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The Type 1 Angiotensin II Receptor Tail Affects Receptor Targeting, Internalization, and Membrane Fusion Properties

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ABSTRACT

Endocytosis modulates cell responses by removing and recycling receptors from the cell surface. Type I angiotensin II receptors (AT_1R) are somewhat unique in that they are expressed at apical (AP) and basolateral (BL) membranes in proximal tubule cells and both receptor sites undergo endocytosis. We analyzed AT_1R cytoplasmic (–COOH) tail deletion mutants to determine whether classic AT_1R endocytosis motifs functioned similarly in polarized cells and simultaneously altered receptor properties. Serially truncating the AT_1R tail had little effect on AP/BL AT_1R distribution as determined by ^{125}I-angiotensin II binding in $LLCPK_{Cl4}$ cells transfected with an AT_1R transcript. AP AT_1R expression required the proximal 12 amino acids in the AT_1R-COOH tail. Deleting all but the proximal 12 aa of the AT_1R-COOH tail (T316L mutant) decreased AP AT_1R internalization at 20 min ($17 \pm 6\%$; $p < 0.05$ versus full-length; $n = 5$) and inhibited AP AT_1R-stimulated arachidonic acid release (counts released per milligram of protein at 20 min: full-length, $18{,}762 \pm 4018$; T316L, 2430 ± 1711; $n = 4$; $p < 0.02$). Endosomal fusion assays were performed using peptide sequences of regions in the AT_1R tail involved in endocytosis (YFLQLLKYIPP [LL] and LSTKMSTLSY [STL]). Peptide STL significantly inhibited endosomal fusion ($22 \pm 10\%$ of control; $n = 5$; $p < 0.05$ versus positive control). Peptide LL had no significant inhibitory effect. AT_1R in polarized cells contain dominant endocytosis signals but these motifs do not correlate with AP or BL AT_1R expression. Moreover, peptide sequences within the AT_1R –COOH tail necessary for endocytosis also modulate endosomal fusion properties.

Proximal tubule epithelial cells are endowed with a significant capacity for endocytosis, scavenging filtered proteins from the tubular lumen before they are lost in the urine. Proteins, lipids, drugs, and other pharmaceuticals are internalized from the apical surface, situated in the proximal tubule lumen, via endocytic uptake for delivery to subcellular compartments. Membrane recycling back to the cell surface must occur to balance membrane loss from internalization. This pattern of endocytosis and recycling is used by many receptor types, including angiotensin II (Ang II) receptors (Hunyady et al., 2000).

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Type 1 Ang II receptors (AT_1R), members of the superfamily of G-protein-coupled receptors, initiate numerous signaling pathways in proximal tubule cells to regulate salt and water reabsorption (Harris et al., 1997). Unlike many other G-protein-coupled receptors, AT_1R are expressed at AP and basolateral (BL) membranes of proximal tubules, and apical AT activation has been shown to modulate proximal tubule function (Brown and Douglas, 1982; Brown and Douglas, 1983). We have previously shown in a model of proximal tubule epithelia that AP AT_1R and BL AT_1R displayed differential rates of endocytosis and recycling (Becker et al., 1995). Moreover, AP Ang II binding and internalization were associated with PLA_2 activation and liberation of arachidonic acid (Becker and Harris, 1996).

Several lines of evidence link the trafficking of organelles containing endocytosed receptors with membrane fusion. Membrane fusion is integral to endocytic-related processes. The molecular machinery involved in the final steps of membrane fusion has been identified, cloned, and reconstituted in

ABBREVIATIONS: Ang II, angiotensin II; AT_1R, type 1 angiotensin II receptor; AT_2R, type 2 angiotensin II receptor; AP, apical; BL, basolateral; DuP753, losartan; CGP 42112A, *N*-nicotinoyl-*N*-(*N*-benzyloxycarbonyl-Arg)Lys-His-Pro-Ile; $AT_{1a}R$, type 1a angiotensin II receptor; aa, amino acid(s); CMV, cytomegalovirus; PBS-A, phosphate-buffered saline with 0.1% albumin; IP, immunoprecipitation; AA, arachidonic acid; RT-PCR, reverse transcription-polymerase chain reaction; N.S., not significant; bp, base pair(s).

vitro (Rothman and Warren, 1994; Witze and Rothman, 2002). However, the mechanisms involved in initiating events regulating the fusion are still incompletely understood for many classes of receptors. The recent demonstration that the single transmembrane domain receptor, megalin (Saito et al., 1994), can modulate the fusion properties of membranes in which it resides suggests that receptor-mediated effects on membrane fusion may be important for receptor trafficking and targeting.

Mutational studies have identified structural motifs in the AT_1R cytoplasmic tail that are critical for Ang II receptor membrane targeting, internalization, and initiation of signaling in nonpolarized cells (Hunyady et al., 1994a,b; Thekkumkara et al., 1995, 1998; Thomas et al., 1995a,b; Thekkumkara and Linas, 2002). However, there are few studies investigating motifs that may mediate differential targeting and/or endocytosis of receptors at AP and BL membranes in polarized epithelia. Furthermore, no previous studies have investigated the potential role that AT_1R may play in regulating membrane fusion. The current study attempts to define this more clearly by examining the effects of peptide motifs in the AT_1R cytosolic tail on receptor endocytosis and membrane fusion reconstituted in vitro.

Materials and Methods

Materials. [^{3}H]Arachidonic acid (100 Ci/mmol), [^{3}H]inulin (500 mCi/g), and ^{125}I-labeled angiotensin II (2200 Ci/mmol) were obtained from PerkinElmer Life and Analytical Sciences (Boston, MA). Angiotensin II, penicillin (10,000 units)-streptomycin (10 mg/ml) solution, trypsin-EDTA, Dulbecco's modified Eagle's medium-Ham's F-12 medium mixture, and bovine serum albumin were obtained from Sigma (St Louis, MO). Geneticin (G-418) was obtained from Invitrogen (Carlsbad, CA). DuP753 (losartan) was provided by DuPont Merck (Wilmington, DE) and CGP 42112A was obtained from Ciba-Geigy (Basel, Switzerland). Fetal calf serum was obtained from Hyclone (Logan, UT). Antisera to the $AT_{1a}R$ third intracellular domain were kindly provided by Ian Phillips (University of Florida, Gainesville, FL).

