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Identification of Ligand Recognition Sites in Heat-Stable Enterotoxin Receptor, Membrane-Associated Guanylyl Cyclase C by Site-Directed Mutational Analysis

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Guanylyl cyclase C (STaR), a receptor protein for heat-stable enterotoxin (STa) elaborated by *Escherichia coli*, is associated with and spans the plasma membrane of mammalian intestinal cells. The extracellular domain functions in the binding of STa and the association of each domain to an oligomeric form. Two amino acid residues, Arg-136 and Asp-347, were identified as the residues binding to STa in the extracellular domain of pig STaR by site-directed mutagenesis and analysis of expression on 293T cells. Replacement of these residues by other amino acid residues resulted in the loss of binding of pig STaR to STa, and as a result, STa-induced guanylyl cyclase activity was eliminated. Furthermore, mutation in a region (from Asp-347 to Val-401) which is close to the transmembrane domain caused a significant reduction in both STa-binding activity and guanylyl cyclase catalytic activity. These results suggest that the region adjacent to the transmembrane domain plays an important role in facilitating a favorable conformation of STaR for STa binding.

Membrane-associated guanylyl cyclase (GC) comprises a family of receptor proteins which consist of three functional domains: an amino-terminal extracellular ligand-binding domain, a transmembrane domain, and a carboxy-terminal cytoplasmic catalytic domain (9). GC C has been identified as the receptor (STaR) for a heat-stable enterotoxin (STa) from pathogenic strains of *Escherichia coli* by cloning and expression analysis of the STaR gene from rat, human, and pig intestinal cDNA libraries (4, 20, 21, 27). The binding of STa to the extracellular domain of STaR triggers a biological response via a single-path transmembrane portion, resulting in the production of cytoplasmic cyclic GMP (cGMP) as the second messenger. A complete understanding of the mechanisms which operate after the production of cGMP is not yet available. Recently, one proposal for the biological mechanism was reported, in which protein kinase A, activated by cGMP, phosphorylates the cystic fibrosis-related chloride channel, thus stimulating chloride secretion (1), which is involved in secretory diarrhea and dehydration in humans as well as in domestic animals (10, 18). STaR also has been shown to be a target protein for an endogenous ligand, guanylin, a 15-residue peptide which can be isolated from rat small intestine (3) and which has a conformation similar to that of the toxic domain of STa (17, 22). Thus, STaR would be expected to interact with STa, as well as guanylin.

STaR is a glycoprotein with a molecular size of 140 kDa (5, 25) and has a topological organization similar to those of the receptor proteins (NPR-A and NPR-B, respectively) for atrial and brain natriuretic peptides (9, 28). While the cytoplasmic domain of STaR exhibits 50 and 35% amino acid sequence identity to NPR-A and NPR-B, respectively (4), the extracellular domain of STaR shows only a weak sequence homology to NPR-A and NPR-B, a fact which reflects the differences in the ligand specificities in these extracellular domains. STaR exists as an oligomeric form under native conditions, and the extracellular domain of STaR contributes to the subunit organization (13). The interaction between the subunits is reinforced and stabilized when binding of STa occurs (26). Thus, the extracellular domain of STaR possesses two functions, namely, oligomer formation and ligand binding. The extracellular domain of NPR-A associates in a ligand-independent fashion and serves to induce a conformational change in the receptor protein by binding with a ligand. The formation of a complex between the extracellular domain and the ligand transduces the extracellular signal into the cytoplasmic domain across the transmembrane region (2). These findings suggest that the extracellular domains of NPR-A and STaR function in similar manners, even though their amino acid sequences are significantly different (4). The identification of the binding region of STaR as well as an understanding of the mechanism of binding of STa to the extracellular domain would be useful in terms of elucidating the mode of signal transduction of STaR as well as defining the interaction regions of the GC family members, including NPR-A, which are also involved in binding to their ligands.

In this paper, we report the site-directed mutational analysis of the extracellular region of pig STaR (pSTaR) in order to define its region of binding to STa. The analysis was done with various pSTaR mutants transiently expressed in 293T cells. In order to examine the binding of pSTaR to STa, a series of mutations was generated at two regions in the extracellular domain of pSTaR, (i) the HC region, which is highly conserved among rats, humans, and pigs, and (ii) the ENTM region, which corresponds to a region in the extracellular domain near the transmembrane region. The latter mutants were generated as a result of a recent finding which showed that Leu-364,

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TABLE 1. Site-directed mutagenesis of pSTaR

