Angiotensin II Receptor Endocytosis Involves Two Distinct Regions of the Cytoplasmic Tail

A ROLE FOR RESIDUES ON THE HYDROPHOBIC FACE OF A PUTATIVE AMPHIPATHIC HELIX*

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Following agonist stimulation, many receptors are rapidly internalized from the plasma membrane via a mechanism which presumably involves recognition motifs within the cytoplasmic domains of the receptor. We have previously demonstrated (Thoman, W. G., Thekkumkara, T. J., Motel, T. J., and Baker, K. M. (1995) J. Biol. Chem. 270, 207–213) that truncation of the angiotensin II (AT1A) receptor, to remove 45 amino acids from the cytoplasmic tail, markedly reduced agonist stimulated receptor endocytosis. In the present study, we have stably and transiently expressed wild type and carboxyl terminus mutated AT1A receptors in Chinese hamster ovary cells to identify regions and specific amino acids important for this process. Wild type AT1A receptors rapidly internalized (t1/2 = 2.5 min; Ymax = 76.4%) after AII stimulation. Using AT1A receptor mutants, truncated and deleted at the carboxyl terminus, two distinct regions important for internalization were identified: one membrane proximal site between residues 315–329 and another distal to Lys333, within the terminal 26 amino acids. Point mutations (Y302A, Y312A, L316F, Y319A, and K325A) were performed to identify residues contributing to the membrane proximal site. Mutation of Y302A, Y312A, and K325A had little effect on the rate (t1/2 = 4.3, 2.8, and 2.8 min) and maximal amount (Ymax = 81.7, 67.8, and 73.5%) of AII induced internalization. In contrast, L316F and Y319A mutations displayed an approximately 2.5-fold reduction in rate (t1/2 = 6.1 and 6.2 min) and L316F a decreased maximal level (Ymax = 38.1 and 71.4%, respectively) compared to wild type. Interestingly, Leu316 and Tyr319 are closely aligned within the hydrophobic aspect of a putative amphipathic helix, possibly representing an internalization motif for the AT1A receptor. We conclude that the AT1A receptor does not use the NPXXY (NPLFY320) motif, first described for the β2-adrenergic receptor, to mediate agonist stimulated endocytosis. Rather, two distinct regions of the carboxyl terminus are utilized: one involving hydrophobic and aromatic residues on a putative α-helix and another serine/threonine-rich domain.

Endocytosis of cell surface receptors is a ubiquitous eukaryotic process. It permits the internalization of extracellular nutrients (e.g. cholesterol via low density lipoprotein receptors), serves to dampen cell responses by removing ligand-activated receptors from the cell surface, and mediates cellular resensitization by recycling functional receptors to the cell surface (1–6). Although some endocytosis occurs constitutively, the rate is increased dramatically following binding of many extracellular ligands to their cognate transmembrane receptors. This process is homologous in that usually only receptors for the stimulating ligand are internalized, and of those, only receptors occupied by ligand are targeted. Homologous endocytosis predicts that receptors inherently contain within their structures and sequences determinants for internalization, and that these determinants are concealed or remain latent until agonist binding. Thus, it is generally assumed that within the cytoplasmic domains of receptors are one or more amino acid codes, or motifs, that are recognized by adaptor complexes which mediate the selective recruitment of receptor-ligand complexes into primarily clathrin-coated vesicles (3, 5, 7, 8) and non-coated vesicles and caveolae (discussed in Ref. 9). Commonly, these motifs include aromatic (predominantly tyrosine) and hydrophobic amino acids, and while phosphorylation of these crucial tyrosines does not necessarily drive the internalization process, phosphorylation at other sites may initiate allosteric changes within the receptor responsible for unmasking endocytotic codes (10–12).

Angiotensin II (AII)3 is a peptide hormone with important actions on blood pressure regulation, water and salt balance, neuromodulation, and cellular growth (13). Two major types of AII receptors, termed AT1 and AT2, and two subtypes of the AT1 receptor, termed AT1A and AT1B, have been identified (14). These receptors are all members of the seven transmembrane, guanyl nucleotide-binding protein (G-protein)-coupled receptor (GPCR) superfamily, but the AT1A receptor, which contains 359 amino acids including approximately 54 amino acids as a carboxyl-terminal cytoplasmic tail, is the principal mediator of the biological actions of AII. All receptors present on cells cultured from various tissues (15–19) and cloned AT1A and AT1B (20–24), but not AT2 (25), receptors expressed in cell systems, rapidly internalize upon AII binding. Internalization of AT1A receptor complexes is independent of G-protein coupling (20, 22) and occurs via a clathrin-dependent process (20), which is the primary route for many, but not all, GPCRs. While the exact cellular processes and receptor motifs that control AT1A receptor endocytosis are unknown, the AT1A carboxyl terminus

