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Functional Domains and Expression of Truncated Atrial Natriuretic Peptide Receptor-A: The Carboxyl-Terminal Regions Direct the Receptor Internalization and Sequestration in COS-7 Cells

KAILASH N. PANDEY, RAVINDRA KUMAR, MING LI, and HUONG NGUYEN

Department of Physiology, Tulane University School of Medicine, New Orleans, Louisiana and Medical College of Georgia, Augusta, Georgia

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ABSTRACT

The objective of this study was to determine the role of cytoplasmic (protein kinase-like homology and guanylyl cyclase catalytic) domains of atrial natriuretic peptide (ANP) receptor-A (Npra) in postbinding events and metabolic turnover of ligand-receptor complexes. Using deletion mutagenesis, the specific regions in the intracellular domains of Npra relevant to the receptor function, namely ligand-binding, cGMP production, and internalization and sequestration of ligand-receptor complexes, have been determined in transiently expressing COS-7 cells. Deletion of 12 aa (aa) at the carboxyl-terminal end of receptor (Δ1045-Npra) affected neither ligand-binding efficiency nor cGMP production. However, deletion of 120 to 170 aa residues (Δ937-Npra, Δ916-Npra, Δ902-Npra, and Δ887-Npra) decreased ligand binding by 16 to 20% and cGMP production by 50 to 90%. Further deletion of 422 aa and 569 aa (Δ635-Npra and Δ488-Npra) reduced ligand binding efficiency by 40% and 90%, respectively. The deletion of 12 aa (Δ1045-Npra) did not affect the internalization of Npra; however, deletions up to 170 aa (Δ937-Npra, Δ916-Npra, Δ887-Npra) reduced the internalization of ligand-receptor complexes by 60%. Cells expressing either full-length (wild-type) Npra or 120 aa deleted receptor (Δ937-Npra) released 40 to 45% ^{125}I-ANP radioactivity into culture medium, but only 10 to 15% radioactivity was released from the cells that expressed Δ635-Npra. Furthermore, 35 to 40% ^{125}I-ANP radioactivity was detected into the intracellular compartments of cells that expressed the wild-type Npra, and only 5 to 10% ^{125}I-ANP radioactivity was observed in cells expressing the Δ635-Npra (−422 aa) or Δ488-Npra (−569 aa) mutant receptors. These results show that specific regions within the intracellular domains of Npra determine the extent of ligand-binding efficiency, cGMP production, endocytosis, and intracellular sequestration of ligand-receptor complexes in cDNA expressing COS-7 cells.

Atrial natriuretic peptide (ANP), an endogenous hormone, is synthesized primarily in the cardiac atrium and exerts profound effects on renal and cardiovascular function, largely directed to the reduction of blood volume and body fluid homeostasis (Brenner et al., 1990; Drewett and Garbers, 1994; Pandey, 1996; Levin et al., 1998). Membrane-bound guanylyl cyclase-A [natriuretic peptide receptor-A (Npra)] is a major natriuretic peptide receptor that synthesizes the intracellular second-messenger cGMP in response to ANP binding (Murad et al., 1987; Garbers, 1992). Npra is considered a primary ANP-signaling molecule because major cellular and physiological responsiveness of hormone is mimicked by cGMP and its cell-permeable analogs (Anand-Srivastava and Trachte, 1993; Foster et al., 1997; Pandey, 1997). The studies with Npra-gene knockout mice have indicated that deficiency of Npra increases blood pressure and causes hypertensive heart disease in mice, similar to those seen in untreated human hypertensive patients (Oliver et al., 1997). The general topological structure of the guanylyl cyclase receptor family is consistent with at least four distinct regions, which include extracellular ligand-binding, transmembrane, and intracellular protein kinase-like homology and guanylyl cyclase catalytic domains. The guanylyl cyclase catalytic domain of Npra has been assigned to a region of approximately 250 aa that presumably contains the catalytic active site of the receptor (Liu et al., 1997; Sunahara et al., 1998; Tucker et al., 1998). Although the transmembrane guanylyl cyclase receptors contain a single cyclase catalytic domain per polypeptide chain, however, they function as a

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ABBREVIATIONS: ANP, atrial natriuretic peptide; Nprx, natriuretic peptide receptor, where *x* is a, b, or c; aa, amino acid(s); KHD, kinase-like homology domain; DMEM, Dulbecco's modified Eagle's medium; FBS, fetal bovine serum; PAGE, polyacrylamide gel electrophoresis; AZB, azidobenzoyl; HBSS, Hanks' balanced salt solution; TCA, trichloroacetic acid.

homodimers (Wilson and Chinkers, 1995; Yang and Garbers, 1997). The protein kinase-like homology domain (KHD) is a region of approximately 280 aa that immediately follows the transmembrane spanning domain of the receptor.

The KHD of Npra is more closely related to protein tyrosine kinases than protein serine/threonine kinases. In fact, it is largely similar to the protein kinase domain of the platelet-derived growth factor receptor, with approximately 31% aa identity between the comparable regions of the kinase domains (Hanks et al., 1988). It has been proposed that the KHD of Npra serves as an important mediatory role in transducing the ligand-induced signals to activate the guanylyl cyclase catalytic domain of the receptor (Garbers, 1992; Duda et al., 1993). It has been suggested that an intervening step involving the KHD is necessary to the cyclase catalytic activation process (Goraczniac et al., 1992; Koller et al., 1992). It has also been suggested that ATP serves as an intracellular allosteric regulator of KHD for the activation of Npra (Larose et al., 1991, Duda et al., 1993). Initially, ATP was considered to function by interacting with KHD because this region contains a glycine-rich nucleotide-binding motif and was postulated to provide the ATP-regulatory module for ANP signaling (Goraczniac et al., 1992; Duda et al., 1993). On the contrary, however, the mutation of all three conserved glycine residues in KHD, as well as conserved lysines carboxyl-terminal to them, did not change the activity of mutant Npra. This suggests that ATP may function in regulating the guanylyl cyclase activity, at least in part, by interacting with regions other than the glycine-rich motif of Npra (Koller et al., 1993). Indeed, previous studies as well as the recent data have indicated that KHD seems to be important for ANP-dependent activation of Npra (Larose et al., 1992; Potter and Hunter, 1998). However, the exact mechanisms of activation and the relay of signals from the KHD to guanylyl cyclase catalytic site of the receptor remain to be established.

To delineate the roles of specific sequence regions in the KHD and guanylyl cyclase catalytic domain, we studied the molecular mechanisms of the receptor function using deletion mutagenesis of murine Npra. Our results suggest that the specific sequence motifs within the intracellular domains of Npra determine specific functions in terms of ligand-binding, potentiation of second-messenger cGMP, and receptor postbinding events (such as internalization, sequestration, and metabolic turnover of ligand-receptor complexes) in intact COS-7 cells.