Mutant	Substitution[a]	Oligonucleotide[b]	Enzyme site[c]
		* ***	
M1	VS(3,4)AG	TGGCCCTCCTCTGCAGGTCAGAATTGC	*Pst*I
		* **	
M2	NE(36,37)TA	AAGCAGTGGTGACTGCAGGAGTGAACATA	*Pst*I
		** ***	
M3	EGL(78–80)AAV	GTAGCACCTGTGCTGCAGTCGACCTCCTCA	*Pst*I
		** * *	
M4	QMY(107–109)AIS	ATTCCACCTTCGCGATATCCCTTGACACA	*Eco*RV
		***	
M5	DT(111,112)AA	AGATGTACCTTGCTGCAGATTTGAACT	*Pst*I
		** * *	
M6	TD(112,113)GA	TGTACCTTGACGGAGCTCTGAACTATCCC	*Sac*I
		* * *	
M7	ET(132,133)GI	GTGACTACAAAGGAATTCTAACCAGGCTG	*Eco*RI
		* * ***	
M8	RLM(136–138)GLV	AAACTTTAACCGPGACTAGTGTCTCCAGCA	*Spe*I
		* * *	
M9	ET(230,231)AA	GTGTGGTGCACCTGCAGCCGTCCACACCC	*Pst*I
		*	
M10	E(243)A	CGGGCAGTGGCTGCAGACACCGTCATT	*Pst*I
		* ***	
M11	NDF(296–298)TAV	CACTGGTAAAAACTGCAGTCACTCTCGCCT	*Pst*I
		** * *	
M12	LEK(317–319)TAE	TGAAGATATTTACTGCAGAGAGAGAAGAC	*Pst*I
		***	
M13	ED(321,322)AH	TTGAAAAGAGAGCTCACGTTACCACCT	*Sac*I
		* * **	
M14	TTS(324–326)AAA	GAGAAGACGTTGCCGCGGCGAAGTTTGCT	*Sac*II
		** *	
M15	DN(347,348)AS	CTGTGACTTTGGCTAGCTGTGGGGATAT	*Nhe*I
		** ***	
M16	VDT(363–365)AAA	TGTACACCTCTGCTGCAGCCAGCAAATACA	*Pst*I
		* ***	
M17	YDT(374–376)SAA	TCCTTTTGACCTCTGCAGCCCGCAAAAACT	*Pst*I
		* ***	
M18	NDI(399–401)TAV	ACAAACTTCCTACTGCAGTTCCAGGCCGGG	*Pst*I

[a] The first letters represent the original amino acid residues, and the last letters represent the substituted residues; the numbers between them correspond to the positions of the replaced residues.
[b] Asterisks indicate positions of the mutated nucleotides.
[c] Newly generated restriction enzyme site.

located near the transmembrane region of NPR-A, plays a critical role in binding to atrial natriuretic peptide and thus functions in signal transduction of atrial natriuretic peptide (6). Mutant proteins were examined for binding affinity to STa, GC activity, and expression on 293T cell surfaces. The results show that the mutation of Arg-136–Leu-137–Met-138 to Gly-Leu-Val in the HC region (M8 in Table 1) and of Asp-347–Asn-348 to Ala-Ser in the ENTM region (M15 in Table 1) is crucial in both the binding to STa and GC activity.

MATERIALS AND METHODS

Materials. STh(1-19) (the full sequence from position 1 to 19 of a heat-stable enterotoxin [STh] produced by a human strain of enterotoxigenic *E. coli*) and *N*-5-azido-2-nitrobenzoyl (ANB)-STh(5-18) were synthesized as described previously (14). Dulbecco's modified Eagle's medium (DMEM) and 3-isobutyl-1-methylxanthine were obtained from GIBCO and Sigma, respectively. $Na^{125}I$ and EXPRE^{35}S^{35}S[^{35}S] Protein Labeling Mix were purchased from Amersham and Du Pont-New England Nuclear, respectively. Restriction endonucleases and DNA ligase were purchased from Takara Shuzo (Kyoto, Japan) and Toyobo (Osaka, Japan), respectively. Oligonucleotides were synthesized by using an Applied Biosystems DNA synthesizer model 391 PCR-MATE and purified on a C_{18} reversed-phase high-performance liquid chromatography (HPLC) column (Nacalai Tesque, Kyoto, Japan) by HPLC. Monoclonal antibody (MAb) 12CA5 and a polyclonal antibody raised against influenza virus hemagglutinin (HA) peptide HA1(75-110) were purchased from BAbCO.