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1The abbreviations used are: AII, angiotensin II; AT1A, angiotensin II receptor subtypes; G-protein, guanyl nucleotide-binding protein; GPCRs, G-protein coupled receptor(s); CHO-K1, Chinese hamster ovary cells; G418, genetin; MEM, minimal essential medium; PCR, polymerase chain reaction.
contains four candidate tyrosine residues (Tyr302, Tyr312, Tyr319, and Tyr339). Interestingly, Tyr302 in the motif NPXXY is analogous to Tyr326 and shown to be important for p2-adrenergic receptor internalization (25), but the contribution of Tyr302 to AT1A receptor endocytosis has not been reported. The serine/threonine-rich portion of the carboxyl terminus has been implicated in AT1A receptor internalization (23), and we recently demonstrated that truncation of the AT1A receptor to delete the carboxy-terminal 45 amino acids markedly reduced agonist-mediated endocytosis (24). In the present study, we have stably and transiently expressed in CHO-K1 cells AT1A receptors containing truncation, deletion, and point mutations of the cytoplasmic tail and determined AT1A stimulated endocytosis. Our data implicate two separate domains within the carboxy-terminal region of the AT1A receptor in endocytosis and identify key amino acids involved in this process.

EXPERIMENTAL PROCEDURES

Reagents and Cell Culture Materials—
Reagents and cell culture materials are detailed in Table 1. Antibodies were from Calbiochem, Santa Cruz, CA; Santa Cruz, CA; and Molecular Probes, Eugene, OR. 125I-AII (specific activity >2000 Ci/mmol) was purchased from DuPont NEN or Hazeltine Laboratories (Vienna, VA). Chinese hamster ovary cells (CHO-K1) were obtained from the American Type Culture Collection (Rockville, MD). The Escherichia coli strain XLI-Blue and pBluescript II vector was obtained from Stratagene (La Jolla, CA). Sequenase 2.0 DNA sequencing kits were from U. S. Biochemical Corp. and the pRC/CMV vector was from Invitrogen (San Diego, CA). α-MEM, OPTI-MEM, fetal bovine serum, the antibiotic genetin (G418), other cell culture additives and plasmid DNA were obtained from Life Sciences. DNA modifying enzymes were from Promega (Madison, WI). All other chemicals were from Fisher or Sigma.

Receptor Constructs and Mutagenesis—We have previously reported the cloning and isolation of a genomic clone for the rat AT1A receptor (12), subcloning of the full-length receptor (coding for 395 amino acids) into pBlueScript II (pB2AT1A) and the subsequent incorporation of the receptor into the pRC/CMV eukaryotic expression vector (pRC2AAT1A) (26). The cloning of the 45-amino acid carboxyl-terminal region of the AT1A receptor into the pRC/CMV vector was carried out primarily to assess the contribution of the carboxyl-terminal region of the AT1A receptor to AT1A mediated endocytosis (24). In the present study, we have stably and transiently expressed in CHO-K1 cells AT1A receptors containing truncation, deletion, and point mutations of the cytoplasmic tail and determined AT1A stimulated endocytosis. Our data implicate two separate domains within the carboxy-terminal region of the AT1A receptor in endocytosis and identify key amino acids involved in this process.

All mutated PCR fragments were cloned into pBlueScript II and the entire coding region and the relevant mutation confirmed by sequencing. Mutated receptor sequences were restriction digested with HindIII and subcloned into pRC/CMV at the same site to yield the expression plasmids pRC/302A, pRC/312A, pRC/319A, and pRC/ K326A.

Stable and Transient Transfections—CHO-K1 cells were maintained in α-MEM containing fetal bovine serum (10%), penicillin G sodium (100 μg/ml), streptomycin sulfate (100 μg/ml), and amphotericin B (0.25 μg/ml) (complete medium). CHO-K1 cells (50% confluent in 100-mm dishes) were transfected with 20 μg of plasmid DNA for wild type or mutated receptors using the Polybrene method as described previously (24). Transfected cultures were maintained in the presence of 0.5 μg G418/ml of complete medium for 10–14 days to select for plasmid containing cells, and individual clones were picked for propagation. Clonal lines were maintained under a selection pressure of 200 μg G418/ml and tested for the capacity to bind 125I-AII as described (24). Multiple clones expressing wild type or mutated receptor were obtained. Results for individual clones, chosen for full characterization and the internalization experiments described herein, were confirmed in one or two other clones.

The authenticity of the data obtained from the clonal lines was also confirmed using transient transfections. For this, CHO-K1 cells at 80% confluence, in 35-mm dishes, were transfected using the lipofectamine method, as described by the manufacturer (Life Sciences). Cells were washed with each well, and fresh medium was added mixed with 8 μl of the lipofectamine reagent (2 mg/ml) in 200 μl of OPTI-MEM for 20 min. The mixture was diluted to 1 ml with OPTI-MEM and placed on the washed cells, which were returned to the incubator for 5 h. The DNA/lipofectamine solution was aspirated and replaced with 3 ml of complete media. Cells were cultured for 48 h and internalization assays performed. For each transfection, 125I-AII binding assays were performed in parallel on some wells to confirm transfection efficiency and receptor expression. The level of receptor expression, extrapolated by assuming similar dissociation constants established for the clonal cell lines, was approximately 400–600 fmol/mg protein for the wild type receptors and 100–400 fmol/mg protein for the mutants.