Experimental Procedures

Materials. ANP (rat-28) was purchased from the Peninsula Laboratories, Inc. (Belmont, CA). Na-^{125}I (100 mCi/ml; carrier free) and L-[^{35}S]methionine (1000 Ci/mmol) were obtained from Amersham Pharmacia Biotech Inc. (Piscataway, NJ). Erase-a-base kit was obtained from Promega (Madison, WI). Restriction enzymes were purchased from Stratagene (La Jolla, CA). The mammalian expression vector p^{cDNA3} was obtained from InVitrogen (San Diego, CA). cGMP radioimmunoassay kit was purchased from the Biomedical Technologies, Inc. (Stoughton, MA). Succinimido-4-azidobenzoate was obtained from Pierce (Rockford, IL). Tissue culture supplies were received from Life Technologies (Grand Island, NY). All other chemicals were molecular biology reagent grade.

Plasmid Construction. Full-length murine Npra cDNA (Pandey and Singh, 1990) was excised from Bluescript vector by digesting with *Not*I and was then subcloned into a site of $p^{GEM5zf(+)}$ vector previously digested by *Not*I. The ligated inserts in opposite orientation (3′-5′ direction) were used for deletion mutagenesis. Plasmid vector $p^{GEM5zf(+)}$ containing the cDNA insert was linearized by *Sph*I restriction enzyme. An adaptor containing two additional restriction sites (*Not*I and *SnB*I) was ligated between *Ata*II and *Sph*I sites in multiple cloning regions of $p^{GEM5zf(+)}$ vector. The primers (5′-c t a c g t a g c g g c c a c a c a t g-3′ and 3′-g t a c g a t g c a t c g c c g g c g t-5′) with 1/2 *Sph*I site were synthesized and annealed to each other to prepare the cassette. Plasmid $p^{GEM5zf(+)}$ was digested with restriction enzyme *Sph*I and the DNA cassette was ligated at the *Sph*I site. After ligation of the cassette to the plasmid, the left *Sph*I site was regenerated; however, the right *Sph*I site was not regenerated because of a single-base mismatch to the original site. Plasmid $p^{GEM5zf(+)}$ was linearized by double digestion using *Sph*I and *Spe*I restriction endonucleases. Restriction enzymes *Sph*I and *Spe*I generated exonuclease III resistant sites at the 3′ and 5′ ends, respectively. A complete series of exonuclease III digestion and S1 nuclease treatment was performed using a Promega Erase-a-base kit, according to the method of Henikoff (1984). The DNA ends were filled using Klenow polymerase and ligated according to the manufacturer's protocols. The plasmids were prepared, and the sizes of deleted cDNAs were confirmed by nucleotide sequencing. All deleted Npra cDNAs were excised from $p^{GEM5zf(+)}$ vector by digesting with restriction enzyme *Not*I and were then subcloned into a site of the mammalian expression vector p^{cDNA3} that had been previously digested by *Not*I. The vector p^{cDNA3} was reconstructed using an adaptor containing the termination codon in all reading frames at the *Xho*I site. The expression vector p^{cDNA3} is designed to function under the control of cytomegalovirus immediate early promoter and contains the simian virus-40 origin of replication to increase the transient expression of encoded protein in transfected cells. Restriction mapping and DNA sequencing to verify the correct reading-frame of ligated cDNA inserts [according to the method of Tabor and Richardson (1987)] identified the plasmids of interest that had inserts in correct orientation.

Production of Rabbit Polyclonal Antibodies. The peptide Lys-Cys-Gly-Phe-Asp-Asn-Glu-Asp-Pro-Ala-Cys-Asn-Gln-Asp-His-Phe-Ser-Thr, corresponding to 18 amino-terminal residues (450–467) in the extracellular domain adjacent to the transmembrane domain of Npra, was conjugated to keyhole limpet hemocyanin. The keyhole limpet hemocyanin-peptide conjugate was injected i.p. in the presence of complete Freund's adjuvant into rabbit 809 and 810 (Immuno-Dynamics, Inc., La Jolla, CA). The rabbits were boosted four times with peptide in Freund's incomplete adjuvant. The antiserum was purified using the Thiopropyl-Sepharose 6B matrix (Pharmacia, Piscataway, NJ).

Transfection and Receptor Binding Assay. COS-7 cells were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS) at 37° in an atmosphere of 5% CO_2 and 95% O_2. Cells were transfected with plasmids containing either full-length or deleted Npra cDNAs. Transfection was performed by electroporation using 20 μg of plasmid DNA at 220 V with a capacitance setting of 960 μF using a gene Pulser (BioRad, Richmond, CA). After electroporation, cells were seeded into 60-mm^2 culture dishes. The medium was changed after 24 h and functional studies were performed 48 h after transfection. Rat ANP-28 was iodinated with Na-^{125}I (100 mCi/ml; carrier free) by the Chloramine-T method as described previously (Pandey et al., 1986, 1988). ANP-binding was measured essentially as described elsewhere (Pandey, 1992, 1993). Briefly, cells were washed three times with assay medium (DMEM containing 0.1% bovine serum albumin) and incubated at 4°C in 2 ml of fresh assay medium, containing 1 nM ^{125}I-ANP in the presence or absence of 100 nM unlabeled ANP. After the binding was completed, the medium was removed and cells were washed four times with ice-cold assay medium and dissolved in 0.5N NaOH. ^{125}I-ANP radioactivity was counted.

Photoaffinity Labeling, SDS-Polyacrylamide Gel Electrophoresis (PAGE), and Autoradiography. The photoaffinity li-

gand azidobenzoyl-^{125}I-ANP (AZB-^{125}I-ANP) was prepared as described previously (Pandey et al., 1986, 1988). Forty-eight hours after transfection, cells were washed with assay medium and then incubated at 4°C in fresh medium containing AZB-^{125}I-ANP in the presence or absence of unmodified ANP for 10 min in dark as described elsewhere (Pandey, 1992, 1993). After binding, cells were washed three times with ice-cold assay medium and photolyzed in fresh medium. Cells were then rewashed four times with assay medium and lysed in a solution containing 0.5% SDS, 1% Triton X-100, 1 mM phenylmethylsulfonyl fluoride, 2 mM *N*-ethylmaleimide, 10 μg/ml leupeptin, and 10 μg/ml aprotinin. The aliquots of the cell lysates were boiled for 5 min with equal volumes of sample buffer and analyzed by SDS-PAGE using 7.5% gels under reduced conditions according to the method of Laemmli (1970). Electrophoresis was carried out at a constant current of 25 mA until the bromphenol blue front reached the bottom of the gel. Proteins in the gel were stained with Coomassie Brilliant blue R-250. After destaining, gels were dried and autoradiographed at −70°C using Kodak X-Omat film and Cronex Lightning Plus (DuPont, Boston, MA) intensifying screen. The proteins used for standard molecular weight (i.e., relative molecular mass) calibration were as follows: myosin (M_r 205,000), β-galactosidase (M_r 116,000), phosphorylase *b* (M_r 97,000), bovine serum albumin (M_r 67,000), ovalbumin (M_r 45,000) and carbonic anhydrase (M_r 29,000).

cGMP Assay. Forty-eight hours after transfection, cells were treated with ANP at 37°C for 10 min in the presence of 0.2 mM 3-isobutyl-1-methylxanthine. Cells were washed three times with PBS and scraped in 0.5N HCl. Cell suspensions were placed in a boiling-water bath for 3 min and centrifuged at 1500*g* for 15 min at 4°C. Supernatants were collected and lyophilized. Samples were reconstituted with acetate buffer and recentrifuged. In supernatants, the cGMP contents were determined by radioimmunoassay kit as described previously (Khurana and Pandey, 1995).

