagged receptors were prelabeled at 4°C with the anti-HA monoclonal antibody and then treated for various times with 100 nM PGE_2. Cells were fixed and processed for detection of surface or intracellular immunofluorescence as described under *Materials and Methods*. Top, distribution of prostaglandin EP2 receptor in the presence of 100 nM PGE_2 or vehicle for various times. Bottom, distribution of intracellular epitope-tagged EP2 receptor in the presence of 100 nM PGE_2 or vehicle over the same time course. Single optical sections obtained by confocal microscopy are shown. Results are representative of three individual experiments.

371, 374, 377, 379, and 382. Mutant S370-382A was impaired in agonist-induced desensitization. However, in the current study we have shown by ELISA analysis that an epitope-tagged form of S370-382A undergoes internalization to the same extent and at the same rate as the native receptor. The finding was confirmed by immunofluorescence microscopy, which showed significant internalization of the mutant S370-382A receptor.

We also mutated the remaining four serines and one threonine residue in the region 350 to 383, which are located between residues 354 and 369. The full-length mutant receptor, designated S354-369A, contained the amino acid substitutions S354A, S359A, S364A, S366G, and T369A. The epitope-tagged mutant, designated HA-S354-369A, underwent internalization to the same extent as the wild-type receptor.

We examined a further mutant, designated S354-382A, containing amino acid substitutions S354A, S359A, S364A, S366G, T369A, S370A, S371A, S374A, S377A, S379A, and S382A. This mutant again was a full-length receptor, combining the mutations in S354-369A with the mutations in S370-382A. Mutant S354-382A, containing a total of 10 serine mutations and 1 threonine mutation in the C terminus, internalized to the same extent as the wild-type. We conclude that the C terminus of the EP4 receptor is involved in internalization; however, serine and threonine residues do not seem to be involved.

In common with findings with numerous G protein-coupled receptors, the results support the idea that the C-tail is an important domain in internalization. In general, receptors with short C-tails do not undergo internalization, whereas receptor forms with long C-tails do internalize. Similarly, truncation of the C-tail often leads to loss of internalization. For example, our results may be compared with those described for the thromboxane A2 receptor (TP). The human TP receptor seems to be encoded by a single gene that can be alternatively spliced in the C-tail to give rise to a short isoform TPα (343 residues) and a long isoform TPβ (407 residues). Benovic and coworkers have examined internalization of the isoforms (Parent et al., 1999). Using ELISA and immunofluorescence microscopy, they demonstrated that TPβ but not TPα underwent agonist-induced internalization when expressed in HEK 293 cells.

Numerous studies have been directed toward identification of particular residues in the C-tail involved in internalization. For example, a short sequence SSNGNTGEQS located in the mid-region of the C-tail of the β2AR has been identified as being potentially important for agonist-mediated sequestration (Hausdorff et al., 1991). Mutation of Ser355, Thr356, Ser360, and Thr364 markedly decreased internalization of

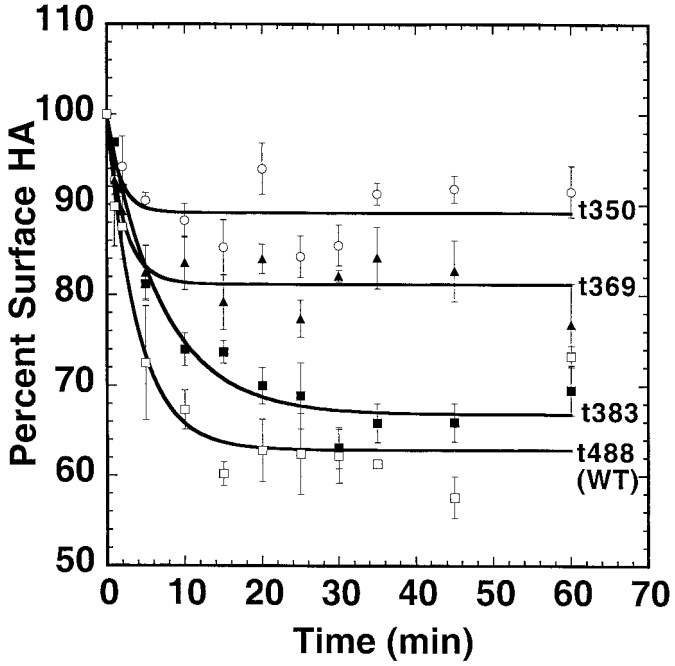

Fig. 5. Time course of PGE_2-induced internalization of 293-EBNA cells stably expressing the wild-type EP4 receptor or each of the deletion mutants. HA-t350 (○), HA-t369 (▲), HA-t383 (■), or HA-488t (□) were stimulated with 1 μM PGE_2 for various times. Incubations were stopped and the percentage of receptors remaining at the cell surface was measured by ELISA analysis as described under *Materials and Methods*. The results represent the mean ± S.E. of three to four experiments, each done in triplicate.