Cell Culture. LLC-PK_{Cl4} cells, an LLC-PK_1 clone, were cultured as described previously (Becker et al., 1995). Cells were seeded at a density of 0.5×10^6 cells/12-mm polycarbonate filter membrane filter (Transwell chambers, 0.4-μm pore size; Corning Costar, Cambridge, MA) and cultured for 3 to 5 days. Before assays were performed, AP surfaces in each well were incubated with [^{3}H]inulin for 1 h at 37°C. Aliquots were removed from BL wells and radioactive leak was measured by scintillation spectrometry. Any well with >3% leak (AP→BL) was discarded from further analysis (Becker et al., 1995).

Site-Directed Mutagenesis. Clone 3, containing the entire open reading frame of the rabbit AT_1R (Burns et al., 1993) was placed into pBluescript SK(−). Site-directed mutagenesis (Altered Sites in vitro mutagenesis system; Promega, Madison, WI) was employed to mutate aa 301 to a stop codon (tat to taa) (T301F). Mutants T316L and T330L were designed by adding a stop codon after the appropriate Leu residue. These sequences were then subcloned into the vector PCRII using the TA cloning kit (Invitrogen). Selected clones were sequenced to confirm the mutation then subcloned into the vector pRC/CMV (Invitrogen) at HindIII and XbaI sites. T345S mutants were self-ligated into a plasmid after exposure to SacI. A stop codon and a NotI site (GCGGCCGCA) were attached to the end of this plasmid template. After confirming the T345S sequence, the insert was subcloned into pRC/CMV at HindIII and Not1 sites.

Primers employed for each mutant were: for T316L: upstream, 5′-ATATCCATCACACTGGCGGC-3′; downstream, 5′-GGCGGCCTAAAGCTGGAGAAAATATTTC-3′; for T330L: upstream, 5′-ATATCCATCACACTGGCGGC-3′; downstream, 5′-CGCCTATAGATTTGAATGGGATTTGGC-3′; for T345S: upstream, 5′-TCGATAAGCTTGATATCG-3′; downstream, 5′-TAATGCGGCCGCGCTCACGTTATCTGAGGG-3′.

Transfection of $LLCPK_{Cl4}$ Cells with Wild-Type or Mutant pRC-CMV-AT_1R Vector Constructs. $LLCPK_{Cl4}$ cells were transfected using modified Ca-PO_4 mediated DNA transfection. Cells at 40 to 50% confluence were exposed to 1 ml of 2× HEPES-buffered saline (42 mM HEPES, 276 mM NaCl, 10 mM KCL, 3 mM Na_2HPO_4, and 11 mM dextrose, pH 7.1 ± 0.05) + 20 μg/ml wild-type or mutant AT_1R pRC-CMV vector + 1 ml 0.25 M $CaCl_2$ and 40 mM chloroquine. Cells were incubated at 23°C for 30 min then 8 ml of growth medium containing 0.005 volumes of 0.25 M $CaCl_2$ and 40 mM chloroquine were added to the suspension and the cells incubated at 37°C for 6 h. Cells then were "shocked" with 5 ml of 20% dimethyl sulfoxide in growth medium for 5 min at 37°C and subsequently washed with 10 ml of growth medium then incubated in growth medium in the culture conditions as described. Medium was changed after 48 h to growth medium + G-418 (500 μg/ml) and medium was then changed every 48 to 72 h.

Reverse Transcription-Polymerase Chain Reaction for AT_1R. RT-PCR was performed as described previously (Cheng et al., 1995) to verify the presence of the transfected rabbit AT_1R transcript. Total RNA was isolated from cells by the acid guanidinium thiocyanate/phenol/chloroform method (Chomczynski and Sacchi, 1987). Five micrograms of total RNA was reverse transcribed using murine reverse transcriptase (First Strand cDNA Synthesis Kit: Amersham Biosciences, Piscataway, NJ) and an AT_1R-specific primer. The resulting single strand cDNA mixture was amplified in a Perkin Elmer GeneAMP 9600 PCR System using Taq polymerase (Perkin Elmer/Cetus). PCR was routinely carried out for 30 cycles at 95°C for 20 sec, 55°C for 30 sec, and 72°C for 90 sec, followed by a 10 min extension at 72°C. Sense and antisense primers were constructed to amplify a 703 bp region of the 5′ coding region of the AT_1R. The employed primers were upstream sense, 5′-TGGGAATATTT GGGAACAGC-3′ and downstream antisense, 3′-GTGAATATTTGGTGGGGAAC-5′. Parallel samples were amplified for 25 PCR cycles with the primers, 5′-AACCGCGAGAAGATGACCCAGATCATGTTT-3′; and 3′-AGCAGCCGTGGCC ATCTCTTGCTCGAAGTC-5′ to assess β-actin expression (14). Samples were analyzed by agarose gel electropheresis (4% agarose).

Specific ^{125}I-Angiotensin II Binding. Binding studies were performed as described previously (Becker et al., 1995). Cells, grown on transwells, were washed with ice-cold phosphate-buffered saline containing 0.1% albumin (PBS-A). The studied surface was incubated with ^{125}I-Ang II [0.1 nM] with or without unlabeled Ang II [0.1 nM-1 μM] in a 300 μl volume for 4 h at 4°C. Cells then were washed with ice-cold PBS-A and lysed with one ml 0.05 M NaOH. An aliquot of lysate was used for protein determination and the remainder placed in a gamma counter (Beckmann 5500) to assess cell-associated radioactivity. Specific binding was determined by subtracting nonspecific binding measured in the presence of Ang II [1 μM] from total ^{125}I-Ang II binding.

^{125}I-Angiotensin II Internalization. Internalization studies were performed as described previously (Becker et al., 1995). After the final binding study wash, a subset of cells was placed in PBS-A at 23°C for 5 to 45 min. PBS-A was then replaced with ice-cold 50 mM acetic acid, pH 3, 150 mM NaCl for 5 min at 4°C. The acid wash was removed and cells lysed with 0.05 M NaOH. An aliquot of lysate was counted in a gamma counter to determine cell-associated radioactivity, a measure of internalized ^{125}I-Ang II. Acid wash radioactivity also was counted and added to the cell-associated radioactivity to determine surface + internalized ^{125}I-Ang II and compared with total specific ^{125}I-Ang II binding at that membrane.