All Binding Studies—All binding studies were performed on cultures of transfected CHO-K1 cells in 12-well culture plates as described previously (28). The AII receptor binding buffer contained 50 mM Tris-HCl, pH 7.5, 120 mM NaCl, 4 mM KCl, 5 μM MgCl2, 1 mM CaCl2, 10 μg/ml Bacitracin, and 2 mg/ml α-glucose. Receptor affinity and density were determined by competition binding studies in the presence of 40 pM 125I-AII and increasing concentrations (1 pM to 10 μM) of unlabeled AII.

Determination of Receptor Internalization—Clonal cell lines, stably expressing wild type or mutated receptors, were grown in triplicate to confluence in 12-well culture plates. The rate and degree of agonist-induced endocytosis was determined as follows: cells were washed three times with Hank's buffered salt solution, covered with 100 μl of AII receptor binding buffer (prewarmed to 37°C), and the plates returned to 37°C for 30 min. 125I-AII, in 100 μl of binding buffer, was added to a final concentration of 0.4–0.6 nM, and the incubation continued at 37°C for 2, 5, and 20 min. At each time point, plates were chilled on ice and washed five times with 1.0 ml of ice-cold binding buffer to prevent further internalization and to remove unbound 125I-AII. Bound 125I-AII associated with non-internalized receptors in the plasma membrane was removed by two 40-s washes with 5 mM ice-cold acetic acid in 150 mM NaCl, pH 2.5. These acid washes were retained, and the internalized radioactivity was collected by adding 1.0 ml of 0.2 M NaOH, 0.25% sodium dodecyl sulfate to each well and washing with an additional 0.5 ml of the same solution. The radioactivity in the acid-insensitive (acid-resistant) fractions was measured with a LKB Compugamma counter. After correction for background radioactivity associated with the parent untransfected CHO-K1 cells performed in parallel, an index of receptor internalization was obtained by expressing the acid-insensitive counts as a percentage of the total binding (acid-insensitive plus acid-sensitive) for each well. For transient transfections, the level of internalization was determined as above, but only at the 10-min time point.

Analysis of the Data—Non-linear regression analysis of the competition binding data was achieved using the computer software GraphPad Prism (GraphPad Software Inc.); dissociation constants (Kd) and receptor density (Bmax) were estimated as described (29). For endocytosis assays, the percentage of internalized receptors was plotted against time, and the curves were analyzed as one phase exponential associations using GraphPad Prism. The half-time (t1/2, in min) to reach a...
Determinants for \( {\text{AT}}_{1\alpha} \) Receptor Internalization

\[ \text{Table I} \]

| Receptor construct | \( K_d \) (nM) | \( B_{\text{max}} \) (fmol/mg protein) |
|---------------------|----------------|-------------------------------------|
| Wild type (T24)     | 0.82a          | 667                                 |
| TL314               | 1.08a          | 560                                 |
| TK33                | 1.34           | 840                                 |
| Del315-329          | 1.16           | 145                                 |
| Y302A               | 0.79           | 209                                 |
| Y312A               | 0.54           | 193                                 |
| Y319A               | 1.39           | 219                                 |
| L316F               | 1.12           | 246                                 |
| K325A               | 0.79           | 527                                 |

*a Determined previously (24).

**RESULTS**

cDNAs encoding wild type and mutated rat \( {\text{AT}}_{1\alpha} \) receptors were subcloned into the pRcCMV mammalian expression vector and transfected into CHO-K1 cells. The various constructs are depicted in Fig. 1. Colonies resistant to neomycin (G418) were selected and propagated, and individual clonal lines expressing functional receptors were selected by the ability to bind \( 125^\text{I}-\text{AII} \). To determine if the expressed mutated receptors were selected and propagated, and individual clonal lines expressing functional receptors were selected by the ability to bind \( 125^\text{I}-\text{AII} \). To determine if the expressed mutated receptors displayed affinities comparable to the wild type receptor, competition binding studies were performed. As summarized in Table 1, the wild type and assorted truncated, deletion, and point substitution mutants all bound \( 125^\text{I}-\text{AII} \) with high affinity (\( K_d \) approximating 1 nM) indicating that these receptors attain a conformation necessary for high affinity recognition of AII. Dissociation constant values in the nanomolar range compare well with those previously reported for \( {\text{AT}}_1 \) receptors expressed in cells and tissues (33). The level of receptor expression for the mutated receptors ranged between 145–840 fmol/mg protein (Table 1).