Mutagenesis. The pCG vector carrying the cytomegalovirus promoter was used for all expression plasmids (8). A 1.7-kb cDNA with a newly generated *Xba*I site at the 5′ end and an *Hin*dIII site at the 3′ end was prepared by using pBS-STaR (27) as a template by PCR and inserted into the pCG vector pCG-pSTaR-1.7. In addition, a cDNA fragment encoding the C-terminal portion of pSTaR with *Hin*dIII sites at both the 5′ and 3′ ends was cut out from the pBS-STaR and inserted into the 3′ site of the pCG-pSTaR-1.7, thus generating an expression plasmid of pSTaR, pCG-pSTaR, which contained the entire reading frame of the gene coding for pSTaR. *E. coli* CJ236 (*dut ung* F′) was transfected with pCG-pSTaR, which carries an f1 origin of replication, and infected by M13KO7 helper phage to generate the single-strand form of the pCG-pSTaR DNA. Site-directed mutagenesis was performed with purified M13 single-stranded DNA as a template and synthetic oligonucleotides as primers as described by Kunkel (15). The primers were selected with the mutation sites as shown in Table 1.

Expression of pSTaR mutants. 293T cells are derived from a human embryonic kidney cell line infected with adenovirus carrying a simian virus 40 large-T-antigen expression plasmid (23). Cells in 60-mm-diameter plates were grown in DMEM supplemented with 10% fetal calf serum. These cells were then transfected with 10 μg of the pCG-pSTaR expression vector or with its mutant genes by the calcium phosphate method (11). For the cGMP assay, cells ($\sim 10^5$) were transferred to 24-well dishes after transfection. Assays were performed 48 h after transfection.

Photoaffinity labeling and binding assay. ANB-STh(5-18) was radioiodinated to a specific activity of approximately 2,000 Ci/mmol with $Na^{125}I$ and chloramine T (12). The radioiodinated compound, ^{125}I-ANB-STh(5-18), was purified by reversed-phase HPLC as described previously (24). The 293T cells ($\sim 10^6$) expressing site-directed mutants of pSTaR were incubated with ^{125}I-ANB-STh(5-18) in the absence or presence of STh(1-19), rinsed twice with cold phosphate-buffered saline (PBS), and subjected to flash photolysis in PBS by UV radiation at 254 nm, as described previously (27). The photoaffinity-labeled and radiolabeled proteins were solubilized in sodium dodecyl sulfate-polyacrylamide gel

electrophoresis (SDS-PAGE) sample buffer containing 20 mM Tris-HCl (pH 7.6), 2% SDS, 4% glycerol, 1.6 mM EDTA, 5.2% dithiothreitol, and 0.05 mg of bromophenol blue per ml, after which the specific activity was measured with a gamma-well counter. These solubilized proteins were subjected to SDS–7.5% PAGE according to the method of Laemmli (16) and analyzed with Fujix Bio-image analyzer BAS 2000 or Kodak X-Omat RP film.

cGMP assay. The assay of cGMP in 293T cells transfected with pCG-pSTaR or its mutant genes was performed at various concentrations of STh(1-19), as described previously (27). Reactions were initiated by the addition of STh(1-19) and terminated by the addition of 10% trichloroacetic acid. The cells were then rapidly frozen at −80°C. After the cells were thawed at room temperature, the cell debris was removed by centrifugation at 15,000 rpm (18,500 × *g*) for 15 min. The resulting supernatants were extracted three times with water-saturated ether (0.5 ml). Aliquots of samples were assayed in duplicate for cGMP content with a radioimmunoassay kit (cGMP assay kit; Yamasa, Tokyo, Japan) according to the manufacturer's specifications.

Preparation of epitope-tagged pSTaR. To prepare epitope-tagged pSTaR, two oligonucleotides (sense, 5′-TATCCTTATGACGTGCCTGACTATGCCTCTG CA-3′; antisense, 5′-GAGGCATAGTCAGGCACGTCATAAGGATATGCA-3′) encoding an epitope (YPYDVPDYA) of influenza virus HA (7) were synthesized and inserted into the *Pst*I site of the M1 mutant (Fig. 1). This construct is hereafter referred to as Ha-pSTaR. The HA epitope inserted into the M1 mutant consists of an 11-amino-acid-residue sequence (YPYDVPDYASA).