Metabolic Labeling of transfected COS-7 Cells with [^{35}S] Methionine. COS-7 cells were transfected with wild-type and carboxyl-terminal deleted Npra cDNAs and plated in 60-mm^2 dishes containing DMEM supplemented with 10% FBS. Twenty-four hours after transfection, a pulse-chase experiment was performed by washing the attached cells twice with Hanks' balanced salt solution (HBSS) and incubating at 37°C with 2 ml of methionine-free DMEM supplemented with 1 μM unlabeled methionine, 10% FBS, and 0.15 mCi/ml L-[^{35}S]methionine. After 30 min, the pulse medium was removed. The cells were washed once with HBSS and 3 ml of chase medium consisting of normal DMEM and 10% FBS and incubated in fresh medium at 37°C for 18 h. After incubation, the chase medium was removed, and the cells were washed with HBSS and scraped in solubilizing buffer (20 mM HEPES, pH 7.4, 150 mM NaCl, 10% glycerol, 1% Triton X-100, 5 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, 20 μg/ml aprotinin, and 20 μg/ml leupeptin). Cell lysate was stirred for 30 min at 4°C and the mixture was centrifuged for 5 min at 1,000*g* to remove the insoluble material and then recentrifuged at 100,000*g* for 60 min to obtain the clear supernatant, which contained the solubilized receptors.

Immunoprecipitation. For immunoprecipitations, equal amounts of proteins (100 μg) obtained from cells expressing the wild-type or carboxyl-terminal deleted receptors were incubated with 1:400 dilution of Npra antiserum at 4°C for 4 h. The immunocomplex was precipitated with protein-A agarose, and nonspecific proteins were removed by washing the precipitated material four times with radioimmunoprecipitation assay buffer containing 20 mM Tris · HCl, pH 7.8, 150 mM NaCl, 1% sodium deoxycholate, 1% Triton X-100, and 0.2% SDS, as described previously (Pandey, 1994). The contents were dissolved in Laemli's sample buffer, boiled for 5 min, and the immunoprecipitated proteins were analyzed by SDS-PAGE and fluorography.

Receptor Internalization and Sequestration. Transfected COS-7 cells were plated in 60-mm^2 dishes and cultured at 37°C in an atmosphere of 5% CO_2 and 95% O_2. Forty-eight hours after transfection, cells were washed three times with assay medium and incubated in the presence of ^{125}I-ANP at 4°C for increasing time periods. The internalization experiments were performed as described previously (Pandey, 1993). The unbound ^{125}I-ANP was removed by extensive washing with cold assay medium and total cell-associated radioactivity was determined by dissolving the cells in 0.5N NaOH, which accounted for the initial zero time control value of 100%. After completion of the binding, cells were quickly warmed to 37°C to allow the internalization of ligand-receptor complexes. At the indicated times, culture dishes were removed from 37°C and placed on ice, and medium was collected to determine the release of both degraded and intact ligands. The cell surface-associated radioactivity was removed by washing the cells with ice-cold acidic buffer (50 mM glycine/100 mM NaCl, pH 3.8) at 4°C. After acid wash, the internalized ^{125}I-ANP radioactivity was measured by dissolving the cells in 0.5N NaOH. To determine the rate of lysosomal degradation of ligand-receptor complexes, the cells expressing the wild-type and truncated receptors were pretreated with the lysosomotropic agent chloroquine (200 μM) at 37°C for 45 min and then allowed to bind ^{125}I-ANP at 4°C for 1 h. The amount of intact and degraded products in the medium was determined by precipitation with 10% trichloroacetic acid (TCA) containing 200 μl of bovine serum albumin (5%) as carrier. The supernatants contained the degraded products and precipitates of TCA retained the intact ^{125}I-ANP.

Results

To characterize the functional properties of the intracellular domains of Npra, a full-length (1057 aa residues) cDNA insert was sequentially deleted from the 3′ end extending to near the transmembrane domain of the receptor. We generated a series of functional truncated Npra inserts by sequen-

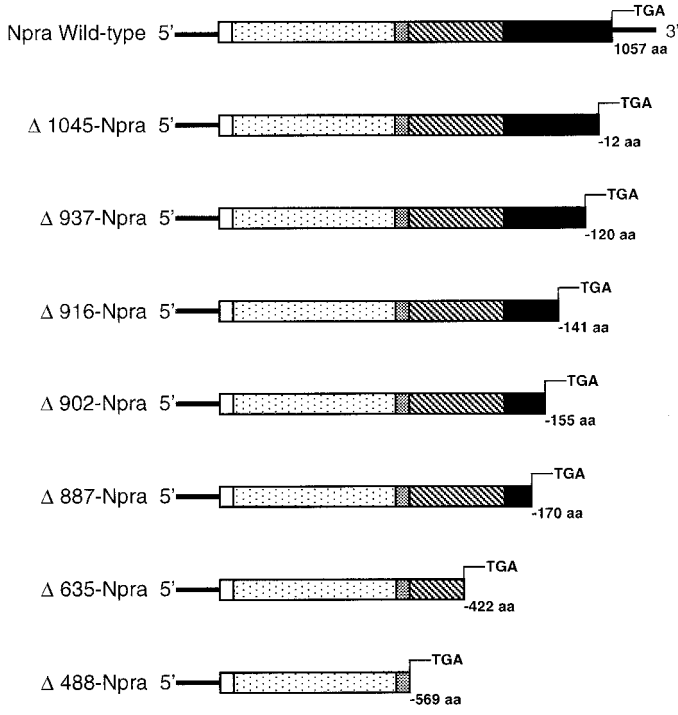

Fig. 1. Schematic diagrams representing the full-length (wild-type) and carboxyl-terminal deletion mutations of Npra. All mutant constructs demonstrate aa deletions at the carboxyl-terminal end of Npra. Truncated mutant cDNAs were ligated into the preconstructed mammalian expression vector p^{cDNA3}, as described under *Experimental Procedures*. The constructs shown above include wild-type Npra (1057 aa) and the sequentially carboxyl-terminal-deleted receptors: −12 aa (Δ 1045-Npra); −120 aa (Δ 937-Npra); −141 aa (Δ 916-Npra); −155 aa (Δ 902-Npra); −170 aa (Δ 887-Npra); −422 aa (Δ 635-Npra); and −569 aa (Δ 488-Npra).