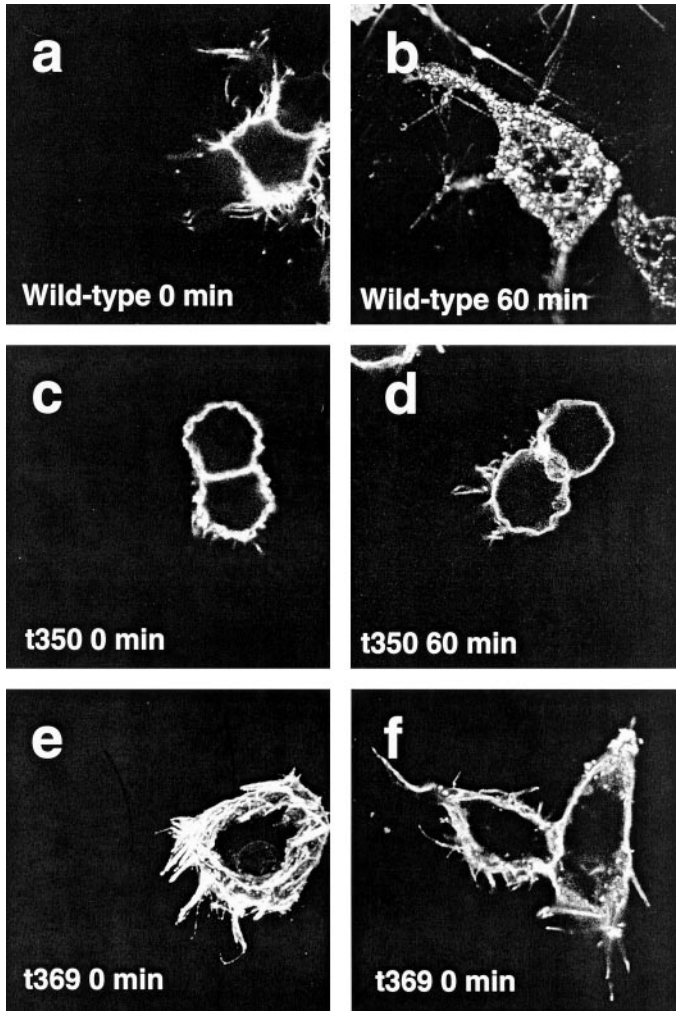

Fig. 6. Confocal microscopy comparison of wild-type HA-tagged receptor with HA-t350 and HA-t369. Top, distribution of wild-type EP4 receptor incubated with vehicle (a) or 100 nM PGE_2 (b) for 60 min. Center, distribution of HA-t350 incubated with vehicle (c) or 100 nM PGE_2 (d) for 60 min. Bottom, distribution of intracellular HA-t369 incubated vehicle (e) or 100 nM PGE_2 (f) for 60 min. Cells were fixed and processed for detection of immunofluorescence as described under *Materials and Methods*. Single optical sections obtained by confocal microscopy are shown. Results are representative of three individual experiments.

the receptor. There is a somewhat homologous sequence (SSQGQDSESV) in the C-tail of EP4. However, this sequence is located close to the distal end of the C terminus and its removal in the truncation t383 has no effect on internalization.

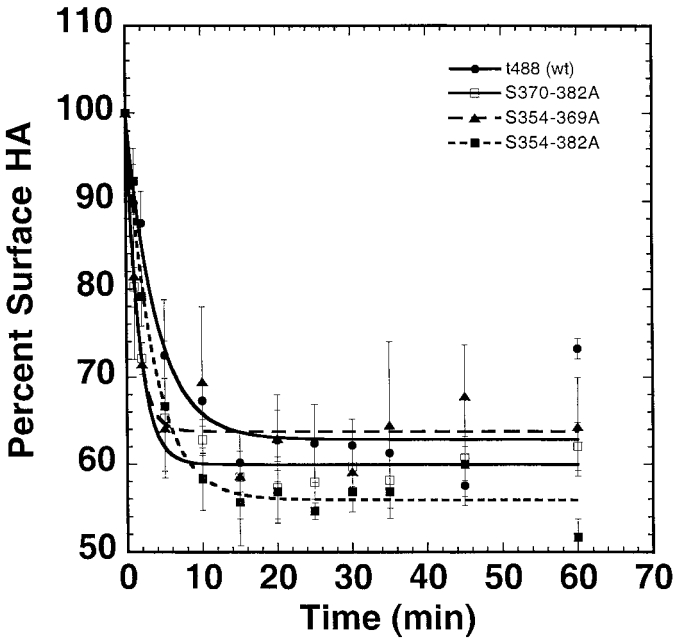

Fig. 7. Time course of PGE_2-induced internalization of wild-type EP4 and mutant receptors. HEK 293 EBNA cells were stably transfected with either HA epitope-tagged wild-type EP4 receptors (●) or mutant S370-382A (□) (S370A, S371A, S374A, S377A, S379A, and S382A), mutant S354-369A (▲) (S354A, S359A, S364A, S366G, and T369A), or mutant S354-382A (■) (S354A, S359A, S364A, S366G, T369A, S370A, S371A, S374A, S377A, S379A, and S382A). Cells were stimulated with 1 μM PGE2 for various times. Incubations were stopped and the percentage of receptors remaining at the cell surface was measured by ELISA analysis as described under *Materials and Methods*. The results represent the mean ± S.E. of three to four experiments, each done in triplicate.