Immunoprecipitation. Immunoprecipitation was performed by procedures similar to those described previously (25). Briefly, 5×10^5 cells transfected with the gene encoding Ha-pSTaR or its mutant genes were washed twice with DMEM (1 ml) in the absence of methionine, incubated at 37°C for 4 h in a mixture (1 ml) of methionine-free DMEM and 10% dialyzed fetal calf serum, and then incubated in the same medium (1 ml) which contained 0.25 mCi of L-[^{35}S]methionine per ml (25 μl). Cells were harvested with TNE buffer (1 ml), containing 40 mM Tris-HCl (pH 7.5), 150 mM NaCl, and 1 mM EDTA, and washed twice with PBS. The cells were then solubilized in 0.5 ml of Sol buffer (50 mM Tris-HCl [pH 7.5], 100 mM NaCl, 10% glycerol, 10 mg of leupeptin per ml, and 1% Triton X-100) for 10 min on ice. After centrifugation for 20 min at 100,000 × *g*, the supernatant (200 μl) was incubated with MAb 12CA5 at 4°C overnight and then with 20 ml of protein A-Sepharose 6MB (Pharmacia) at 4°C for 1 h. Immunoprecipitates were washed four times with 0.5 ml of Sol buffer containing 1% sodium deoxycholate and 0.1% SDS, solubilized by heating with 0.2 ml of SDS-PAGE sample buffer, and analyzed by SDS-PAGE on 7.5% gels. The gels were dried, treated with 1 M sodium salicylate, and exposed to Kodak X-Omat RP film.

Cell surface localization of Ha-pSTaR and its mutants. Transfected cells were harvested with TNE buffer (1 ml) and washed twice with PBS. The proteins localized on the cell surface were biotinylated according to the manufacturer's specifications (Protein biotinylation module; Amersham). Biotinylated cells were lysed and immunoprecipitated by a polyclonal antibody raised against influenza virus HA peptide HA1(75-110). The immunoprecipitates were analyzed by Western blotting (immunoblotting) with horseradish peroxidase-conjugated streptavidin (Amersham) followed by ECL detection (Amersham).

RESULTS

Transient expression of recombinant pSTaR on 293T cells. To establish a simple transient-expression system for the gene encoding pSTaR, the pCG-pSTaR expression vector was constructed. The expression of the recombinant pSTaR in 293T cells transfected with the pCG-pSTaR was estimated by SDS-PAGE of the products labeled with ^{125}I-ANB-STh(5-18) followed by autoradiography, as shown in Fig. 2. Distinct protein bands were detected at positions corresponding to 145, 130, and 85 kDa, which represent molecular sizes similar to those described for the human STaR stably expressed in 293 cells (5) and for pSTaR in CHO cells (27). These protein bands were not observed in the presence of nonradiolabeled STh(1-19) or in 293T cells transfected with the pCG vector alone, confirming that they represented gene products of pSTaR. A recombinant human STaR has been expressed in 293 cells as 153- and 133-kDa proteins, which differ in degree of N glycosylation, with degradation products corresponding to 81, 56, and 49 kDa (5, 21). Therefore, we conclude that the 145- and 130-kDa proteins cross-linked with ^{125}I-ANB-STh(5-18) probably correspond to the intact pSTaR and differ in degree of N glycosylation and that the protein observed at 85 kDa corresponds to a degradation product(s) arising from the 145- and/or the 130-kDa proteins. The binding affinity of pSTaR expressed in 293T cells and the GC activity were comparable to those determined previously (5, 20, 27) (data not shown). These results confirm that the expression system, which consisted of the pCG-pSTaR vector and 293T cells, can be used to evaluate the biochemical properties of pSTaR and its mutant proteins.