Arachidonic Acid Release. Arachidonic acid (AA) release assays were performed as described previously (Becker and Harris, 1996). Cells were incubated in serum-free medium for 48 h. Cells were then washed three times in medium supplemented with 1

mg/ml bovine serum albumin then incubated overnight in one ml of medium containing [^{3}H]AA (4 μCi/ml). After this incubation, medium was aspirated and cells were washed five times with nonradioactive medium then incubated in nonradioactive medium for 30 min at 37°C. Cells then were treated with Ang II [100 nM] in AP or BL medium for the indicated times. Previous studies had indicated that this concentration of Ang II yielded a maximal AA release response in this model (Becker and Harris, 1996). After the assay period, an aliquot of AP or BL medium was removed and centrifuged at 12,000*g* to pellet cellular debris. The supernatant was transferred to a scintillation vial with 3 ml Aquasol and radioactivity released into the medium determined by liquid scintillation spectrometry. The remaining media was aspirated, cells were washed and then digested with the addition of 0.05M NaOH. An aliquot of the digest was used for protein determination and the remainder was assayed for total incorporated radioactivity.

Preparation of Rat Renal Cortical Endosomes. Rat renal intermicrovillar clefts were prepared from kidneys harvested from anesthetized rats, using Percoll gradient centrifugation and magnesium precipitation (Hammond et al., 1994a; Hammond et al., 1994b). Colocalization of apically derived enzymes and glycoprotein receptors suggests this is an apically derived membrane fraction. This fraction consists of intermicrovillar clefts (Hammond et al., 1994a; Hammond et al., 1994b), based upon heavy enrichment in cleft elements such as H^+-ATPase, clathrin, cubulin and megalin and uptake of dyes added to the homogenate. Methods to fuse these membranes have been optimized and validated (Hammond et al., 1994b).

In Vitro Reconstitution of Endosomal Fusion. Rat renal heavy endosomal fusion was reconstituted in vitro in an *N*-ethylmaleimide-sensitive, ATP- and cytosol-dependent process. Dynamic fluorescent signatures were used to assay renal endosomal fusion (Hammond et al., 1994b; Jo et al., 1995). The "spectroscopic" ruler effects of energy transfer between two different fluorescent dextrans makes it useful for fusion assays. If energy transfer is observed, the donor and acceptor molecules lie within 1 to 6 nm in the same membrane–bound compartment. Energy transfer will not occur across a 7.5 nm lipid bilayer membrane. Fusion can be assayed in a vesicle population in a cuvette, or on a vesicle-by-vesicle basis using small particle flow cytometry analysis. To test whether peptide sequences corresponding to putative AT_1R internalization motifs affected endosomal fusion, aliquots of endosomes were preincubated with active, reverse or random sequence peptides [1 pM -1 μM] or mastoparan in the same concentrations for at least 30 min on ice, then assayed in the aforementioned fashion.

Immunoblot Studies: Endosomes. Highly purified rat renal cortical AP endosomes, isolated by Percoll gradient centrifugation, were analyzed by immunoblot as described previously (Hammond et al., 1997). Aliquots of purified endosomes, brush border membrane vesicles and lysosomes were separated by sodium dodecyl sulfate-polyacrylamide gel electropheresis (10% acrylamide) using 4 μg of protein per lane and a 1:1000 dilution of rabbit polyclonal anti-$AT_{1a}R$ antibody. The gel was revealed with the peroxidase-base enhanced chemiluminescence system (ECL, Amersham, Springfield, IL).

Immunoblot of $LLCPK_{Cl4}$ Cells with Wild-Type or pRC-CMV-AT_1R Vector Constructs. $LLCPK_{Cl4}$ cells were transfected as described previously (Becker et al., 1995). Protein aliquots were then obtained and separated by SDS-polyacrylamide gel electrophoresis (10% acrylamide) using 30 μg of protein per lane and a 1:500 dilution of rabbit polyclonal anti-AT_1R antibody (Chemicon, Temecula, CA). The gel was revealed with the peroxidase-base enhanced chemiluminescence system (ECL; Amersham Biosciences).

Immunoblot of Protein Tyrosine Phosphorylation in $LLCPK_{Cl4}$ Cells with Wild-Type or Mutant pRC-CMV-AT_1R Vector Constructs. $LLCPK_{Cl4}$ cells were transfected as described. We noted previously that Ang II treatment leads to AT_1R-mediated protein tyrosine phosphorylation (Becker et al., 1999). To validate whether internalization was important in this cell model, transfected cells were treated with sodium vanadate (1 nM) for 30 min before Ang II treatment (0.1–100 nM) for 5 to 30 min at 37°C. Cells were then washed with 3× PBS and exposed to 0.5 ml of ice-cold immunoprecipitation (IP) buffer (1% Triton X-100, 150 mM NaCl, 10 mM Tris, pH 7.4, 1 mM EDTA, 1 mM EGTA, 0.2 mM sodium vanadate, 0.2 mM phenylmethylsulfonyl fluoride, and 0.5% Nonidet P-40) for 30 min at 4°C. Cell lysate was passed through a 26-gauge needle and centrifuged at 2500*g* for 15 min at 4°C. Protein (250 mg) was added to 400 ml of water, 500 ml of 2× IP buffer, and 2.5 mg of rabbit polyclonal anti-PTyr antisera (Zymed Laboratories, South San Francisco, CA). This solution was vortexed and incubated overnight at 4°C. Fifty milliliters of 10% protein A (Pansorbin; Calbiochem, San Diego, CA) was added, and the solution incubated at 4°C for 60 min. Samples were centrifuged at 2500*g* for 10 min, 4°C, and the pellet washed 3× with 500 ml of IP buffer. After the final wash, the pellet was resuspended in 30 ml of 2× Laemmli buffer, boiled for 5 min, and centrifuged for 5 min at 2500*g*, 4°C. Twenty micrograms of protein then was separated by SDS-PAGE (10% acrylamide) and transferred to polyvinylidene difluoride membranes. Membranes were incubated with blocking buffer (Tris-buffered saline/0.3% Tween 20/3% bovine serum albumin) overnight at 4°C, then incubated with PY20 monoclonal antiphosphotyrosine antibody at a 1:750 dilution overnight at 4°C. Membranes were then washed with buffer (Tris-buffered saline/0.3% Tween 20) five times and incubated with goat anti-mouse secondary antibody for 1 h at 23°C. Membranes were again washed five times with buffer and immunoblots were developed by the ECL system (Amersham Biosciences).

Protein Determination. Proteins were quantified by the method of Smith et al. (1985) using bicinchoninic acid assay protein reagents (Pierce, Rockford, IL).

