Role of the Thrombin Receptor’s Cytoplasmic Tail in Intracellular Trafficking

DISTINCT DETERMINANTS FOR AGONIST-TRIGGERED VERSUS TONIC INTERNALIZATION AND INTRACELLULAR LOCALIZATION

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The G protein-coupled thrombin receptor is activated by an irreversible proteolytic mechanism and, perhaps as a result, exhibits an unusual trafficking pattern in the cell. Naive receptors tonically cycle between the cell surface and a protected intracellular pool, whereas receptors cleaved and activated at the cell surface internalize and move to lysosomes. Toward understanding how these trafficking events are regulated, we examined a series of receptor mutants. A receptor with alanine substitutions at all potential phosphorylation sites in the cytoplasmic tail failed to display agonist-triggered internalization but, like wild type receptor, displayed robust signaling, tonic cycling, and localization to both the cell surface and an intracellular pool. A truncation mutant that lacked most of the cytoplasmic tail also signaled robustly, lacked phosphorylation, and was defective in agonist-triggered internalization. However, in contrast to the specific phosphorylation site mutant, the truncation mutant did not display tonic cycling and localized exclusively to the cell surface. An analysis of a series of truncation mutants localized residues important for receptor trafficking to a 10-amino acid stretch in its cytoplasmic tail. These data suggest that phosphorylation may trigger internalization of activated thrombin receptors but that a second phosphorylation-independent signal mediates tonic internalization of naive receptors. They further suggest that maintenance of the intracellular pool of naive thrombin receptors requires tonic receptor internalization.

The human thrombin receptor is a seven-transmembrane domain G protein-coupled receptor (GPCR) that is activated by a unique mechanism (1–5). Thrombin, a serine protease, cleaves its receptor’s amino-terminal exodomain (Fig. 1), unmasking a new amino terminus that then acts as a tethered ligand by binding to the body of the receptor and causing receptor activation. Limited proteolysis is irrevocable, and the tethered ligand that is unmasked by this proteolysis cannot diffuse away from its receptor. The irreversibility of this activation mechanism contrasts with that of classical reversibly liganded GPCRs and begs questions regarding the fate of activated thrombin receptors and the mechanisms by which cells regain thrombin responsiveness.

In fibroblasts and endothelial cells, the thrombin receptor resides both on the cell surface and in a substantial intracellular pool (6–8). Upon exposure to thrombin, receptors on the cell surface are cleaved and internalized and then traffic predominantly to lysosomes (7, 9–11). When thrombin is removed, uncleaved receptors from the intracellular pool appear at the cell surface. This trafficking behavior differs from that of the classical GPCR, the β₂-adrenergic receptor (12, 13), even when the two are expressed in the same cell (7). In the absence of agonist, the reversibly liganded β₂-adrenergic receptor resides primarily on the cell surface; it internalizes in response to agonist but recycles back to the cell surface after agonist removal. The trafficking of activated thrombin receptors to lysosomes and the existence of a protected intracellular pool may be necessitated by the receptor’s irreversible activation mechanism, providing for disposal of spent receptors and a means of restoring sensitivity to thrombin without waiting for new receptor synthesis.

Recent work revealed that in the absence of agonist, thrombin receptors tonically cycle between the cell surface and the intracellular pool. In these studies, cell surface receptors were labeled by binding antibody to an epitope on the receptor’s amino terminus at 4 °C. Upon subsequent incubation at 37 °C and in the absence of agonist, the amount of antibody bound to the cell surface declined to a new steady state of approximately half the initial level within 60 min. This decrease in the level of surface antibody was interpreted to reflect tonic cycling of the receptor leading to a lower steady state level of antibody on the cell surface once the rates of internalization and recycling of antibody-labeled receptors became equal. Indeed, the decline in antibody at the surface coincided with its appearance in an intracellular pool, and reappearance of such internalized receptor-bound antibodies on the cell surface was easily detected after removal of surface antibodies. The thrombin receptor can be activated without cleavage by the agonist peptide SFLLRN, which mimics the tethered ligand unmasked by thrombin (1). When agonist peptide was included in these experiments, the amount of antibody at the cell surface declined at a greater rate and to a greater extent than in the absence of agonist. These data suggest a working model in which the thrombin receptor exhibits two kinds of internalization: agonist-independent in-

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ternalization that is part of tonic cycling and agonist-triggered internalization.