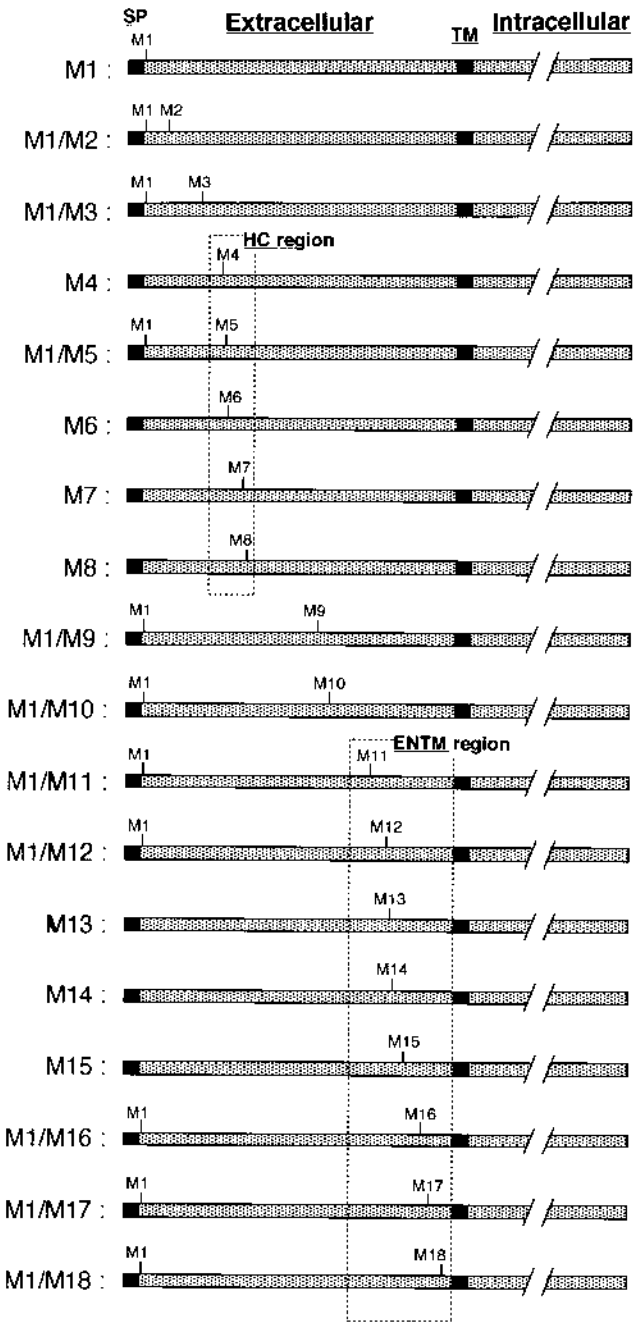

FIG. 1. Schematic representation of mutant pSTaRs (M1 to M18) prepared for this study. Positions of the mutations are shown. Amino acid substitutions in pSTaR mutant proteins are summarized in Table 1. A peptide epitope (HA epitope) was introduced into the M1 site. SP, signal peptide; TM, transmembrane domain; HC region, highly conserved region; ENTM region, extracellular domain near the transmembrane region.

Mutational sites on the extracellular domain of pSTaR. By employing the recombinant pSTaR expression system, the effect of amino acid substitutions on the function of pSTaR was

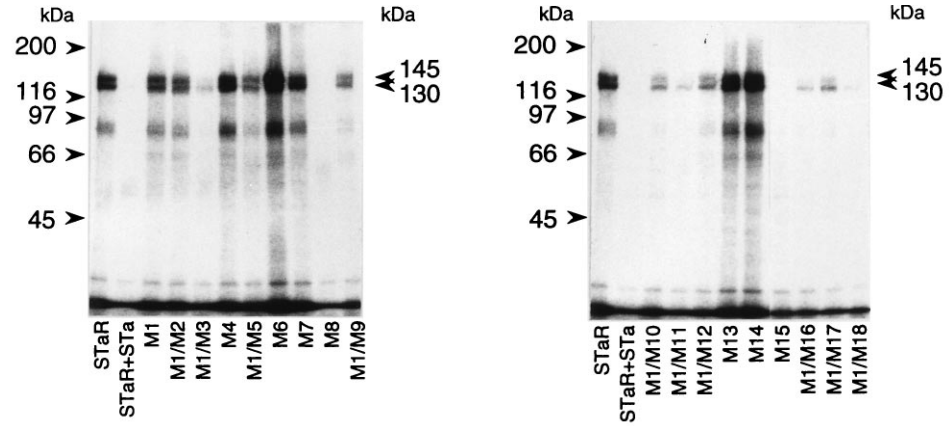

FIG. 2. SDS-PAGE and autoradiographic analysis of pSTaR and its mutant proteins transiently expressed on 293T cells and photoaffinity radiolabeled with ^{125}I-ANB-STh(5-18). Photolysis and SDS-PAGE experiments were performed as described in Materials and Methods. The molecular masses of the photolabeled proteins are indicated on the right. The molecular masses of marker proteins (Bio-Rad) are indicated on the left.

examined along with the ligand-binding affinity and cGMP catalytic activity. Site-directed mutation of the extracellular domain of pSTaR was performed in two regions. The first region was from Arg-91 to Asn-155, which is highly conserved among rat, pig, and human STaRs (HC region; 65 amino acid residues). The sequence from Arg-91 to Asn-155 in pSTaR has 92 and 91% identity, respectively, to those of the human and rat STaRs, while the predicted amino acid sequence of the complete extracellular domain of pSTaR shows 76 and 71% identity, respectively. The second region was from Leu-274 to Leu-409 of the extracellular domain, which is in the direct vicinity of the transmembrane domain (ENTM region; 136 amino acid residues). Figure 1 shows a schematic diagram of the mutation of pSTaR. The amino acid substitutions and their positions in pSTaR mutants are summarized in Table 1. Site-directed mutants for the HC region were M4, M1/M5, M6, M7, and M8, and those for the ENTM region were M1/M11, M1/M12, M13, M14, M15, M1/M16, M1/M17, and M1/M18. Other mutants with mutations in the N-terminal region were M1, M1/M2, and M1/M3, and those with mutations between the HC and ENTM regions were M1/M9 and M1/M10. As for most pSTaR mutants, charged amino acid residues (especially Asp and Glu) were replaced by neutral amino acid residues (Ala or Gly), since charged residues are generally located on the molecular surface of proteins and therefore may interact with ligand.