Statistical Analysis. Results are presented as mean ± S.E.M. Results of ^{125}I-Ang II binding assays are reported as counts bound per milligram of protein. Results of ^{125}I-Ang II internalization assays were normalized as percentage of specific ^{125}I-Ang II binding at the studied membrane surface. Analysis of ^{125}I-Ang II binding as a function of Ang II concentration was determined with Prism (GraphPad Software, Inc., San Diego, CA). Statistical comparisons used analysis of variance and student's *t* test with the Bonferroni correction when indicated. A p value < 0.05 was considered statistically significant.

Results

^{125}I-Ang II Binding and Internalization. Transfected $LLCPK_{Cl4}$ cells grown on permeable supports were shown by RT-PCR analysis to express the full-length or truncated rabbit AT_1R transcripts (Fig. 1). These cells were assayed for AP- and BL-specific ^{125}I-Ang II binding. Cells expressing full-length AT_1R bound 8.3 ± 4.2 fmol/mg of protein (AP) and 18 ± 5.6 fmol/mg of protein (BL), with slightly different K_d values at AP and BL surfaces (AP, 6.4 ± 0.8 nM; BL, 3.2 ± 1 nM) ($n = 6$). Nontransfected cells did not display any specific ^{125}I-Ang II binding ($n = 3$). Previous studies had demonstrated that 1 μM losartan inhibited specific ^{125}I-Ang II binding in transfected $LLCPK_{Cl4}$ cells (Becker et al., 1995).

Cells expressing AT_1R lacking the distal 15 aa of the AT_1R carboxy-terminal tail including the putative palmitoylation site, T345S cells, had similar K_d values at AP and BL surfaces (AP K_d, 5.1 ± 1.7 nM; BL K_d, 1.9 ± 1 nM) ($n = 8$). T330L cells, transfected with AT_1R that contained approximately half of the carboxy-terminal tail, also displayed similar AP (8.0 ± 1.4 nM) and BL (4.1 ± 2.2 nM) K_d values ($n = 3$), respectively.

T316L cells, lacking the distal 80% of the AT_1R cytoplasmic tail, maintained the same relative AP/BL distribution for specific ^{125}I-Ang II binding but with slightly higher K_d values

(AP, 10 ± 1.9 nM; BL, 5.8 ± 2.7 nM; $n = 6$). T302F cells, expressing mutant AT_1R lacking the entire cytoplasmic tail, had no specific binding at the AP surface ($n = 6$); although they did manifest specific BL ^{125}I-Ang II binding (10.6 fmol/mg of protein; K_d, 10.1 ± 3.7 nM; $n = 6$).

Full-length AP AT_1R internalized the majority of bound ^{125}I-Ang II within 10 min (82 ± 7%) (Fig. 2A). BL AT_1R internalized significantly less bound ^{125}I-Ang II (10 min, 32 ± 7%; 20 min, 27 ± 6%; $p < 0.002$ versus AP; $n = 6$) (Fig. 2B). The internalization pattern of the T345S mutants was not distinguishable from that of the full-length receptor (AP-bound ^{125}I-Ang II: 10 min, 67 ± 12%; 20 min, 75 ± 8%; N.S. versus full-length; $n = 5$. BL internalization: 10 min, 30 ± 6%; 20 min, 29 ± 5%; $n = 7$; N.S. versus full-length) (Fig. 2, A and B). Compared with the full-length receptor, T330L cells internalized less ^{125}I-Ang II from the apical surface (10 min, 25 ± 15%; 20 min, 31 ± 9%; $p < 0.05$, 20 min versus full length; $n = 4$); interestingly, however, BL ^{125}I-Ang II internalized in T330L cells was similar to full-length AT_1R and T345S mutants (10 min, 21 ± 8%; 20 min, 25 ± 7%; $n = 5$; N.S. versus full length).

Deleting the distal 44 aa of the AT_1R cytoplasmic tail significantly abrogated ^{125}I-Ang II internalization at both apical and basolateral surfaces (T316L AP: 10 min, 10 ± 6%; 20 min, 17 ± 6%. BL: 10 min, 5 ± 5%; 20 min, 15 ± 4%; $n = 5$; $p < 0.05$ versus full length). Minimal basolateral ^{125}I-Ang II internalization was seen in the mutants with the cytoplasmic tail completely deleted (T302F BL: 10 min, 7 ± 6%; $n = 6$; 20 min, 12 ± 4%; $n = 7$; $p < 0.05$ versus full-length BL) (Fig. 2, A and B).

Arachidonic Acid Release. We assessed arachidonic acid release after AP or BL Ang II treatment for each of the AT_1R mutant clones. We previously demonstrated arachidonic acid release occurs as a delayed event after AP Ang II binding in this model and that it was a signaling event that correlated with AT_1R endocytosis (Becker and Harris, 1996). Full-length AT_1R, when exposed to Ang II (100 nM) at the AP surface, increased [^{3}H]AA release (counts released per milligram of protein at 20 min: 18,762 ± 4018; $n = 5$) (Fig. 3). BL Ang II stimulated only minimal [^{3}H]AA release at 20 min: 4417 ± 1426 ($n = 3$). T345S mutants, similar to full-length AT_1R, stimulated [^{3}H]AA release after AP Ang II treatment (15,986 ± 3611; $n = 8$; N.S. versus full length) (Fig. 3). AP Ang II treatment for 20 min also stimulated [^{3}H]AA release in T330L cells (10,145 ± 4611; $n = 4$; N.S. versus full length) (Fig. 3). As expected, AP Ang II treatment in T302F-expressing cells did not lead to any significant [^{3}H]AA release after 20 min (1577 ± 2046; $n = 3$; $p < 0.02$ versus full length) (Fig. 3). In addition, AP Ang II treatment also did not stimulate

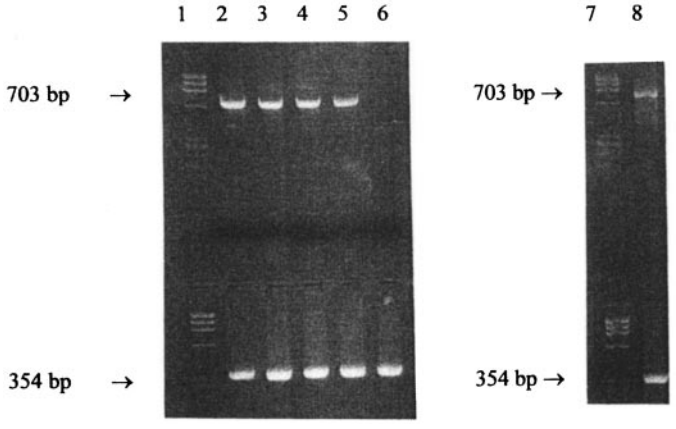

Fig. 1. RT-PCR amplification of full-length and mutant AT_1R mRNA in transfected and nontransfected $LLCPK_{Cl4}$ cells. Total RNA (5 μg) from transfected and nontransfected $LLCPK_{Cl4}$ cells was reverse-transcribed then subjected to PCR amplification as described under *Materials and Methods*. The amplified AT_1R fragment was 703 bp. For normalization, parallel samples were analyzed for β-actin expression (354 bp). Lane 1, molecular weight markers; lane 2, full-length AT_1R transfectants; lane 3, T345S transfectants; lane 4, TL 316 transfectants; lane 5, T302F transfectants; lane 6, control nontransfected $LLCPK_{Cl4}$ cells; lane 7, MW markers; lane 8, T330L transfectants. T330L mutant AT_1R transcript represents RT-PCR analysis from a separate assay.