The signals that specify these distinct trafficking patterns are unknown. A possible role for phosphorylation is suggested by the receptor’s rapid phosphorylation upon activation (14). Beyond terminating signaling (8, 14), might receptor phosphorylation also serve to mark activated thrombin receptors for agonist-triggered internalization? If so, is this signal distinct from that which specifies tonic agonist-independent internalization? We examined the trafficking of a series of mutant receptors to begin to address these questions.

EXPERIMENTAL PROCEDURES

Materials—M1 anti-FLAG monoclonal antibody, which binds the epitope DYKDDDDK, was purchased from Eastman Kodak Co. (8, 15). Horseradish peroxidase (HRP)-coupled goat anti-mouse antibody was from Bio-Rad and was used at a 1:1000 dilution unless otherwise indicated. Cell culture medium was obtained from the University of California San Francisco cell culture facility. In all procedures, Dulbecco’s modified Eagle’s medium (DMEM) was supplemented with 3 mg/ml glucose, 100 units/ml penicillin, 100 μg/ml streptomycin, and 2.5 μg/ml Fungizone. For stably transfected cells, 900 μg/ml G418 (Life Technologies, Inc.) was added. Phosphate-buffered saline (PBS) contained 0.1 mg/ml CaCl₂ and MgCl₂, 0.2 mg/ml KH₂PO₄ and KCl, 8 mg/ml NaCl, and 2.2 mg/ml Na₂HPO₄. PBS/EDTA contained 0.4 mg/ml EDTA and the same components as PBS except CaCl₂ and MgCl₂. The thrombin receptor agonist peptide, SFLLRN, was synthesized with a carboxyl amide and purified by high pressure liquid chromatography.

Plasmid Construction—All mutant receptors were derived from WT5, an otherwise wild type human thrombin receptor containing a FLAG epitope at its amino terminus (8, 16), and were subcloned into the mammalian expression vector pBJ1 (provided by Mark Davis, Stanford University, Stanford, California). Construction of C-tail:S/T→A was described previously (14). Stop codons were introduced into the receptor by site-directed mutagenesis (17) using the oligos 5'-ACTTGCCATCAACTACCCACTGCTGTT-3' (Y420Z), 5'-TAACAGCTTTT-3' (K407Z), 5'-GCTGGCATACACTACCCACTGCTGTT3' (Q402Z), and 5'-CCCACTGCTGTTTAACTGGGATC-3' (Y397Z).

Transient Transfection—To improve cell adherence in antibody binding assays, Primaria (Falcon) 24-well dishes (2 cm²/well) were incubated with 5 μg/well fibronectin (Sigma) for 45 min. Cos7 cells were split into these dishes and grown overnight in DMEM supplemented with 10% calf serum. For each well to be transfected, 0.4 μg of DNA was incubated with 6.8 μg of LipofectAMINE (Life Technologies, Inc.) for 30 min and then diluted to a volume of 0.5 ml in OptiMEM I (Life Technologies, Inc.). Cells were incubated with this mixture for 6 h at 37 °C, and then it was replaced with DMEM supplemented with 10% calf serum. Cells were then incubated at 37 °C for 48 h. For assays of inositol phosphate hydrolysis, Cos7 cells were transfected in 10-cm dishes using 10 μg of DNA and 180 μg of LipofectAMINE and later split into 24-well dishes.

Stable Transfection—Rat1 cells were stably transfected, and individual clones expressing various receptors were isolated as described previously (8).