tially deleting the aa residues at the carboxyl-terminal end of the receptor (e.g., −12 aa, −120 aa, −141 aa, −155 aa, −170 aa, −422 aa, or −569 aa). The stop codon was provided at the carboxyl-terminal end of each insert after the aa residue; 1045, 937, 916, 902, 887, 637, or 488, respectively, as stated under *Experimental Procedures*. The resultant truncated mutant receptors were designated as Δ1045-Npra, Δ937-Npra, Δ916-Npra, Δ902-Npra, Δ887-Npra, Δ635-Npra, and Δ488-Npra, respectively, and are represented diagrammatically in Fig. 1. Both the wild-type and mutant cDNAs were transiently expressed into COS-7 cells, and 48 h after transfection, functional studies were carried out. Deletion of 12-aa residues (Δ1045-Npra) at the carboxyl-terminal end of the receptor did not affect the ligand binding (Fig. 2). However, the removal of most of the guanylyl cyclase catalytic domain, up to 120 to 170 aa of receptor (Δ937-Npra, Δ916-Npra, Δ902-Npra, and Δ887-Npra), exhibited approximately 20% reduction in ANP-binding compared with wild-type or 12-aa-deleted (Δ1045-Npra) receptors. The deletion of 422 aa residues (Δ635-Npra), including the guanylyl cyclase catalytic domain and the KHD, resulted in almost 50% reduction in ligand-binding capacity of the receptor. Approximately, 85 to 90% of ^{125}I-ANP binding was reduced in COS-7 cells expressing the −569 aa truncated (Δ488-Npra) receptor (Fig. 2).

To determine the binding of AZB-^{125}I-ANP to wild-type or truncated mutant receptors, we performed photoaffinity labeling of expressed receptors in COS-7 cells. Consistent with ^{125}I-ANP binding assay, photoaffinity labeling of ligand-receptor complexes indicated a reduced binding of AZB-^{125}I-ANP to Δ635-Npra compared with the wild-type receptor protein (Fig. 3). The AZB-^{125}I-ANP-labeled recombinant wild-type Npra, and the truncated (Δ635-Npra) mutant receptors were separated by SDS-PAGE as discrete protein bands with the expected molecular masses of 135 kDa and 81 kDa, respectively. The expression of wild-type Npra in COS-7 cells resulted in an ANP-dependent accumulation of intracellular cGMP by more than 35-fold. The deletion of 12 aa residues at the carboxyl-terminal end (Δ1045-Npra) did not show any discernible effect on cGMP production in response to ANP stimulation (Fig. 4). Nevertheless, deletion of 120 aa (Δ937-Npra) in the guanylyl cyclase catalytic domain of Npra resulted in a 40 to 45% reduction in the intracellular accumulation of cGMP. Further deletion of 141 aa residues (Δ916-Npra) in the guanylyl cyclase catalytic domain blocked more than 95% of ANP-dependent intracellular accumulation of cGMP. To assess the specificity of cGMP production by wild-type or carboxyl-terminal-deleted cDNA constructs, we used the Npra antagonist A-71915. The receptor antagonist

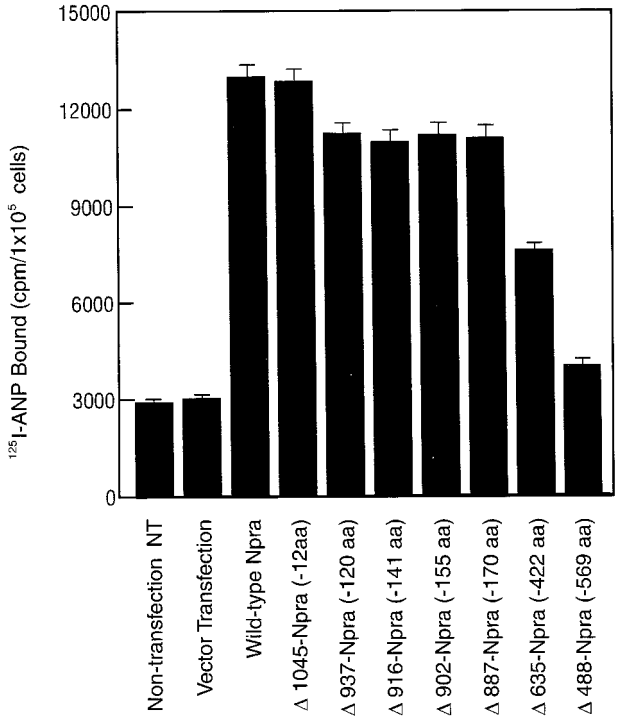

Fig. 2. Binding of ^{125}I-ANP in COS-7 cells expressing the with wild-type and carboxyl-terminal-deleted mutant receptors. Transfection of wild-type (full-length) and carboxyl-terminal- deleted Npra cDNAs was carried out by electroporation as described under *Experimental Procedures*. Forty-eight hours after transfection, cells were washed with assay medium and incubated with 1 nM ^{125}I-ANP in the presence or absence of 100 nM unlabeled ANP for 1 h at 4°C. At the end of the incubation period, cells were washed four times with ice-cold HBSS, dissolved in 0.5N NaOH, and cell-bound radioactivities were determined. The data represent the mean ± S.E. of three to four independent experiments.

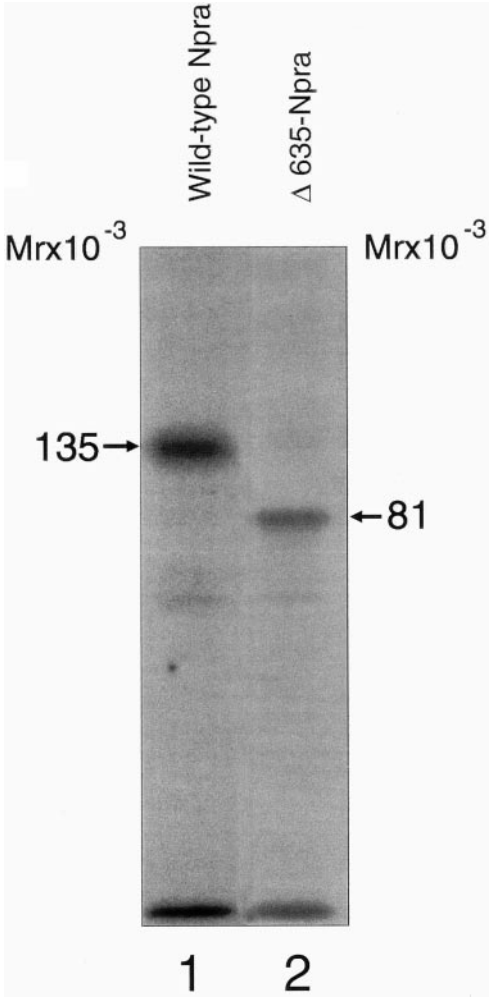

Fig. 3. Photoaffinity-labeling of expressed wild-type and truncated (Δ635-Npra) receptors in COS-7 cells. Forty-eight hours after transfection, COS-7 cells were photoaffinity-labeled as described under *Experimental Procedures*. The expressed wild-type and Δ635-Npra receptor proteins were separated by SDS-PAGE and autoradiography. The wild-type and Δ635-Npra receptors migrated as 135-kDa and 81-kDa protein products, respectively. The corresponding protein bands are indicated by arrows.