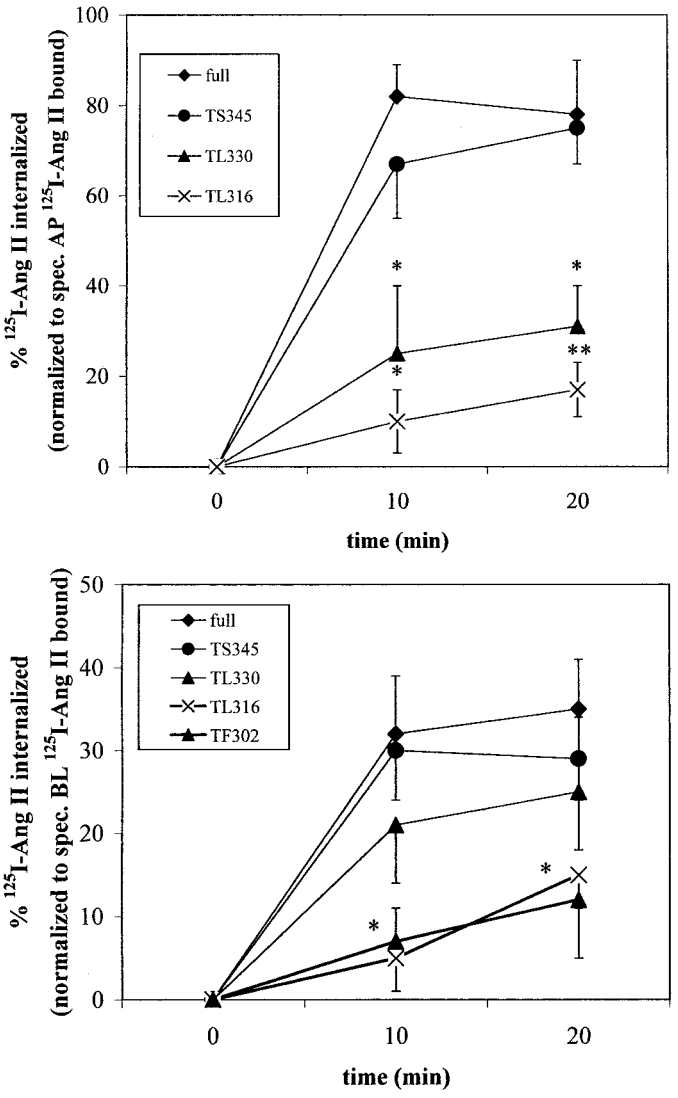

Fig. 2. A and B, AP and BL ^{125}I-Ang II internalization in transfected $LLCPK_{Cl4}$ cells. ^{1125}I-Ang II internalization studies were performed as described under *Materials and Methods*. Cells were exposed to ^{125}I-Ang II per binding study protocol then cells were washed three times with PBS/0.1% albumin. Some cells were lysed with 0.05 M NaOH, and an aliquot was counted to determine specific ^{125}I-Ang II binding. Other cells were incubated in PBS/0.1% albumin for 0 to 20 min. These cells were then acid-washed to remove surface-bound ligand, then lysed with 0.05 M NaOH. An aliquot of lysate was saved for protein determination and the remainder counted in a γ counter to determine internalized, cell-associated radioactivity. Data are expressed as a percentage of total specific ^{125}I-Ang II binding at that membrane surface. Results are expressed as mean ± S.E.M. for each time point ($n = 4$–7 for each). *, $p < 0.05$ versus full-length AT_1R; **, $p < 0.03$ versus full-length AT_1R.

[^{3}H]AA release at 20 min in T316L-expressing cells despite the presence of specific AP ^{125}I-Ang II binding (2430 ± 1711; $n = 4$; $p < 0.02$ versus full-length) (Fig. 3).

Peptide Studies. These data suggested that motifs in the AT_1R cytoplasmic tail played a significant role in receptor-mediated endocytosis and the lipid-related signaling response that occurred after this event. To determine whether the regions of the AT_1R tail that affected endocytosis also influenced endosomal fusion, endosomal fusion assays were performed in the presence or absence of peptides that contained putative internalization motifs within the AT_1R cytoplasmic tail. These two peptides were: LSTKMSTLSY (peptide STL, aa 330–339) or YFLQLLKYIPP (peptide LL, aa 311–322). Peptide LL did not significantly affect in vitro fusion of rat renal cortical endosomes (80 ± 16% of control; $n = 5$; N.S.) (Fig. 4). However, peptide STL significantly inhibited endosomal fusion (22 ± 10% of control; $n = 5$; $p < 0.05$ versus positive control) (Fig. 4). A random sequence peptide that incorporated the residues of peptide STL, TMTYSSLLSK, did not significantly affect endosomal fusion (84 ± 20% of control; $n = 2$; data not shown). Similarly, two peptides matching derivatives of the internalization sequence of the transferrin receptor and the Walsh inhibitor had no effect on fusion (NTKANVTKPKR, 101 ± 6%; DNNTKANVTKPKR, 103 ± 7%; $n = 6$ for each, N.S.).

Polyclonal antiserum raised to a fusion protein of the entire cytosolic tail of the rat $AT_{1a}R$ was also tested in the fusion assay. At peak binding dilution, determined by flow cytometry binding curves, the antiserum inhibited membrane fusion (23 ± 6% of control; $n = 4$; $p < 0.05$). Antisera to myosin I and clathrin, both of which bound the membrane on flow cytometry analysis, had no effect on membrane fusion (102 ± 7% and 98 ± 4%, respectively; $n = 4$, N.S.). Control antisera to the exofacial domains of the AT_1R also had no significant effect on membrane fusion ($n = 4$). Because AT_1R-mediated membrane fusion may be associated with G-protein-coupled receptor activation, mastoparan was also tested in varying concentrations (1 pM–1 μM) to determine whether it affected endosomal fusion. Mastoparan had no significant effect at any of the concentrations tested ($n = 8$).