Assay of Surface Antibody Levels—Assay media were DMEM supplemented with 1 mg/ml bovine serum albumin (BSA) and 10 mM HEPES buffer, pH 7.4. For each well to be assayed, cells were washed with media and incubated at 4 °C for 5 min. Cells were then incubated with 0.25 ml of media containing 3 μg/ml anti-FLAG antibody at 4 °C for 1 h. They were then washed twice with media and incubated at 37 °C for various times. Next, cells were fixed in 0.5 ml of 4% paraformaldehyde dissolved in PBS at 4 °C for 5 min and then washed twice with PBS. Cells were next incubated for 1 h at room temperature in 0.25 ml of media containing HRP-coupled goat anti-mouse antibody, then washed thrice with PBS and incubated for 20 min in One Step ABTS solution (Pierce), which contains the substrate for HRP. The A of this solution at 405 nm was measured using a Molecular Devices microplate spectrophotometer. For time course studies, the start times for the initial incubation with anti-FLAG antibody were staggered so that all procedures after the incubation at 37 °C were performed simultaneously in each experiment.

Assay of Intracellular Antibody Levels—Stably transfected cells were split into Primaria 24-well dishes (1 x 10⁶ cells/well) and incubated for 24 h at 37 °C in DMEM supplemented with 10% calf serum. Cells were incubated with anti-FLAG antibody at 4 °C, washed, and then incubated at 37 °C after the procedure described above for measuring surface antibody. Next, surface antibody was removed by a brief incubation in PBS/EDTA (binding of the M1 anti-FLAG antibody requires calcium (15)). The cells were then lysed in a 0.1-ml solution containing 50 mM Tris, pH 7.4, 100 mM NaCl, 5 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, 3% BSA, and 1% Triton X-100. The amount of anti-FLAG antibody in each lysate was then determined by an enzyme-linked immunosorbent assay. To determine the amount of antibody initially
Thrombin Receptor Internalization

Fig. 2. Agonist-triggered and agonist-independent internalization of wild type and phosphorylation site mutant thrombin receptors. Disappearance of receptor-bound antibody from the surface of Cos7 cells transiently transfected with FLAG epitope-tagged wild type (WT5, circles) or C-tail:S/T→A mutant (squares) thrombin receptor was measured. Cells were incubated with anti-FLAG antibodies at 4 °C and then, after removal of unbound antibody, incubated for the indicated times at 37 °C with (○, ○) or without (□, □) 50 μM SFLLRN agonist peptide. Cells were then fixed, and surface-bound antibody was quantitated (see “Experimental Procedures”). No detectable receptor internalization occurred during the 4 °C incubation. Antibody binding to cells transfected with pBJ1 only (empty vector) was typically less than 5% of that seen in transfected cells. For each time point, this nonspecific binding was determined and subtracted from the corresponding values obtained from receptor-transfected cells. Specific binding at each time point was then normalized to specific binding before the incubation at 37 °C (0 min point) to derive the fraction of initial surface cohort remaining. Bars, S.D. of triplicate points. The assay shown is representative of an experiment repeated three times. The initial levels of surface expression of wild type and C-tail:S/T→A mutant receptor were similar in these experiments. In this experiment, these values were 1.00 for cells transfected with WT5, 0.75 for the C-tail:S/T→A mutant, and 0.02 for pBJ1 only (∄ normalized to WT5).

bound to the surface, lysates were prepared from cells washed in PBS lacking EDTA.

A sandwich enzyme-linked immunosorbant assay (18) was used in which anti-FLAG antibody was allowed to bind to immobilized goat anti-mouse antibody and was detected by free goat anti-mouse antibody coupled to HRP. The 96-well plates were coated with 1 μg/well goat anti-mouse antibody (Pierce) in 0.1 ml of PBS overnight at 4 °C. Each well was next incubated with 0.25 ml of 3% BSA in PBS for 3 h to block nonspecific binding. Lysates were applied to the plates, which were then incubated for 2 h at room temperature and then washed thrice in PBS. Each well was then incubated for 1 h with 0.5 μg of HRP-coupled goat anti-mouse antibody in 0.1 ml of PBS containing 3% BSA. After five washes in PBS, 0.2 ml of HRP substrate was added, and HRP activity was determined as above. The amount of antibody typically detected in cell lysates was within the linear range of the assay determined by direct application of anti-FLAG antibody.