A-71915 blocked the generation of cGMP in response to ANP-treatments in COS-7 cells expressing both the wild-type and mutant receptors (Fig. 4). The results of immunoprecipitation studies indicated that wild-type and carboxyl-terminal-deleted receptors are expressed on the cell surface without a significant decrease in the protein contents of mutant receptors (Fig. 5). The wild-type and several mutant receptors showed almost equivalent levels of protein expression in immunoprecipitates, except that the expression of Δ488-Npra was significantly reduced compared with all other truncated mutant receptors.

We examined whether guanylyl cyclase catalytic and/or protein KHDs are required for internalization and intracellular sequestration of Npra. For this purpose, we determined the cell-surface associated, internalized and released ^{125}I-ANP radioactivity in COS-7 cells expressing the wild-type or sequentially truncated cDNA inserts. We distinguished between the surface-associated, internalized and released ^{125}I-ANP radioactivity by acid wash procedure as described under *Experimental Procedures*. Forty-eight hours after transfection, cells in 60-mm^2 culture dishes were allowed to bind ^{125}I-ANP at 4°C for 1 h. After this, unbound ^{125}I-ANP was removed by washing the cells with assay medium, and cells in culture dishes were then incubated in fresh medium at 37°C to allow the internalization of ligand-receptor complexes inside the cell. At the indicated time periods, culture dishes in replicates were removed from 37°C, and media was collected. Cells were washed once with HBSS and then treated with glycine acidic buffer (pH 3.8) at 4°C to release the cell-surface associated ^{125}I-ANP (Fig. 6a). At the initial internalization period (zero time), approximately 85% of the cell-surface bound ^{125}I-ANP radioactivity was released by acid-treatment of cells expressing the wild-type or 12 aa deleted (Δ1045-Npra) receptors. However, a continued reduction in cell-surface binding was observed with all other carboxyl-terminal-deleted receptors. Approximately 60 to 65% of ^{125}I-ANP binding was observed in cells expressing the Δ937-Npra or Δ887-Npra, and only 40% of ^{125}I-ANP binding was detected in cells that expressed the Δ635-Npra compared with wild-type receptors (Fig. 6a). After the acid wash, the cells in culture dishes were dissolved in 0.5 N NaOH to quantify the internalized ^{125}I-ANP. We observed that after 5 min incubation at 37°C, approximately 35 to 40% ^{125}I-ANP remained inside the cells that expressed wild-type receptors, and only 20 to 25% ^{125}I-ANP was localized in cells expressing the Δ937-Npra or Δ887-Npra mutant receptors (Fig. 6b). However, an almost negligible amount of ^{125}I-ANP was detected inside the cells that expressed either Δ635-Npra or vector DNA. The data presented in Fig. 6c show that after a 30-min incubation at 37°C, cells expressing the wild-type Npra or Δ1045-Npra released almost 80% internalized ^{125}I-ANP. The release of ^{125}I-ANP was reduced to 60% in cells that expressed Δ937-Npra or Δ887-Npra; however, only 10 to 15% ^{125}I-ANP was released from the cells expressing the Δ635-Npra.

The COS-7 cells expressing the wild-type or mutant receptors were pretreated with the lysosomotropic agent chloro-

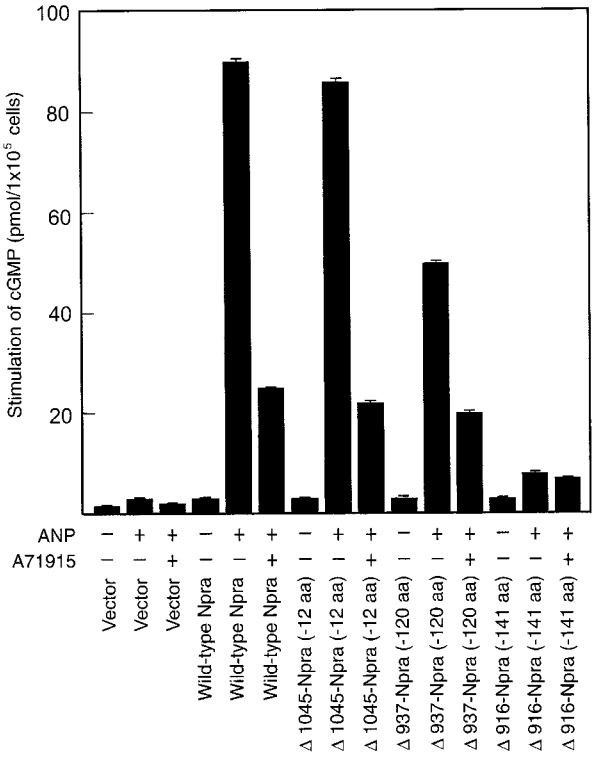

Fig. 4. Stimulation of cGMP in COS-7 cells expressing the wild-type and carboxyl-terminal-deleted mutant receptors. Forty-eight hours after transfection, cells were washed four times with assay medium and treated with 100 nM ANP for 10 min at 37°C in the presence of 0.2 mM 3-isobutyl-1-methylxanthine. A parallel set of dishes also received Npra antagonist A71975. The intracellular accumulation of cGMP was measured by radioimmunoassay as described under *Experimental Procedures*. The bars represent the mean ± S.E of four to six separate determinations.