Immunoblot Studies. After Ang II treatment, full-length AT_1R internalized as demonstrated by the immunoblot studies (Fig. 5a). Moreover, an event related to internalization, receptor-mediated protein tyrosine phosphorylation is contingent upon the presence of the bulk of the tail (Fig. 5b). To verify that this process actually occurs in vivo we assessed the presence of AT_1R in AP endosomes. Highly purified rat renal cortical AP endosomes were isolated by Percoll centrifugation and subjected to immunoblot analysis for the rat $AT_{1a}R$. An anti-$AT_{1a}R$ rabbit polyclonal antibody raised to a fusion protein representing 57 amino acids of the C terminus of the receptor was used for immunoblot studies. As shown in Fig. 6, the rabbit polyclonal anti-$AT_{1a}R$ antibody recognized two distinct bands in endosomes, approximating 68 and 79 kDa. These bands matched the molecular mass of bands recognized in brush-border membrane vesicles. Lysosomes were negative for the receptor.

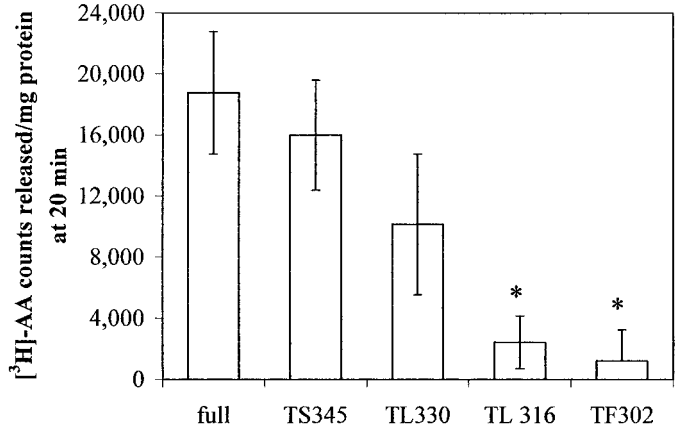

Fig. 3. Apical Ang II-stimulated [^{3}H]arachidonic acid release in transfected $LLCPK_{Cl4}$ cells. Cells were loaded with [^{3}H]arachidonic acid (4 μCi/ml) overnight as described under *Materials and Methods*. After this incubation, radioactive medium was aspirated, and cells were washed five times with serum-free, nonradioactive medium, then incubated in nonradioactive medium for 30 min at 37°C. Cells were then treated with apical 100 nM Ang II for 20 min, after which an aliquot of apical or basolateral medium was removed and centrifuged at 12,000*g* to pellet cellular debris. The supernatant was transferred to a scintillation vial with 3 ml of Aquasol and radioactivity released into the medium determined by liquid scintillation spectrometry. The remaining media was aspirated, cells were washed and then digested with addition of 0.05 M NaOH. An aliquot of the digest was used for protein determination and the remainder assayed for total cellular incorporated radioactivity. Results shown are mean ± S.E.M. for each ($n = 3$–8 for each). *, $p < 0.02$ versus full-length AT_1R.

Discussion

Ang II is a peptide hormone that regulates blood pressure, salt and water balance, and cell growth. It acts through two major receptor types, AT_1R and AT_2R. AT_1R, the predominant receptor type in renal tissue, is a member of the G-protein-coupled receptor superfamily. Two AT_1R subtypes (AT_{1a} and AT_{1b}) are present in rodent kidney (Iwai and Inagami, 1992). Only one subtype, however, has been identified in human, rabbit, and ovine renal tissue (Burns et al., 1993; Speth et al., 1995; Wolf et al., 1997). Whereas AT_2R is an important receptor in fetal kidney tissue, AT_1R is responsible for the vast majority of Ang II-related physiologic effects in adult kidney.

The AT_1R carboxy-terminal tail seems to be important in

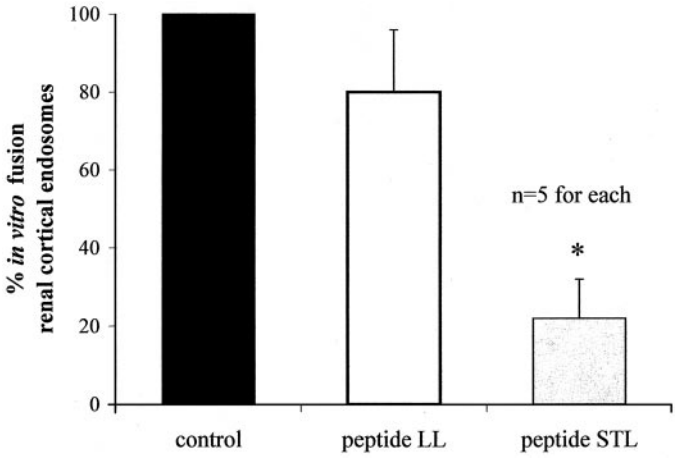

Fig. 4. Peptide inhibition of in vitro rat renal cortical endosomal fusion. Aliquots of endosomes were preincubated with either peptide LL (1 μM) or peptide STL (1 μM) for 30 min on ice, then assayed as described under *Materials and Methods*. Results shown are mean ± S.E.M. for peptide LL and peptide STL versus positive control ($n = 5$ for each); *, $p < 0.05$ versus positive control.