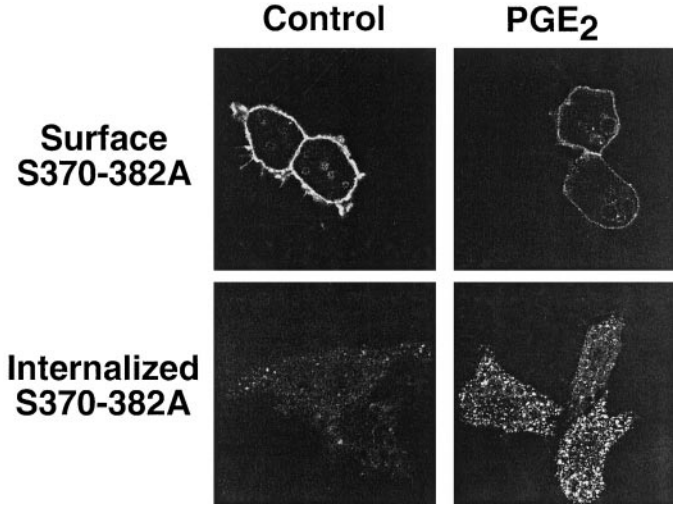

Fig. 8. Immunofluorescence visualization of surface and intracellular receptors on cells expressing mutant S370-382A. 293-EBNA cells expressing the receptors were incubated with anti-HA monoclonal antibody for 30 min at 4°C and then treated with or without 100 nM PGE_2 for 60 min. Reactions were terminated and cells were fixed and processed for detection of either surface or intracellular immunofluorescence. Detailed procedures are described in *Materials and Methods*. Top, surface detection of mutant S370-382A with vehicle or PGE_2. Bottom, intracellular detection of S370-382A incubated with vehicle or 100 nM PGE_2 for 60 min at 37°C. Immunofluorescence was analyzed by confocal microscopy. Images represent single optical sections.

In the AT1A angiotensin receptor, the motif Ser-Thr-Leu (residues 335–337) seems to be involved (Thomas et al., 1995), whereas in the m3 muscarinic receptor, Thr550, Thr553, and Thr554 are implicated (Yang et al., 1995). In the case of the gastrin-releasing peptide receptor, multiple serine and threonine residues distal to a putative palmitoylated cysteine residue have been shown to regulate internalization rate (Benya et al. 1993). In the case of the EP4 receptor, mutation of serine (and threonine) residues in two groups of six and five had no effect on internalization.

Although specific amino acid residues and sequence motifs have been implicated in G protein-coupled desensitization and internalization, numerous studies similar to ours seem to indicate that the mechanisms responsible for the two processes are distinct. For example, substitution of four serine and threonine residues in the carboxyl terminus of the *N*-formyl peptide chemoattractant receptor resulted in a mutant that was unable to desensitize but continued to internalize in a manner similar to that of the wild-type receptor (Maestes et al., 1999). This was also true for the parathyroid hormone receptor, in which alanine mutagenesis of six serine residues abolished phosphorylation but had no effect on the rate and extent of internalization (Malecz et al., 1998). Similarly, certain residues can be mutated in the C-tail of the β-adrenergic receptor that affect desensitization without affecting sequestration (Campbell et al., 1991), and mutation of individual serine residues in the C-terminal tail of the lutropin/choriogonadotropin receptor shows distinct structural requirements for agonist-induced uncoupling and agonist-induced internalization (Lazari et al., 1998). Pals-Rylaarsdam et al. (1995) showed that desensitization and internalization of the m2 muscarinic acetylcholine receptor are directed by independent mechanisms. In the case of the prostaglandin EP4 receptor, mutation of serine residues in the region 370 to 382 abolishes desensitization (Bastepe and Ashby, 1999) but has no effect on internalization.

Residues other than serine and threonine have been shown to be involved in internalization. Dileucine motifs located in the C terminus have been implicated in internalization of certain receptors, including the β-adrenergic receptor (Gabilondo et al., 1997). There are three dileucine motifs in the EP4 receptor C-tail: Leu 395/396, Leu 414/415, and Leu 452/453. However, all of these residues are eliminated by truncation of the receptor at residue 383 with little effect on internalization, indicating that none is important for this process.

Lack of involvement of serine and threonine residues tends to imply a mechanism of internalization distinct from that described for the β-adrenergic receptor. The latter involves agonist-induced phosphorylation of the receptor, followed by binding of β-arrestin, binding of the phosphorylated receptor-arrestin-complex to clathrin and endocytosis by clathrin-coated vesicles (Ferguson et al., 1998; Gagnon et al., 1998; Lefkowitz et al., 1998). Other mechanisms, represented by angiotensin II type 1A receptor, do not require β-arrestin or dynamin I (Zhang et al., 1996; Ferguson et al., 1998) and internalization seems to proceed via noncoated vesicles (caveolae). Studies are underway in this laboratory to determine the pathway of internalization of the prostaglandin EP4 receptor.

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