Assays of Inositol Phosphate Hydrolysis—Thirty-six h after transfection, Cos7 cells were incubated overnight in DMEM containing 1 mg/ml BSA and 2 μCi/ml tritiated myoinositol (Amersham Corp.), and the hydrolysis of inositol phosphate was measured as described previously (19). The expression of receptor on the surface of cells transfected in parallel was determined as above except that cells were fixed before incubation with anti-FLAG antibody for 1 h at room temperature.

Receptor Phosphorylation—As described previously (14), stably transfected cells were labeled with [32P]orthophosphate, and their receptors were immunoprecipitated using a rabbit polyclonal antibody to an epitope carboxyl-terminal to the thrombin cleavage site. Immunoprecipitates from unlabeled cells were prepared in parallel and subjected to immunoblotting with a monoclonal antibody to the same epitope.

Immunostaining and Microscopy—Stably transfected cells were plated on glass coverslips and cultured at 37 °C for 2 days in DMEM supplemented with 10% calf serum. Subsequent incubations were at room temperature. Cells on the coverslips were fixed in 4% paraformaldehyde for 5 min and then washed thrice in PBS containing 0.1% nonfat dry milk and 150 mM NaOAc, pH 7.0. Cells were then incubated for 15 min in the same buffer (nonpermeabilized cells) or in the same buffer with 0.5% Triton X-100 (permeabilized cells). Next, cells were washed three times in PBS containing 0.1% nonfat dry milk (wash buffer). The cells were then incubated for 1 h in wash buffer containing 10 μg/ml anti-FLAG antibody, washed thrice in wash buffer, and incubated for 1 h in wash buffer containing 5 μg/ml fluorescein isothiocyanate-coupled goat anti-mouse antibody (Life Technologies, Inc.). After four washes in PBS, Slowfade Reagent (Molecular Probes) was applied to each coverslip. Cells were photographed on a Nikon Microphot-FXA fluorescence microscope.

RESULTS AND DISCUSSION

Thrombin receptor internalization was first assayed in transiently transfected Cos7 cells expressing receptors tagged with an amino-terminal FLAG epitope (Fig. 1). Anti-FLAG antibody was bound to cell surface receptors at 4 °C. Cells were then warmed to 37 °C, and the decrease in surface-bound antibody was followed as an index of receptor internalization. Previous studies showed that this epitope-tagged wild type receptor trafficked like untagged wild type receptor (7), and antibody binding was transfection-dependent in this system (see the legends to Figs. 2 and 3). In the absence of agonist, the amount of antibody bound to the surface of cells expressing epitope-tagged wild type thrombin receptor decreased to a new steady state level that was 50% of initial surface binding in 1 h (Figs. 2 and 3). Antibody bound to the surface of cells expressing epitope-tagged wild type thrombin receptor decreased to a new steady state level rather than continuing to decrease with time.
suggest that antibody dissociation from the receptor was not significant in these studies and that even in the absence of agonist the wild type thrombin receptor internalized more rapidly than other membrane proteins. It is unlikely that internalization of the thrombin receptor was caused by binding of the anti-FLAG antibody, given that little internalization of ATE-CD8 was detected despite antibody binding and that agonist-independent internalization of the receptor was nearly eliminated by mutation of its cytoplasmic tail (see below). Addition of the agonist peptide SFLLRN caused the wild type receptor to internalize still more rapidly than it did in the absence of agonist (Figs. 2 and 3).