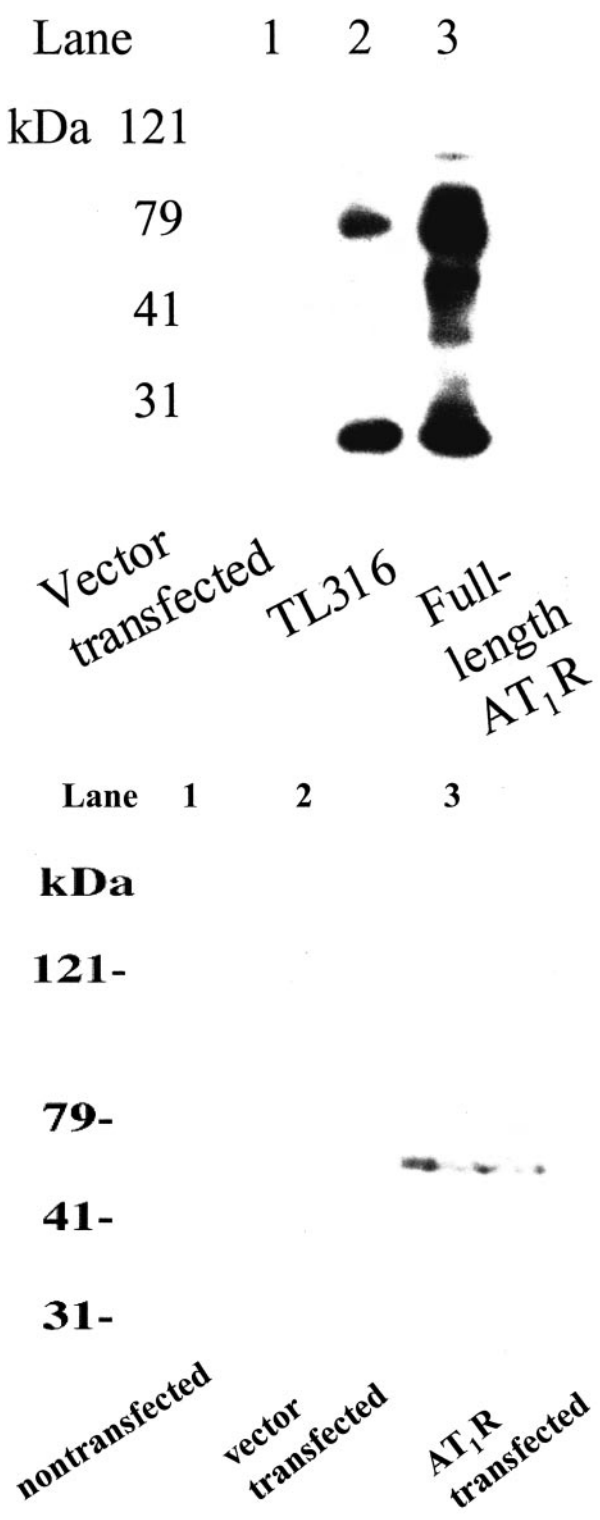

Fig. 5. a, immunoblot of $LLCPK_{Cl4}$ cells with wild-type or pRC-CMV-AT_1R vector constructs. Protein aliquots from nontransfected (lane 1), vector-transfected (lane 2), and full-length AT_1R-transfected (lane 3) were assayed as described under *Materials and Methods*. The full-length AT_1R-transfected cells demonstrate the presence of AT_1R. This is a representative assay ($n = 3$). b, immunoblot of protein tyrosine phosphorylation in $LLCPK_{Cl4}$ cells with wild-type or mutant pRC-CMV-AT_1R vector constructs. Protein aliquots from vector-transfected (lane 1), T316L (lane 2), and full-length AT_1R-transfected cells (lane 3) were assayed as described under *Materials and Methods*. The full-length AT_1R-transfected cells demonstrated more protein tyrosine phosphorylation than T316L-expressing cells. This is a representative assay ($n = 3$).

regulating Ang II-mediated transcellular sodium transport (Thekkumkara and Linas, 2002). Because AT_1R are found on both AP and BL proximal tubule membranes in vivo (Burns et al., 1993). One of the goals of these studies was to investigate whether any cytoplasmic tail domains were involved in membrane localization. Unexpectedly, our studies indicated dissociation between tail domains involved in targeting and internalization. Furthermore, these studies suggest different domains of AT_1R are involved in apical and basolateral targeting, because deletion of the proximal 12 aa of the cytoplasmic tail abolished apical receptor expression but did not affect basolateral expression. Further studies will be required to determine which amino acids in this region mediate the apical expression and whether they allow for either random AT_1R delivery or microtubule-dependent delivery to the apical surface (Saunders and Limbird, 1997).

Endocytosis after ligand binding is a key property of AT_1R, and recent studies have elucidated aspects of the complex structure-function relationship between motifs and residues in the AT_1R tail and endocytosis and signaling. A number of studies have determined various endocytosis motifs within the AT_1R cytoplasmic tail involved in endocytosis in nonpolarized cells (Fig. 6) (Hunyady et al., 1994b; Thomas et al., 1995a; Thekkumkara et al., 1998; Hunyady et al., 2000; Thekkumkara and Linas, 2002). The AT_1R carboxy-terminal tail contains serine and threonine residues (Thr332, Ser335, Thr336, and Ser338) that play a role in receptor phosphorylation (Thomas et al., 1995a, 1998, 2000). Moreover, in combination or in aggregate, these residues seem to influence receptor internalization (Thomas et al., 1998). Interestingly, in polarized $LLCPK_{Cl4}$, the full-length clone demonstrated internalization and activation of protein tyrosine phosphorylation. Deletion of part of the receptor tail (T330L) partially inhibited apical internalization but did not affect rates of basolateral internalization. However, deletion of more of the receptor tail (T316L) markedly reduced internalization and abrogated AT_1R-mediated protein tyrosine phosphorylation.

Our studies also indicated that deletion of all but the proximal 12 aa of the cytoplasmic tail inhibited initial rates of apical internalization by >85%. Interestingly, this mutant (T316L) also inhibited the initial rate of basolateral internalization to a similar degree. The T316L mutant was also

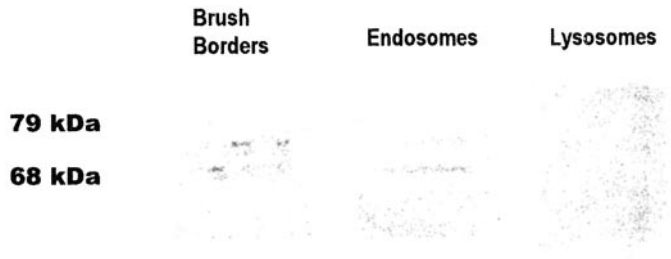

Fig. 6. Immunoblot analysis of $AT_{1a}R$ in rat renal cortical apical endosomes. Purified rat renal cortical endosomes were isolated as described under *Materials and Methods*. Brush-border membrane vesicles, prepared by magnesium precipitation and lysosomes, prepared by density-gradient centrifugation were analyzed in parallel renal cortical apical endosomes for the presence of $AT_{1a}R$ by immunoblot as described under *Materials and Methods*. Lane 1, molecular mass markers; lane 2, brush-border membrane vesicles; lane 3, apical endosomes; lane 4, lysosomes. The anti-$AT_{1a}R$ antibody recognized two distinct bands in the endosomes. These bands matched the molecular mass of similar bands recognized in the brush-border membrane vesicles. Lysosomes were negative for the receptor.

incapable of initiating arachidonic acid release. This finding is consistent with our previous studies, in which pharmacologic inhibition of full-length AT_1R internalization also prevented arachidonic acid release (Becker et al., 1995). Alternatively, tail residues involved in regulating arachidonic acid release may exist between aa 316 and 330, or the lack of arachidonic acid activation may be the result of a conformational change in the receptor based on its mutant structure. Such a mechanism has previously been described to explain the discrepancies between agonist-induced AT_1R phosphorylation occurring distinct from inositol phosphate signaling (Thomas et al., 2000).