Using the appearance of acid-resistant radioactivity, after addition of \( 125^\text{I}-\text{AII} \) at 37°C as an index of receptor internalization, we observed that wild type \( {\text{AT}}_{1\alpha} \) receptors expressed in CHO-K1 cells underwent rapid endocytosis from the plasma membrane (Fig. 2). Shown in Fig. 2 is the internalization profile for four separate clonal lines (T3, T5, T11, and T24) expressing the wild type receptor. Although the level of expression in these clones varies over a 10-fold range (T3, 3400 fmol/mg protein; T24, 667 fmol/mg protein; T11, 330 fmol/mg protein; T5, 310 fmol/mg protein), the kinetics of \( 125^\text{I}-\text{AII} \) internalization, fitted as one phase exponential association curve.

The top left-hand region of the AT1A receptor was obtained via the PHDsec computer modeling–secondary structure predictions for the carboxyl-terminal region of the AT1A receptor (Table II) on the wild type sequence. For the wild type receptor, the specific residue changed is underlined.

**Fig. 2.** Endocytosis of the \( {\text{AT}}_{1\alpha} \) receptor is independent of receptor density but dependent upon an intact carboxyl terminus. CHO-K1 cells stably expressing varying densities of wild type \( {\text{AT}}_{1\alpha} \) receptor (T3, 3400 fmol/mg protein, filled circles; T5, 310 fmol/mg protein, open circles; T11, 330 fmol/mg protein, filled squares; T24, 667 fmol/mg protein, open squares) or a carboxyl-truncated AT1a receptor (TL314, crosses) were incubated with \( 125^\text{I}-\text{AII} \) for 2–20 min at 37°C. At the indicated times, surface-bound and internalized \( 125^\text{I}-\text{AII} \) were determined by acid washing as described under "Experimental Procedures." An index of internalization was calculated by expressing the internalized radioactivity (acid-resistant) as a percentage of the total binding (acid-resistant plus acid-susceptible). Data are means ± S.D. for three (TL314), six (T3, T5, and T11) or nine (T24) determinations.

maximal level of internalization (\( Y_{\text{max}} \) in %) was determined for each association curve.

Computer Modeling—Secondary structure predictions for the carboxy-terminal region of the AT1A receptor were obtained via the PHDsec server (E-Mail: Predictprotein@EMBL-Heidelberg.DE). Full description of the server and the prediction algorithm have been published (30–32).
and appropriate activation of conventional signaling pathways (24).

To further define sites important for internalization (within this carboxyl-terminal region), we evaluated the capacity of another truncated receptor, TK 333, to undergo AII-mediated endocytosis (Fig. 3). This mutant displayed a slower ($t_{1/2}$ 6.1 min) and reduced amount ($Y_{\text{max}}$ 46.3%) of internalization compared to the wild type receptor ($t_{1/2}$ 2.5 min, $Y_{\text{max}}$ 76.4%), implicating the terminal 26 amino acids in the internalization process. However, the level of internalization observed for TK 333 was consistently greater than that observed for TL314 ($Y_{\text{max}}$ 46.3 versus 16.2% (see Fig. 2)), which suggested that the region between Lys333 and Tyr312 also contained an endocytotic determinant. To confirm this, a deletion mutant was constructed in which the terminal 30 amino acids (330–359) were fused to the first 314 residues, deleting amino acids 315–329. As shown in Fig. 3, 125I-AII was slowly ($t_{1/2}$ = 8.6 min) internalized by this mutant, to a degree (90.9%) slightly less than that displayed by TK 333. These observations suggest that there are two sites important for endocytosis within the carboxyl terminus of the AT1A receptor; one distal to Lys333 and another in the region 315–329.

While these observations were being made, Hunyady et al. (23) reported the inhibition of AT1A receptor internalization by mutation of amino acids within a carboxyl-terminal serine/threonine-rich region, in particular, residues Ser335, Thr336, and Leu337. The location of these residues distal to our truncation of the receptor at Lys393 explained the diminished internalization observed for TK 333. We reasoned, however, that this “STL motif” alone was not sufficient to direct internalization because our deletion mutant (Del315–329) displayed markedly reduced endocytosis ($t_{1/2}$ = 8.6 min, $Y_{\text{max}}$ = 40.9%), despite the presence of this tripeptide sequence as well as other contributing residues, Thr332 and Ser331 (23). Thus, we focused on identifying specific residues in the more proximal regions of the carboxyl-tail. The first point mutants constructed were Tyr302 to alanine (Y302A) and Lys325 to alanine (K325A). The rationale for altering these specific residues was that Tyr302 is a highly conserved residue in many G-protein-coupled receptors and because the analogous Tyr326 in the $\beta_2$-adrenergic receptor has been identified as a key residue in the internalization process (25). The K325A mutant was engineered because: 1) this amino acid is present in the configuration AKS which is similar to the internalization motif DAKSS (34), with its crucial central lysine, found in the yeast $\alpha$-factor receptor, also a seven transmembrane spanning protein like AT1A; and 2) Lys325 is located within the region (315–329) identified as important for AT1A receptor endocytosis. Shown in Fig. 4 are the internalization profiles for Y302A and K325A compared to the wild type receptor. The internalization kinetics for the K325A mutant were equivalent ($t_{1/2}$ 2.8 min, $Y_{\text{max}}$ 73.5%) to the wild type receptor, suggesting that this residue is not critical for internalization. The Y302A mutation had little effect on AT1A internalization; the rate was slightly reduced ($t_{1/2}$ 4.2 min), but the maximal level of receptor endocytosis was comparable ($Y_{\text{max}}$ 81.7%) to wild type.