To examine the role of phosphorylation in receptor internalization, we examined a mutant receptor designated C-tail:S/T→A (14, 19) in which alanines were substituted for all serine and threonine residues in the carboxyl tail of the receptor (Fig. 1). This mutant receptor was not phosphorylated upon activation (14) and showed more robust signaling to thrombin than the wild type receptor (19) (see below). In the absence of agonist, C-tail:S/T→A internalized with kinetics identical to the wild type receptor (Fig. 2). However, in contrast to wild type receptor, addition of agonist did not accelerate the rate of C-tail:S/T→A internalization (Fig. 2). At face value, these data suggest that phosphorylation of the receptor’s carboxyl tail is required for agonist-triggered internalization but not for tonic cycling.

To determine whether sequences important for tonic cycling might also be located in the receptor’s carboxyl tail, we analyzed a series of carboxyl tail truncation mutants (Figs. 1 and 3). Mutants were designated by the amino acid corresponding to the codon that was replaced by a stop (single-letter amino acid code; residue number in the receptor (1); Z for stop). Each mutant was expressed on the surface of transfected Cos7 cells at a level similar to the wild type receptor (Fig. 3 legend). Y397Z, a receptor in which most of the carboxyl tail is missing (Fig. 1), showed the most dramatic phenotype. The decrease in surface-bound antibody over 1 h at 37 °C seen with Y397Z was indistinguishable from that seen with ATE-CD8 (Fig. 3), thus Y397Z was completely defective in agonist-independent internalization (also see Fig. 4). In the presence of agonist, over 80% of wild type receptor internalized compared to less than 40% of Y397Z; thus, Y397Z also displayed a defect in agonist-triggered internalization. The less drastic truncation mutant K402Z showed no defect in agonist-independent internalization and a partial defect in agonist-triggered internalization. K407Z and Y420Z, mutants with still smaller truncations, had no defects in either tonic cycling or agonist-triggered internalization (Fig. 3 and data not shown). Thus, residues between 397 and 407 in the carboxyl tail of the thrombin receptor are required for both agonist-triggered and agonist-independent internalization.

In addition to measuring loss of surface-bound antireceptor antibody, we assayed accumulation of intracellular antireceptor antibody as an index of receptor internalization (Fig. 4). After 1 h, approximately 35% of anti-FLAG antibody initially bound to the surface of cells expressing epitope-tagged wild type thrombin receptor was recovered inside the cells (Fig. 4); as expected, addition of agonist increased the rate at which antibody was internalized, and no internalized receptor was detected in untransfected cells (data not shown). These data strongly suggest that accumulation of intracellular antibody in this assay was the result of thrombin receptor internalization. Consistent with our loss-of-surface bound antibody experiments (Fig. 2), agonist-independent internalization of the C-tail:S/T→A mutant and wild type thrombin receptors was indistinguishable (Fig. 4). In striking contrast, no antibody accumulated intracellularly in cells expressing Y397Z, again consistent with the loss-of-surface bound antibody experiments (Fig. 3) and further demonstrating that Y397Z is completely defective in agonist-independent internalization. These experiments utilized stably transfected Rat1 cells. Similar results

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Fig. 4. Internalization of wild type and mutant thrombin receptors: intracellular accumulation of receptor bound antibody. Stably transfected Rat1 cells expressing WT5 (●), Y397Z (●), or C-tail:S/T→A, S42P (▲) were examined. Because we were unable to obtain a cell line stably expressing C-tail:S/T→A, a receptor with the additional mutation of serine 42 to proline (S42P) was used. This cleavage site mutant cannot be activated by thrombin but is still activated by agonist peptide (1). Cells were incubated with anti-FLAG antibody at 4 °C, washed, and then shifted to 37 °C for various times. Antibody bound to the cell surface was removed by a brief incubation with EPTA (binding of the anti-FLAG antibody requires calcium; Refs. 8 and 15) and the amount of internalized antibody was determined by enzyme-linked immunoabssorbant assay (see “Experimental Procedures”). The level of nonspecific receptor-independent internalization seen in untransfected Rat1 cells, typically less than 5% of that seen in transfected cells, was also determined in each experiment and subtracted to yield a value reflecting receptor-dependent internalization. Lysates prepared from cells not treated with EDTA and not incubated at 37 °C were used to determine the amount of antibody initially bound to the surface of each cell type. The values shown represent the fraction of internalized receptor-bound antibody recovered within the cell after various times at 37 °C. Bars, S.D. of triplicate points. The assay shown is representative of an experiment repeated three times. The initial value for surface expression of wild type and mutant receptors in this experiment was 1.00 for WT5 cells, 0.30 for Y397Z cells, 1.65 for C-tail: S/T→A, S42P cells, and 0.02 for Rat1 cells (A normalized to WT5 cells).