In polarized epithelia, discrete subcellular domains express different endocytotic machinery and signaling molecules, and the presence or absence of these elements may affect AT_1R internalization. This may account for the quantitative and qualitative differences observed for AP and BL AT_1R by ourselves and others (Becker et al., 1995; Becker and Harris, 1996; Thekkumkara and Linas, 2002). The importance of a polarized cellular environment may be significant in that G-proteins and cytoskeletal molecules involved in the endocytic process may be distributed to distinct cellular domains. In this regard, it will be of interest in future studies to determine whether the AT_1R-associated protein, which has been invoked as a mediator of AT_1R internalization (Cui et al., 2000), is preferentially localized to apical domains in $LLCPK_{Cl4}$ cells.

In nonpolarized cells, AT_1R internalization has been postulated to occur through a clathrin-dependent-process (Anderson et al., 1993; Anderson and Peach, 1994). Adaptor proteins recognize endocytosis motifs and thereby mediate ligand-bound receptor aggregation into clathrin-coated pits. Electron microscopy studies indeed have suggested Ang II aggregates into clathrin-coated pits in vascular smooth muscle cells (Anderson et al., 1993; Anderson and Peach, 1994). However, it is still unclear whether AT_1R use clathrin-associated endocytosis machinery exclusively or even predominantly to internalize in polarized cells. Dynamin is a key component of this machinery. Although several studies suggest that dynamin and dynamin GTPases are integral to AT_1R internalization (Werbonat et al., 2000; Szaszak et al., 2002), AT_1R internalization remained unaltered in dynamin-deficient cells (Zhang et al., 1996). Thus, the role of dynamin in AT_1R internalization remains to be fully defined and may not be an absolute requirement for AT_1R internalization in polarized cells.

Membrane fusion, an important feature of the endocytotic process, may also be dependent on local molecules and proteins that can alter fusion properties. The demonstration that similar peptide motifs in the AT_1R cytosolic tail may influence both internalization and membrane fusion expands the observations of receptor-mediated regulation of membrane fusion to include G-protein-coupled receptors. Interestingly, recent data suggest that AT_1R mutants, truncated in the carboxy-terminal tail, demonstrate differential interactions with heterotrimeric G proteins, and this may facilitate Rab5a-dependent fusion of endocytic vesicles (Seachrist et al., 2002). Our data suggest, however, that this process is not entirely G-protein dependent, given the lack of effect of mastoparan. This raises the possibility that other regulatory signaling pathways may be dependent in part on the ability of the intact motif to interact with the surrounding membrane (Llorente et al., 2000). Such a hypothesis has been proposed in a recent study. A caveolin-binding motif in the –COOH-terminal AT_1R tail seems to serve as a "docking" site for proteins involved in receptor targeting and functionality (Leclerc et al., 2002).

Ultimately, receptor localization and internalization govern the physiologic functions of these proteins. Immunoblotting demonstrated that AT_1R are present in brush-border membrane and, once internalized, are evident in AP endosomes. Recent studies have also indicated that endosomes contain angiotensin peptides and angiotensin-converting enzyme, consistent with the hypothesis that AT_1R signaling is not limited to plasma membrane-associated receptors (Imig et al., 1999).

In summary, in polarized epithelia, biologically active and physiologically relevant AT_1R are found on both apical and basolateral membranes in the renal proximal tubule. Furthermore, there is suggestive evidence that the full array of AT_1R signaling may require successful endocytosis after ligand binding. Therefore, the current studies provide preliminary evidence for the identification of specific tail sequences

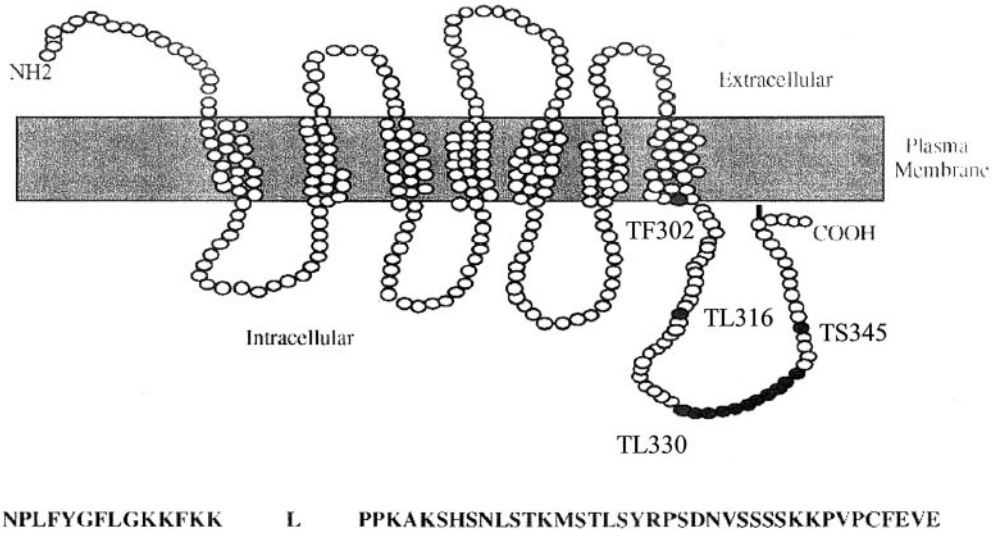

Fig. 7. Schematic representation of the rabbit AT_1R noting residues with truncation mutations at their specific sites in the receptor carboxyl-terminal tail. Peptide sequences used in endosomal fusion studies are noted (Peptide LL and Peptide STL).

in the AT_1R cytoplasmic tail that are involved in apical expression, endocytosis, and membrane fusion properties.

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