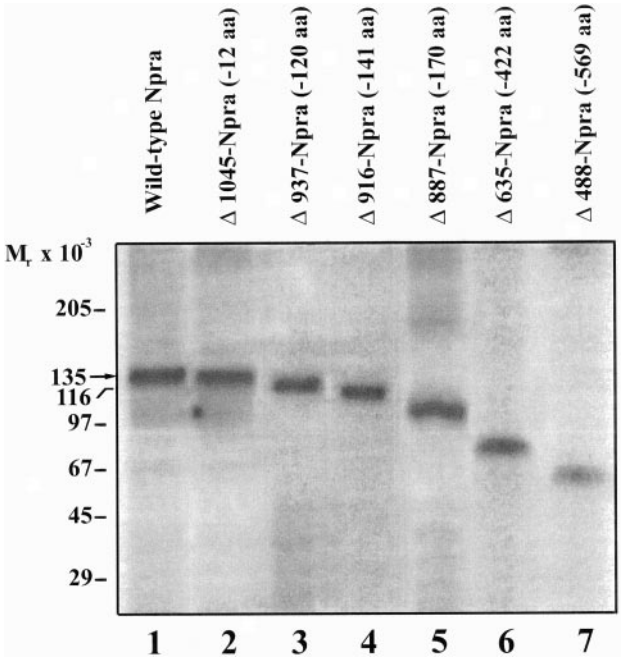

Fig. 5. Autoradiograph showing the ^{35}S-labeled wild-type and carboxyl-terminal-deleted receptors. COS-7 cells were transfected with wild-type and carboxyl-terminal-deleted Npra cDNAs and labeled with ^{35}S-methionine. Cleared cell lysates (200 μg of protein) were immunoprecipitated using the site-directed polyclonal antibodies against the Npra and analyzed on a 7.5% polyacrylamide gel as described under *Experimental Procedures*. Lanes 1–7 show the correspondingly labeled protein bands of expressed wild-type and carboxyl-terminal-deleted mutant receptors in transfected COS-7 cells. Arrow indicates the molecular weight of wild-type receptor. The positions of high molecular mass markers are shown in thousands.

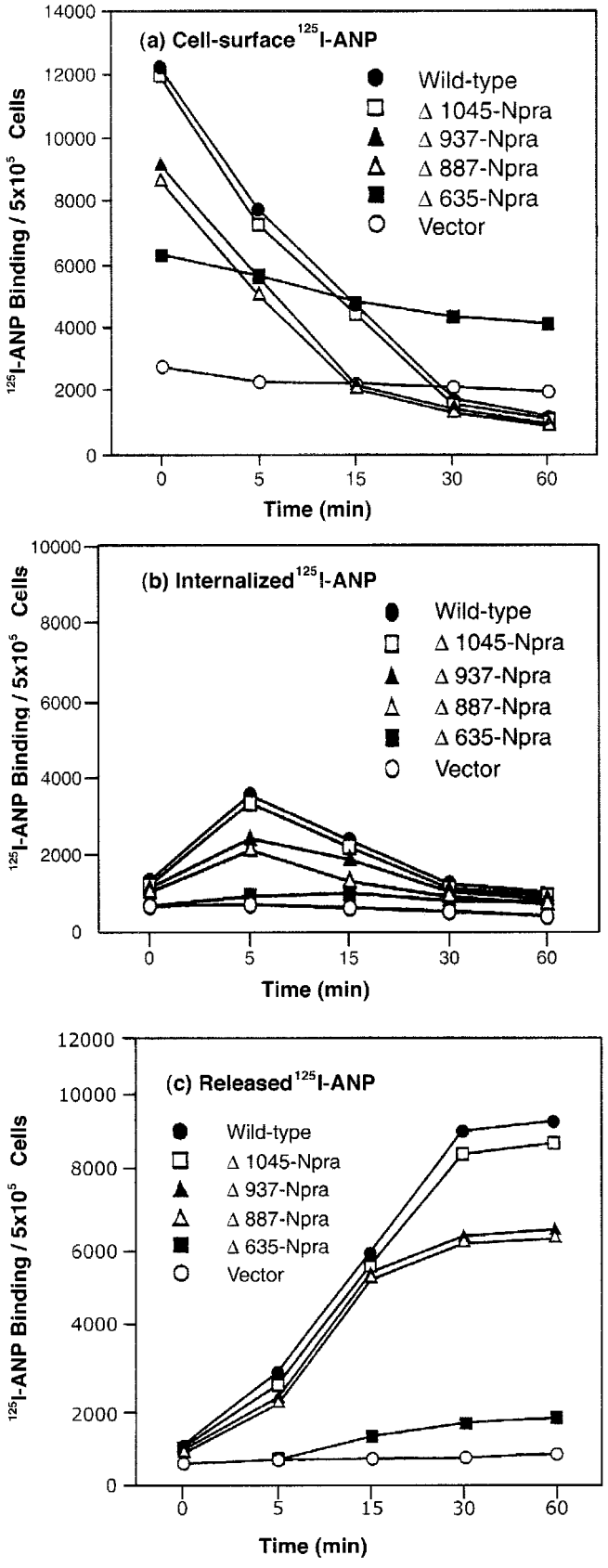

Fig. 6. Quantitative analysis of cell-surface-associated, internalized and released ^{125}I-ANP radioactivity in COS-7 cells expressing the wild-type and carboxyl-terminal truncated receptors. Forty-eight hours after transfection, cells in culture dishes were allowed to bind ^{125}I-ANP at 4°C for 1 h, after which cell monolayers were washed four times with ice-cold assay medium to remove the unbound ligand and then reincubated in fresh medium at 37°C for indicated time periods. Medium was collected and the release of ^{125}I-ANP radioactivity was determined. Cell surface-associated ^{125}I-ANP radioactivity was quantified in acid eluates and cell extracts (0.5 N NaOH) to determine the cell-surface-associated and internalized ligand-receptor complexes, respectively, as described under *Experimental Procedures*. The distribution of ^{125}I-ANP radioactivity is represented as (a) cell-surface associated, (b) internalized into the intracellular compartments, and (c) released into culture media. Each point represents the average value of three separate determinations.

quine at 37°C for 30 min. The release of ^{125}I-ANP in culture media of cells, expressing the wild-type receptors, was blocked by chloroquine during a 15-min incubation (Fig. 7a). However, after an extended period of incubation (30–60 min), the effect of chloroquine diminished, and a large amount of internalized ^{125}I-ANP radioactivity was released into culture media (Fig. 7b). We observed that with increasing deletions of aa residues at the carboxyl-terminal end of receptor, a large proportion of ligand-receptor complexes did not internalize and remained on the cell-surface (Fig. 7, a and b). The quantitative assessment of the intact and degraded ligand was determined by measuring the solubility of ^{125}I-ANP products in 10% TCA. The TCA precipitates (intact ligand) and supernatants (degraded ligand) were separated by centrifugation. The released ^{125}I-ANP consisted of higher amounts of degraded products and a lesser amount of intact ligand in culture media of the cells expressing either wild-type or carboxyl-terminal truncated mutant receptors (Fig. 8, a and b).