Given the prevalence of tyrosine residues in the endocytotic motifs for many receptors (3, 5), we next mutated Tyr312 and Tyr319 separately to alanines. Also, for some receptors (e.g. insulin (35)), dileucine residues within the cytoplasmic regions are important for efficient receptor internalization. Since the AT1A receptor carboxyl terminus includes a dileucine pair (Leu316Leu317) and because these leucines are located between residues 315–329, we decided to test the involvement of this putative motif by mutating Leu316 to another hydrophobic residue, phenylalanine (L316F). Shown in Fig. 5 are the internalization profiles for Y312A, L316F, and Y319A. Mutation Y312A internalized at a rate ($t_{1/2}$ 2.8 min) and to a degree ($Y_{\text{max}}$ 67.8%) comparable with the wild type receptor. Mutation L316F displayed a significantly reduced capacity for endocytosis with a 2.5-fold reduction in rate ($t_{1/2}$ 6.1 min) and a diminished maximal level ($Y_{\text{max}}$ 38.1%). The internalization rate ($t_{1/2}$ 6.2 min) for Y319A was also reduced, but the $Y_{\text{max}}$ at 71.4% was similar to that of the wild type receptor.

The results presented above implicate L316F and Tyr319 in the control of AT1A receptor endocytosis. These observations were confirmed in other clonal lines expressing these two mutations, but as further confirmation that our observations are not the result of aberrations arising from clonal selection, we performed transient transfections for selected constructs. CHO-K1 cells were transfected using lipofectamine reagent with pRcAT1A (wild type) or DNA for mutated receptors (pRc/TL314, pRc/TK333, pRc/L316F, and pRc/Y319A), and 48 h after transfection internalization assays were performed. The amount of internalization was determined at 10 min, the time of maximal endocytosis for the wild type receptor. As shown in Fig. 6, the results of transient assays closely mirrored the data obtained with the clonal cell lines. Thus, compared to wild type (66.8%), TK333 (28.1%) exhibited reduced endocytosis, but not to the level displayed by TL314.
Determinants for AT₁A Receptor Internalization

The rapid (t₀ ~ 2.5 min) internalization of all AT₁A receptor complexes observed in this study reflects the dramatic agonist-stimulated endocytosis observed with many GPCRs, including endogenous and cloned AII receptors (15–24). It is generally accepted that internalizing receptors contain motifs or “codes” (3,5) that are recognized by adaptor proteins which mediate the selective aggregation and association of ligand-bound receptors into clathrin-coated pits. These pits then invaginate to form vesicles which fuse with endosomes/lysosomes where low pH dissociates ligand from receptor. The receptor and ligand are then either degraded, or the receptor is recycled back to the cell membrane and the ligand, in the case of AII, may serve to activate putative cytoplasmic (36) or nuclear (37) receptors. While alanine scanning has identified some residues that are required for efficient internalization of some GPCRs, no consensus motif has emerged. In this study, we have identified two disparate regions of the AT₁A receptor involved in AII-stimulated endocytosis. One region is approximately 10 amino acids from the cytoplasmic face of the membrane and involves the hydrophobic residue Leu316 and the aromatic Tyr319, while the other site is more distally located within the serine/threonine-rich region of the receptor tail.

Our observation that two distinct regions of the cytoplasmic tail are important for internalization corroborates accumulating evidence for multiple endocytotic codes in other receptors. For example, the cytoplasmic domain of the insulin receptor contains two tyrosine-based internalization motifs (GPLY and NPEY) and a juxtamembrane dileucine motif (EKITTLL) (35). Similarly, the carboxyl-terminal region of the epidermal growth factor receptor contains at least three endocytotic codes as well as distinct regions for lysosomal targeting (11). Endocytosis of the mannos-6-phosphate receptor requires two separate regions containing aromatic residues (38). In addition to receptors with two or more positive internalization codes, some receptors also display both positive and negative regulatory motifs (39–41). Our observation of two positive internalization “motifs” for the AT₁A receptor most readily compares to another GPCR, the thyrotrophin-releasing hormone receptor (42). In this receptor, two dissimilar positive sites are also important for endocytosis: one membrane proximal and another more distal site involving serine and threonine residues. The similarity of AT₁A and thyrotrophin-releasing hormone receptors also includes the spacing from the membrane of the first site (~10 amino acids) and distance of the two sites from each other in the cytoplasmic tail (~20 amino acids). Whether this arrangement is exploited by other GPCRs and the possible interaction between these two sites and the endocytic machinery remains to be established.