were obtained in transiently transfected Cos7 cells (data not shown); thus, the distinct trafficking patterns of C-tail:S/T→A and Y397Z were not due to idiosyncrasies of individual clonal cell lines.

Like the C-tail:S/T→A mutant (14, 19), Y397Z was not phosphorylated upon activation (Fig. 5) and stimulated more phosphoinositide hydrolysis than did wild type receptor at all agonist concentrations tested (Fig. 6). These results are consistent with the known role of phosphorylation in terminating signaling by the thrombin receptor (14) and other GPCRs (21–23). More importantly, the robust signaling of the Y397Z mutant (Fig. 6) demonstrates that the inability of this mutant to internalize was not due to a global defect in Y397Z receptor function. The observation that Y397Z and C-tail:S/T→A mutant receptors both shared defective phosphorylation and agonist-triggered internalization suggests that receptor phosphorylation may mark activated thrombin receptors for internalization (see below).

To determine the impact of receptor internalization defects on subcellular localization, we examined Rat1 cells stably transfected with wild type, C-tail:S/T→A, and Y397Z receptors by immunofluorescence staining. In cells expressing the wild type receptor, prominent receptor staining was seen on the cell surface (Fig. 7A) and in perinuclear vesicles (Fig. 7B). Plasma membrane staining was much less apparent after permeabilization, perhaps because exposure to detergent removed much of the receptor in this compartment. These results are consistent with previous observations (7, 8). As expected (7, 8), thrombin treatment, which removes the FLAG epitope, eliminated the surface staining of wild type receptor-expressing cells but did not alter staining of the intracellular pool when applied before permeabilization (data not shown). The distribution of the C-tail:S/T→A mutant receptor was indistinguishable from that of the wild type receptor; cells expressing this mutant displayed clear surface (Fig. 7F) and intracellular (Fig. 7G) staining; Y397Z displayed surface staining (Fig. 7C) that was eliminated by thrombin (data not shown). However, in striking contrast to cells expressing the wild type and C-tail:S/T→A receptors, no intracellular staining was detectable in cells expressing Y397Z (Fig. 7D). These data suggest that the Y397Z mutant does not localize to an intracellular pool.
Rat1 cells expressing wild type (A and B), Y397Z (C, D, and E), and C-tail:S/T→A, S42P (F and G) thrombin receptors were examined by immunofluorescence microscopy for the FLAG epitope (see Fig. 1 and “Experimental Procedures”). Nonpermeabilized cells are shown in A, C, and F. Permeabilized cells are shown in B, D, E, and G. All panels show cells photographed with a ×100 objective and under fluorescence illumination except E. E is the identical field of permeabilized Y397Z cells shown in D but photographed under phase-contrast illumination. Exposure times for the immunofluorescence photos were 7, 5, 12, and 12 s (A-D, respectively), 2 s (F), and 5 s (G). Note the striking difference in the relative intensities of surface versus intracellular staining for Y397Z versus the other receptors. The minimal fluorescent staining shown in D was similar to that observed with untransfected Rat1 cells (data not shown). The cells shown are representative of hundreds examined, and similar results were obtained in four separate immunofluorescence studies.