Analysis of mutants. The affinities of binding of the mutant pSTaR proteins to STa were examined by photoaffinity radiolabeling with ^{125}I-ANB-STh(5-18) and subsequent SDS-PAGE, as shown in Fig. 2. The levels of STa binding of the mutants were compared with those of wild-type pSTaR, as shown in Fig. 3A. Nonspecific binding of STa to 293T cells was estimated by using 293T cells transfected with the pCG vector alone and was not significant. Among five mutants with mutations in the HC region, mutant M8 completely lost its ability to bind to ^{125}I-ANB-STh(5-18), whereas three mutants (M4, M1/M5, and M7) showed binding patterns which were identical to that of wild-type pSTaR. The M6 preparation showed binding to ^{125}I-ANB-STh(5-18) approximately twofold higher than that of the wild-type pSTaR. Whether the mutation in M6 is functionally relevant, however, is not known. Among the mutants with mutations in the ENTM region, one mutant (M15) showed no visible bands, but five mutants (M1/M11, M1/M12, M1/M16, M1/M17, and M1/M18) demonstrated faint bands in the binding reaction with ^{125}I-ANB-STh(5-18) at the same positions on SDS-PAGE as those with wild-type pSTaR. Two mutants (M13 and M14) with mutations in the ENTM region showed a binding pattern comparable to that of wild-type

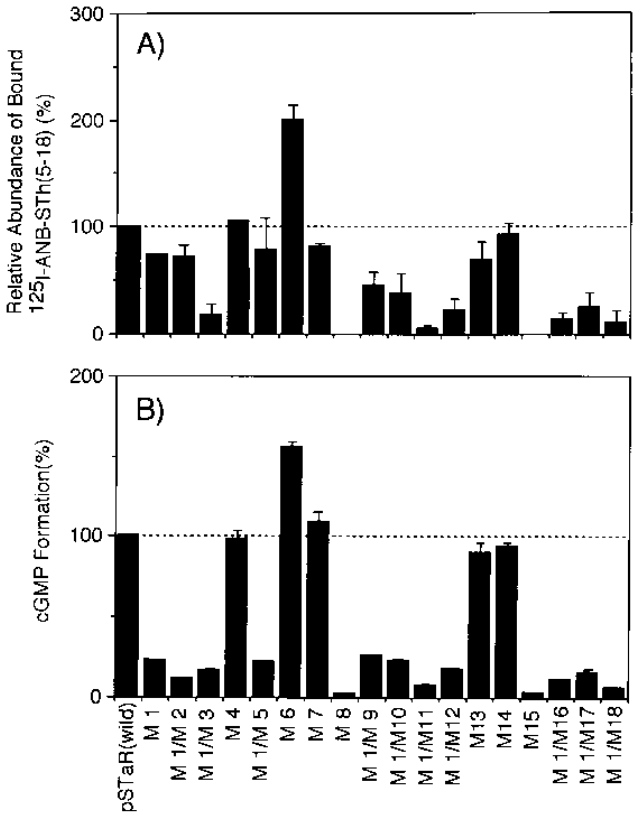

FIG. 3. Binding to STa (A) and STa-dependent accumulation of cGMP (B) of pSTaR and its mutant proteins expressed in 293T cells. The quantity of STa bound to 293T cells ($\sim 10^6$ cells) which express those mutant proteins was measured as described in Materials and Methods and was calculated as a percentage of that for wild-type pSTaR. The values for cGMP formation by the mutant proteins after STa stimulation were calculated as percentages of that for the wild-type pSTaR. Both sets of data are expressed as the means ± standard deviations for at least two separate determinations.