Discussion

To investigate the structure-function relationship of the KHD and guanylyl cyclase catalytic domain of Npra, we studied whether the sequential deletion of aa from the carboxyl-terminal end of Npra would render the overall function undetected. We made nested sets of sequential deletions throughout the intracellular region covering both the guanylyl cyclase catalytic and protein KHDs of Npra to determine the specific sequence regions within these domains that should be important for modulation of the functional activity of this receptor protein. The data show that deletion of carboxyl-terminal regions of Npra affects the ligand-binding activities, cGMP production, and internalization and sequestration of ligand-receptor complexes in COS-7 cells expressing the mutant receptors. The present studies have relied on the loss of function of deletion mutations to identify the regions within the KHD and guanylyl cyclase catalytic domain of Npra for postbinding events and receptor function. The guanylyl cyclase/natriuretic peptide receptors (Npra, Nprb) are unique in nature because they contain distinct ligand-binding, transmembrane, protein kinase-like homology and guanylyl cyclase catalytic domains (Garbers, 1992). The natriuretic peptide receptor-C (Nprc) which also contains a single transmembrane domain with a short (37 aa residues) cytoplasmic tail, is considered to clear ANP from the circulation; hence, it is named the clearance receptor (Maack, 1992). However, recent studies with Nprc-gene-knockout mice have shown that Nprc might also be involved in certain critical biological functions displayed by ANP (Matsukawa et al., 1999). All three natriuretic peptide receptors have several common characteristics in the extracellu-

lar ligand-binding domains (Itakura et al., 1994, 1997; Takashima et al., 1995).

Although considerable efforts have been made to define the structure-function relationship of Npra, sufficient studies have not been carried out using the sequential deletion strategy of the carboxyl-terminal end to define the high-order structure characteristics of this receptor protein. In the present studies, we have identified regions in KHD and guanylyl cyclase catalytic domain of Npra by deletion mutagenesis that seem to play important roles in ligand-binding, cGMP production, and metabolic turn-over of this receptor protein. In COS-7 cells expressing the recombinant Npra, ^{125}I-ANP binds to cell-surface receptors, enters through the process of receptor-mediated endocytosis, and are rapidly released into culture media, which is a process similar to that observed with endogenous native receptors (Pandey, 1993). Our results show that deletion of a large part of guanylyl cyclase domain did not seem to have a major effect on the affinity of ANP-binding. However, cells expressing Δ635-Npra or Δ488-Npra mutant receptors showed approximately 50% and 80% reduction in ^{125}I-ANP binding, respectively, compared with the cells expressing full-length wild-type receptor protein. The immunoprecipitation studies indicated that wild-type and carboxyl-terminal-deleted receptors are expressed on the cell surface without a significant decrease in protein contents of mutant receptors, except that the expression of Δ488-Npra was significantly reduced. These results establish that the reduction in binding affinity of Δ937-Npra, Δ916-Npra, Δ887-Npra, or Δ635-Npra is primarily caused by a loss of specific sequence regions in the intracellular domain of Npra. However, the expression of Δ488-Npra protein was slightly reduced; therefore, it can be assumed that the reduction in ligand-binding affinity of Δ488-Npra might be contributed, because of a loss of specific sequence region of KHD in the amino terminus of Npra, and partly because of its reduced expression on the cell surface.

Deletion of 12 aa residues (Δ1045-Npra) at the carboxyl-terminal end of receptor did not seem to affect ligand binding, cGMP stimulation, or internalization of Npra. However, further deletions at the carboxyl-terminal end of Npra dramatically diminished the extent of ligand binding, cGMP production, and internalization of ligand-receptor complexes in COS-7 cells expressing the mutant receptors. Previous studies have suggested that endogenous native ANP receptors Nprc (Nussenzveig et al., 1990; Pandey, 1992; Rathinavelu and Isom, 1991, 1993) as well as Npra (Pandey, 1993) are rapidly internalized in intact cells. Nevertheless, the exact mechanism of endocytosis of these receptors remains to be established. Our deletion mutagenesis studies, aimed at identifying the specific functional regions, revealed that the sites within the intracellular KHD and guanylyl cyclase catalytic domain of Npra seem to play an important role in endocytosis and intracellular sequestration of this receptor protein. The ligand-binding kinetics of wild-type recombinant Npra showed that the ligand-receptor complexes were rapidly internalized and both the intact and degraded ligands were released into culture media in a manner similar to those of native endogenous receptors (Pandey, 1993). Similarly, it has been shown that endocytotic rates of transfected recombinant Nprc into Chinese hamster ovary cells were comparable with that described for native Nprc (Cohen et al., 1996). These authors suggested that the internalization signal for Nprc is located in the cytoplasmic domain and complete deletion of this domain virtually disrupted the internalization of the receptor, reducing the net endocytotic rate by approximately 10-fold compared with the full-length wild-type receptors.