The results presented here demonstrate that the cytoplasmic tail of the thrombin receptor is critical for regulation of internalization. Normal agonist-triggered internalization seems to require phosphorylation of the receptor’s carboxyl tail. This is demonstrated by the Y397Z and C-tail:S/T→A mutants, in which substantial defects in agonist-triggered internalization were produced by the elimination of phosphorylation sites by either a truncation or multiple point mutations (Figs. 2 and 3). Recent reports suggest that phosphorylation promotes the internalization of the β2-adrenergic receptor and the muscarinic acetylcholine receptor (24–26). In each case, mutation of phosphorylation sites impaired internalization and overexpression of G protein-coupled receptor kinase 2 enhanced internalization. Moreover, a dominant negative G protein-coupled receptor kinase 2 mutant prevented internalization of each receptor. Coexpression of β-arrestin, which binds activated and phosphorylated GPCRs, enhanced agonist-triggered internalization of the β2-adrenergic receptor (27). Thus, receptor phosphorylation may mediate not only receptor uncoupling but also the internalization of activated receptors by promoting the binding of β-arrestin, a molecule previously shown to be required for receptor shut-off (21, 22, 28). The activated thrombin receptor is also a substrate for G protein-coupled receptor kinases, and available data suggest that receptor phosphorylation is an important mechanism for terminating signaling by this receptor (14, 19).

Our studies with the Y397Z and C-tail:S/T→A mutants suggest that, like the β2-adrenergic and muscarinic acetylcholine receptors, phosphorylation of the thrombin receptor’s carboxyl tail may mark activated receptors for internalization as well as shut-off. Once internalized, additional properties must specify the distinct trafficking of activated thrombin receptors, which, unlike β2-adrenergic receptors, move preferentially to lysosomes rather than back to the cell surface (see Introduction).

A more novel feature of thrombin receptor trafficking is its apparent ability to tonically cycle between the cell surface and a sizable intracellular pool (7). If newly synthesized receptors were always delivered to the cell surface, the existence of the intracellular pool would be a consequence of agonist-independent receptor internalization, and a mutant receptor defective in agonist-independent internalization would not localize to an intracellular pool. This prediction is born out by the Y397Z mutant. On the other hand, a receptor with an isolated defect in agonist-triggered internalization would still be expected to localize to an intracellular pool as long as it internalized in the absence of agonist, a prediction born out by the C-tail:S/T→A mutant. Thus, the results presented here connect two unusual properties of the thrombin receptor, agonist-independent internalization and localization to an intracellular pool.

The failure of Y397Z but not K407Z to exhibit agonist-independent internalization (Figs. 3 and 4) shows that residues between positions 397 and 406 are important for tonic cycling of the thrombin receptor. Whether this receptor sequence is directly recognized by a protein that mediates agonist-independent internalization or merely contributes to a structure required for this behavior remains to be determined. Similarly, whether the signals for agonist-triggered and agonist-independent internalization are completely distinct is unknown. Our data show that agonist-independent internalization can occur in the setting of defective agonist-triggered internalization, but the converse remains to be established.

How is it that activated thrombin receptors traffic predominantly to lysosomes, whereas naive receptors cycle back to the surface? The observation that the sites necessary for agonist-triggered internalization (putative phosphorylation sites) and agonist-independent internalization are both located in the thrombin receptor’s carboxyl tail prompts speculation regarding the mechanism by which this trafficking decision might be made. Internalization of activated thrombin receptors (destined to go to lysosomes) may be mediated by receptor phosphorylation and the subsequent binding of arrestin or another protein to the receptor’s tail. Internalization of naive receptors (destined to recycle to the surface) may be mediated by the binding of a distinct protein to a nearby or even overlapping site. Receptor phosphorylation and/or binding of arrestin or another mediator of agonist-triggered internalization might mask this latter site, effecting diversion of activated receptors from the recycling pool to lysosomes. This hypothesis will be tested in future studies.

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