In the pursuit of a universal GPCR internalization motif, Barak et al. (25) recently reported that Tyr326, located near the junction of the seventh transmembrane segment and the cytoplasmic tail, is a key residue for the rapid internalization of β₂-adrenergic receptors. This observation has particular relevance because many GPCRs possess an analogous tyrosine residue within a highly conserved NPₓₓY motif (where X is any amino acid). This motif is similar to the NPXY internalization motif, first described for the low density lipoprotein receptor (43), which is present within the cytoplasmic domains of many receptors. However, we observed in this study that mutation of the corresponding residue (Tyr302) in the AT₁A receptor had little effect on the kinetics of AII-stimulated internalization. This observation suggests that Tyr302 (NPLFY302) does not input significantly into AII-stimulated AT₁A receptor endocytosis, and given that a similar motif (NPFLY318) is maintained in the AT₂ receptor, which does not internalize (23), provides further evidence that other sites are responsible for internalization of AT₁A receptors. Indeed, the NPₓₓY motif may only function for a subset of GPCRs because the comparable tyrosine mutation in the gastrin releasing peptide receptor was also ineffective in modulating endocytosis (44), and because a subgroup of GPCRs (e.g. receptors for secretin, glucagon, parathyroid hormone, calcitonin, etc.) which lack the NPₓₓY motif display rapid internalization in response to agonist.

Our observation that one of the two sites important for internalization is located distal to Lys333 within the terminal 26 amino acids complements the recent findings of Hunyady et al. (23). Using serial truncations and point mutations of the AT₁A receptor, they identified an important contribution of residues in the region Thr333-Ser336 between, with a major involvement of the tripeptide sequence Ser335-Thr336-Leu337. However, our observation that TK333 (removal of 26 amino acids) still

Fig. 5. Comparison of endocytosis for wild type AT₁A receptors and Y312A, L316F and Y319A mutants. Internalization kinetics for CHO-K1 cells stably expressing the wild type (filled circles), Y312A (open circles), L316F (open squares) and Y319A (filled squares) AT₁A receptors were determined as described in the legend to Fig. 1. Data are the means ± S.D. from three separate experiments performed in duplicate.

Fig. 6. Endocytosis of the wild type AT₁A receptor and various truncated, deleted, and point mutants following transient transfection in CHO-K1 cells. CHO-K1 cells were transfected with plasmid DNA encoding wild type (pRc2A/AT₁A), truncated (pRc/TL314 and pRc/TK333), deleted (pRc/Del315–329), and point mutated (pRc/L316F and pRc/Y319A) AT₁A receptors, and 48 h later internalization assays were performed as described under "Experimental Procedures." The percentage internalization for mutant receptors was determined 10 min after addition of 125I-AII at 37°C, a time where internalization of the wild type was maximal. Data are the means ± S.D. from three separate experiments performed in triplicate.

(9.6%) and Del315–329 (6.8%). Moreover, the single point mutations L316F (26.8%) and Y319A (46.1%) showed diminished internalization which was comparable to that observed in clonal counterparts at 10 min.

**DISCUSSION**

The rapid (t₀ ~ 2.5 min) internalization of all AT₁A receptor complexes observed in this study reflects the dramatic agonist-stimulated endocytosis observed with many GPCRs, including endogenous and cloned AII receptors (15–24). It is generally accepted that internalizing receptors contain motifs or “codes” (3,5) that are recognized by adaptor proteins which mediate the selective aggregation and association of ligand-bound receptors into clathrin-coated pits. These pits then invaginate to form vesicles which fuse with endosomes/lysosomes where low pH dissociates ligand from receptor. The receptor and ligand are then either degraded, or the receptor is recycled back to the cell membrane and the ligand, in the case of AII, may serve to activate putative cytoplasmic (36) or nuclear (37) receptors. While alanine scanning has identified some residues that are required for efficient internalization of some GPCRs, no consensus motif has emerged. In this study, we have identified two disparate regions of the AT₁A receptor involved in AII-stimulated endocytosis. One region is approximately 10 amino acids from the cytoplasmic face of the membrane and involves the hydrophobic residue Leu316 and the aromatic Tyr319, while the other site is more distally located within the serine/threonine-rich region of the receptor tail.