Data presented herein demonstrate a differential pattern

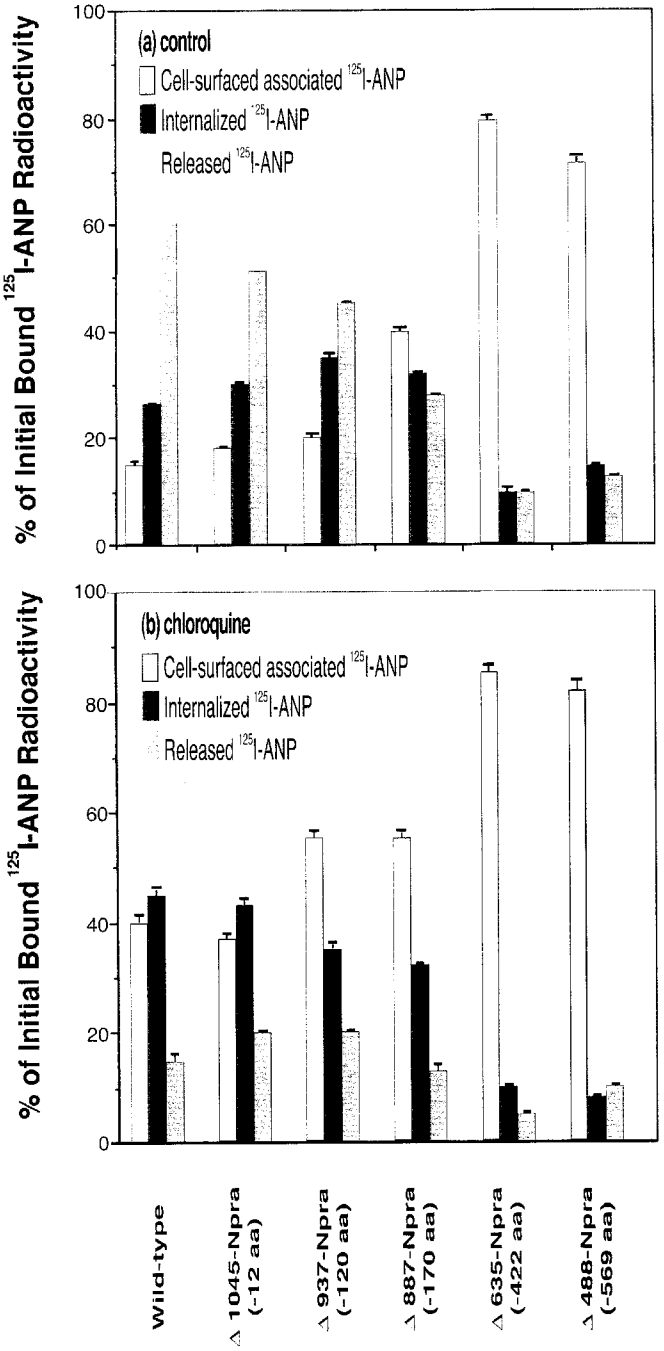

Fig. 7. Quantitative assessment of cell-surface-associated, internalized and released ^{125}I-ANP radioactivity in control and chloroquine-treated COS-7 cells expressing the wild-type Npra and truncated receptors. Forty-eight hours after transfection, cells were washed and pretreated in the presence or absence of 200 μM chloroquine at 37°C for 30 min. ^{125}I-ANP binding was performed at 4°C for 1 h, after which cells were washed with ice-cold assay medium to remove the unbound ligand and then reincubated at 37°C for 15 min in the absence (a) or presence (b) of chloroquine. The cell-surface associated, internalized and released ^{125}I-ANP was determined as described under *Experimental Procedures*. The bars represent the mean ± S.E. of three to four determinations.

of receptor-mediated lysosomal hydrolysis of ^{125}I-ANP in COS-7 cells expressing the recombinant wild-type Npra and the carboxyl-terminal truncated forms of mutant receptors. Our results suggest that truncation of Npra at the carboxyl-terminal end significantly reduced the hydrolysis of ligand-receptor complexes compared with the wild-type receptor. For example, the expression of Δ937-Npra and Δ916-Npra in COS-7 cells resulted in a release of approximately 70% of ^{125}I-ANP in culture media, whereas only 30% of ^{125}I-ANP was released into culture media from COS-7 cells expressing the Δ635-Npra. Complete deletion of both KHD and guanylyl cyclase catalytic domain of Npra abolished the release of internalized ^{125}I-ANP into culture media. Interestingly, most of the internalization signals have been reported to reside in the cytoplasmic domains of endocytosed receptors (Parez et al., 1993; Sorokin et al., 1994; Haft et al., 1994; Huang et al., 1995). Previous studies have indicated that intracellular guanylyl cyclase catalytic domains of natriuretic peptide receptors (Npra and Nprb) are linked to a KHD-dependent mechanism that probably controls the guanylyl cyclase catalytic activity and the production of intracellular cGMP (Koller et al., 1992; Duda et al., 1993; Jewett et al., 1993). It has been suggested that KHD is critical for receptor function; however, the exact mechanism by which it controls the guanylyl cyclase catalytic activity or ligand-binding affinity of the receptor has not been clearly established. Our results demonstrate that sequential deletion of aa residues at the carboxyl-terminal end consisting of KHD and guanylyl cyclase catalytic domain of Npra determine the ligand-binding affinity, generation of second-messenger cGMP, and the metabolic turnover of ligand-receptor complexes in COS-7 cells.

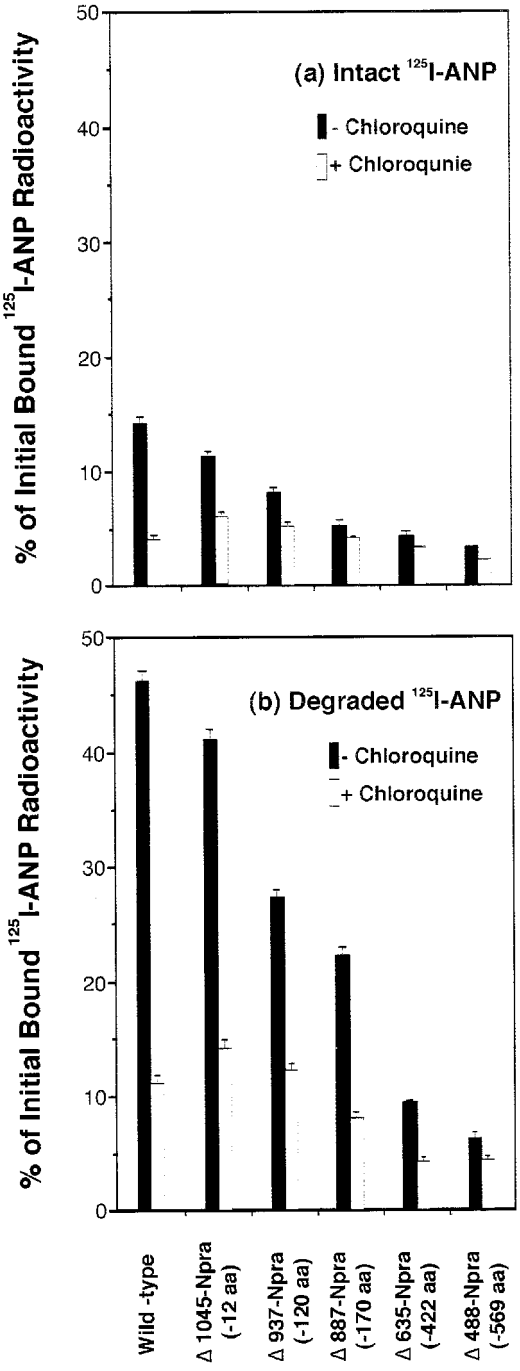

Fig. 8. Quantitative analysis of degraded and intact ^{125}I-ANP released into culture medium of COS-7 cells expressing the wild-type Npra and truncated mutant receptors. Forty-eight hours after transfection, COS-7 cells were washed and preincubated in the absence (a) or presence (b) of the lysosomotropic agent chloroquine (200 μm) at 37°C for 30 min. At the end of preincubation period, cells in culture dishes were allowed to bind ^{125}I-ANP at 4°C for 1 h, after which cells were washed with ice-cold assay medium to remove the unbound ligand and then reincubated in fresh assay medium at 37°C for 15 min. At the end of incubation periods, the culture media were collected and the degraded as well as intact ligands were quantified as described under *Experimental Procedures*. Data represent the mean ± S.E. of three independent determinations.

Our deletion mutagenesis strategy has allowed us to alter selectively and sequentially the carboxyl-terminal end of the receptor to examine the structure-function relationship of Npra in terms of its binding affinity, catalytic activation, and trafficking of ligand-receptor complexes in COS-7 cells. These findings demonstrate that the specific sequence regions within the carboxyl-terminal domains of Npra determine the multiple functional properties of the expressed receptor protein, namely the extent of ligand-binding efficiency, generation of second-messenger cGMP, and the internalization and sequestration of ligand-receptor complexes in the intracellular compartments of intact COS-7 cells.

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Send reprint requests to: Kailash N. Pandey, Ph.D., Department of Physiology, SL-39, Tulane University School of Medicine, 1430 Tulane Ave., New Orleans, LA. E-mail: kpandey@mailhost.tcs.tulane.edu