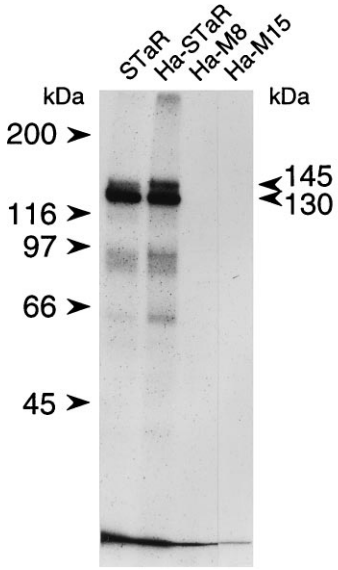

FIG. 4. SDS-PAGE and autoradiographic analysis of Ha-pSTaRs. The analysis was performed under the same conditions as described for Fig. 2.

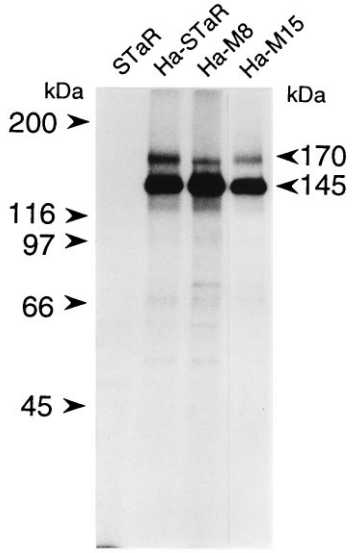

FIG. 5. Immunoprecipitation of Ha-pSTaRs with MAb 12CA5. Mutants of Ha-pSTaR expressed on 293T cells were immunoprecipitated with MAb 12CA5 as described in reference 11 and analyzed by SDS-PAGE as described for Fig. 2.

pSTaR. The other mutations (M1/M16, M1/M17, and M1/M18) in the ENTM region, which are closer to the transmembrane region, cause a significant reduction in STa-binding activity compared with the mutations in other regions. These data suggest that the region (from Asp-347 to Val-401) which was assigned from the M15 mutational site to the M18 mutational site is important for STa binding.

The response of GC activation by pSTaR mutants as a result of exposure to STa was examined by assaying the cGMP content of 293T cells expressing these mutant proteins. The values for cGMP formation by the mutant proteins were compared with that for wild-type pSTaR, as shown in Fig. 3B. The GC catalytic activities of the mutant proteins tested in this study correlated well with their STa-binding activities. In addition, no increase in intracellular cGMP content was observed in the M8 and M15 mutant proteins, which showed no measurable binding to STa.

Characterization of HA epitope-tagged pSTaR. Two pSTaR mutants (M8 and M15) lacked the ability to bind to STa, as described above, raising the question of whether these mutant proteins were truly expressed in their transfected cells. In order to address this issue, protein expression was estimated. A synthetic oligonucleotide encoding an HA epitope (YPYDVPD YASA) was introduced into the *Pst*I restriction enzyme site between Ala-3 and Gly-4 in the M1 site so that the protein would be detectable by immunoreaction with an anti-HA epitope peptide antibody as reported by Field et al. (7).

Figure 4 shows an autoradiogram of SDS-PAGE of HA-tagged proteins (Ha-pSTaR and its mutant proteins) expressed in 293T cells and photoaffinity radiolabeled with ^{125}I-ANB-STh(5-18). The insertion of the HA epitope at the N-terminal region of pSTaR did not interfere with the binding to STa, as judged by the specifically Ha-pSTaR-bound ^{125}I-ANB-STh(5-18). The binding was competitively inhibited by nonradiolabeled STh(1-19) in a manner similar to that for pSTaR. A 9 nM concentration of STh(1-19) was required to achieve half-maximal binding of ^{125}I-ANB-STh(5-18) to Ha-STaR, which was comparable to that observed for pSTaR (data not shown). In addition, there was a significant increase in intracellular cGMP levels in 293T cells which had been transfected with Ha-pSTaR, in an STh(1-19) concentration-dependent manner. However, the intracellular cGMP level was approximately 20% of that of pSTaR with 10^{-5} M STh(1-19), while the 50% effective concentration of STh(1-18) for cGMP formation was approximately 300 nM and nearly the same as that of wild-type pSTaR, as observed for mutant M1.

The HA epitope-tagged M8 and M15 (Ha-M8 and Ha-M15) proteins, did not bind ^{125}I-ANB-STh(5-18) (Fig. 4). In order to confirm the expression of Ha-pSTaR mutants in transfected 293T cells, the Ha-pSTaR mutant proteins were metabolically labeled with [^{35}S]methionine and treated with an MAb (MAb 12CA5) against the HA epitope. As shown in Fig. 5, these Ha-pSTaR mutant proteins were specifically recognized by MAb 12CA5 and immunoprecipitated at 145 kDa (major portion) and 170 kDa (minor portion), whereas the pSTaR gene product from the cells transfected with the pSTaR gene lacking the HA epitope was not immunoprecipitated.