Our observation that two distinct regions of the cytoplasmic tail are important for internalization corroborates accumulating evidence for multiple endocytotic codes in other receptors. For example, the cytoplasmic domain of the insulin receptor contains two tyrosine-based internalization motifs (GPLY and NPEY) and a juxtamembrane dileucine motif (EKITTLL) (35). Similarly, the carboxyl-terminal region of the epidermal growth factor receptor contains at least three endocytotic codes as well as distinct regions for lysosomal targeting (11). Endocytosis of the mannos-6-phosphate receptor requires two separate regions containing aromatic residues (38). In addition to receptors with two or more positive internalization codes, some receptors also display both positive and negative regulatory motifs (39–41). Our observation of two positive internalization "motifs" for the AT₁A receptor most readily compares to another GPCR, the thyrotrophin-releasing hormone receptor (42). In this receptor, two dissimilar positive sites are also important for endocytosis: one membrane proximal and another more distal site involving serine and threonine residues. The similarity of AT₁A and thyrotrophin-releasing hormone receptors also includes the spacing from the membrane of the first site (~10 amino acids) and distance of the two sites from each other in the cytoplasmic tail (~20 amino acids). Whether this arrangement is exploited by other GPCRs and the possible interaction between these two sites and the endocytic machinery remains to be established.

In the pursuit of a universal GPCR internalization motif, Barak et al. (25) recently reported that Tyr326, located near the junction of the seventh transmembrane segment and the cytoplasmic tail, is a key residue for the rapid internalization of β₂-adrenergic receptors. This observation has particular relevance because many GPCRs possess an analogous tyrosine residue within a highly conserved NPₓₓY motif (where X is any amino acid). This motif is similar to the NPXY internalization motif, first described for the low density lipoprotein receptor (43), which is present within the cytoplasmic domains of many receptors. However, we observed in this study that mutation of the corresponding residue (Tyr302) in the AT₁A receptor had little effect on the kinetics of AII-stimulated internalization. This observation suggests that Tyr302 (NPLFY302) does not input significantly into AII-stimulated AT₁A receptor endocytosis, and given that a similar motif (NPFLY318) is maintained in the AT₂ receptor, which does not internalize (23), provides further evidence that other sites are responsible for internalization of AT₁A receptors. Indeed, the NPₓₓY motif may only function for a subset of GPCRs because the comparable tyrosine mutation in the gastrin releasing peptide receptor was also ineffective in modulating endocytosis (44), and because a subgroup of GPCRs (e.g. receptors for secretin, glucagon, parathyroid hormone, calcitonin, etc.) which lack the NPₓₓY motif display rapid internalization in response to agonist.

Our observation that one of the two sites important for internalization is located distal to Lys333 within the terminal 26 amino acids complements the recent findings of Hunyady et al. (23). Using serial truncations and point mutations of the AT₁A receptor, they identified an important contribution of residues in the region Thr333-Ser336 between, with a major involvement of the tripeptide sequence Ser335-Thr336-Leu337. However, our observation that TK333 (removal of 26 amino acids) still dis-
played some internalization, at a level greater than observed for TL314 (deletion of 45 amino acids), suggested that more proximal sites in the carboxyl terminus were also involved. We therefore engineered a deletion mutant in which the terminal 30 amino acids of the AT1A receptor, including all residues (Thr322-Ser338) identified as important by Hunyady et al. (23), were joined to the receptor truncated after Leu314. We hypothesized that if the Thr322-Ser338 region was sufficient for endocytosis, then attachment of these sequences to the noninternalizing truncated receptor would rescue the internalization response. Such transplantation of putative internalization motifs onto internalization incompetent receptors has been previously used to positively identify such sites (45–47). In contrast, we noted that the endocytosis of this deletion mutant, while apparent, was markedly reduced in comparison to wild type and was slightly less than TK333. These results suggest that the region between Gln315 and Ser329 is as important as the more distal STL motif and also raise the possibility that the two regions may be cooperative for endocytosis.

Our observation of a second site within the AT1A receptor tail is novel. Moreover, we have identified a key role for residues Leu316 and Tyr319 which is consistent with the use of aromatic and hydrophobic residues for internalization motifs (3, 5). Single point mutation of Leu316 potently inhibited AII-stimulated endocytosis with equivalent efficacy to the large deletion (Del315–329) and truncation (TK333) mutants. Thus, this and surrounding residues represent attractive candidates for the AT1A internalization motif. What is particularly interesting is that Leu316 is part of a dileucine pair (Leu316-Leu317), which is a motif utilized by insulin receptors (35) and the IgG receptor FcγRI (48) for endocytosis. A role of Leu316-Leu317 is supported by the observation that this dileucine pair is not conserved in the noninternalizing rat AT2 receptor (23). Further studies will be required to determine whether this region is the actual motif engaged by the endocytic machinery or whether the deleterious effect of this mutation is secondary to conformational changes which prevent interaction with other sites.

Based on computer modeling and NMR analysis of peptide sequences, internalization motifs are proposed to involve a type I β-turn with an exposed aromatic (tyrosine) residue (5). We have used the computer modeling method of Rost and Sander (30–32) to predict the secondary structure of the cytoplasmic tail of the AT1A receptor. The seventh transmembrane segment of the 359 amino acid AT1A receptor exits the cell membrane around Leu305 leaving a cytoplasmic carboxyl terminus of 54 amino acids. The region immediately adjacent to the membrane, between Lys307 and Ile320, displayed a high probability of forming an α-helix which was terminated by consecutive proline residues (Pro329-Pro332), while the remainder of the cytoplasmic tail was predicted to form an extended loop structure. As shown in Fig. 7, plotting residues Lys307 through Ile320 as a helical wheel reveals an amphipathic nature to this putative helix. The helix divides symmetrically into one side containing exclusively hydrophobic and aromatic side chains (Leu, Ile, Phe, and Tyr) with the other half containing mostly basic (Lys) amino acids. It is interesting that residues Leu316 and Tyr319, which we have shown are important for AT1A receptor endocytosis, are closely positioned (within 60°) toward the end of this helix. The hydrophobic residues on this aspect would be expected to be protected from the aqueous environment by burying them away from the surface. Alternatively, these hydrophobic residues may be concealed through interaction with other proteins, since amphipathic helices are often involved in protein-protein interactions. Presumably, ligand binding leads to allosteric changes which expose these sites to the endocytic machinery.

![Image](https://example.com/image.png)

**Fig. 7.** Helical wheel representation of the region Lys307 through Ile320 in the carboxyl terminus of the AT1A receptor. The wheel was constructed assuming a 100° turn residue, based on the prediction that this region is α-helical. Note that the helix can be divided into predominately basic and hydrophobic aspects, and the close proximity of Leu316 and Tyr319 (boxed) on the hydrophobic side. Amino acids are represented by the single-letter code (K = Lys, Q = Gln, L = Leu, Y = Tyr, F = Phe, and I = Ile).

While a helical prediction for this region of the AT1A receptor contradicts the current dogma of a tyrosine-based β-turn as the internalization motif, such a possibility is supported by the recent NMR data of Wilde et al. (49). These authors synthesized a 21-amino-acid peptide corresponding to the carboxyl terminus of TGN38, a type I integral membrane protein that recycles between the plasma membrane and the trans-Golgi network. This peptide which contained the sequence YQRL analogous to the common internalization motif YXXΦ (Φ = hydrophobic amino acid) displayed a NMR spectra consistent with a helical, not β-turn, conformation. Regardless of secondary structure, the region corresponding to the terminal end (Pheε-Pro225) of this putative AT1A receptor helix, as noted by Hjorth et al. (Fig. 3 of 50), is preserved across species and among subtypes of AT1 receptors, which is indicative of conserved function. Based on our mutational data and given that this region is not conserved in the noninternalizing AT2 receptor (23), the function of this region may be internalization.

Recent studies demonstrating the association of epidermal growth factor receptors with the adaptor complex AP2 of the endocytic machinery (10,12), and another using kinase-defi
cient epidermal growth factor receptors (11), have provided evidence that receptor endocytic motifs are masked in the absence of ligand binding. Occupancy of receptors by ligand promotes an allosteric change which favors exposure of internalization motifs to the endocytic apparatus and also causes autophosphorylation which amplifies the conformational changes and internalization. Although GPCRs lack intrinsic kinase activity, many are rapidly phosphorylated by cellular kinases on serine, threonine, and tyrosine residues in response to agonist (51), and there is evidence suggesting a role for phosphorylation in endocytosis. For example, mutation of serine/threonine residues in the β2-adrenergic receptor inhibits internalization (52), the serine/threonine region of the gastrin releasing peptide receptor is required for endocytosis (53), and phosphorylation of M2 receptors by the specific receptor kinase GRK2 coincides with increased rates of internalization (54). Thus, in the general context of an internalization mechanism, phosphorylation of GPCRs appears to augment endocytosis. One interpretation of our data, which identified two carboxyl-terminal regions (one hydrophobic and one serine/threonine-rich) important for AT1A receptor endocytosis, could be that upon AII binding a conformational change occurs which leads to exposure of the hydrophobic residues and initiates internalization. In addition, this conformational change presumably promotes phosphorylation of the distal phosphate acceptor residues which may maintain this exposed state. Conversely, an allosteric change in this putative helical region may be required for phosphorylation of distant sites, permitting the
phosphorylated residues to interact directly with the endocytic machinery. However, the propensity of hydrophobic and tyrosine residues in the internalization motifs of many receptors predicts that the former possibility is more likely.

In summary, we have identified two distinct regions of the AT1A receptor necessary for the efficient endocytosis of AT1A receptor complexes and demonstrated a central role for Leu\textsuperscript{316} and Tyr\textsuperscript{319} in this process. These residues are densely aligned on the hydrophobic face of a putative \(\alpha\)-helix. Future studies will focus on identifying a role for AT1A receptor phosphorylation in internalization and the possible interaction of the AT1A receptor with the adaptor complexes that initiate the clustering and endocytosis process. The truncated, deleted, and point-mutated AT1A receptors described in this study should prove useful in delineating these processes for the AT1A receptor and may provide a more general understanding of the process of endocytosis for other GPCRs.

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