The molecular sizes of the Ha-pSTaR precipitated with MAb 12CA5 were different from those observed in the reaction with ^{125}I-ANB-STh(5-18) (Fig. 4). However, the major band at 145 kDa was detected in both experiments, indicating that the 145-kDa protein was almost surely generated from the gene encoding Ha-pSTaR. Insertion of the HA epitope peptide into the N-terminal region of the extracellular domain of pSTaR could affect the migration of pSTaR on SDS-PAGE. Characterization of the protein having a molecular mass of 170 kDa is currently under way.

Location of Ha-pSTaR mutants on 293T cell surfaces. To examine the localization of Ha-pSTaR mutant proteins in 293T cells, the cell surface proteins were biotinylated and then immunoprecipitated with the polyclonal anti-HA antibody. The protein bands of Ha-pSTaRs were separated on SDS-PAGE and visualized by Western blot analysis using horseradish peroxidase-conjugated streptavidin (Fig. 6). Intense bands were observed for Ha-M8, Ha-M15, and Ha-pSTaR at a position of 145 kDa, whereas no band was detected for pSTaR (Fig. 6). The other protein bands observed in Fig. 6 can be attributed to their nonspecific interaction with protein A-Sepharose which was used for the immunoprecipitation, since these protein bands were detected when protein

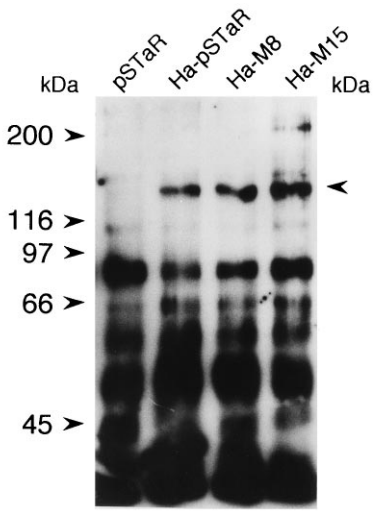

FIG. 6. Western blot of biotinylated cell surface proteins which were immunoprecipitated by polyclonal anti-HA antibody. Ha-pSTaRs expressed on 293T cells were detected by biotinylation of cell surface proteins, immunoprecipitation with polyclonal anti-HA antibody, and Western blotting with horseradish peroxidase-conjugated streptavidin. The arrowhead at the right shows the positions of Ha-pSTaRs on the gels.

A-Sepharose and the cell lysates were mixed in the absence of the polyclonal anti-HA antibody. Thus, Ha-M8 and Ha-M15 displayed cell surface localization at magnitudes similar to that of Ha-pSTaR. These results demonstrate that both Ha-M8 and Ha-M15 lacked STa-binding capabilities in spite of their expression on the surfaces of 293T cells.

DISCUSSION

STaR is a member of a family of single-transmembrane-spanning receptors possessing an extracellular ligand-binding domain and an intracellular GC domain (20). To identify the STa-binding region on the extracellular domain of STaR and to elucidate the association function of the extracellular domain for signal transduction of STa, we prepared a large number of mutants with mutations in the extracellular domain of pSTaR. We introduced a *Pst*I site into the wild-type pSTaR gene in order to construct the mutant STaR genes, resulting in the induction of multiple mutations. This method enabled us to easily construct the mutant pSTaR genes and quickly determine the amino acids in pSTaR important for STa binding.

We found two mutant proteins, M8 and M15, which were completely lacking in STa-binding ability and in the response of intracellular cGMP accumulation to STa stimulation, even though they were expressed and localized on the cell surfaces (Fig. 2, 3, and 6). Both mutant proteins contain two amino acids substituted in the amino acid sequence of pSTaR. The loss of the ability to bind to STa of the M8 mutant, in which Arg-136 and Met-138 were replaced by Gly and Val, respectively, is attributed to the replacement of Arg-136, a basic residue, by Gly, a small neutral residue. Arg-136 is conserved among the amino acid sequences of rat, human, and pig STaRs, while rat STaR contains Leu instead of the Met at position 138 in human and pig STaRs. In addition, the replacement of Met-138 by a similar hydrophobic residue, Val, appears to have no effect on binding. Therefore, one can conclude that Arg-136 is important for STa binding. In M15, which lacked the ability to bind STa, Asp-347 and Asn-348 were replaced, respectively, with Ala and Ser. These residues are in the C-terminal portion of the extracellular domain of pSTaR, which is in close proximity to the transmembrane region. Asp-347 is a conserved amino acid in the amino acid sequences of rat, human, and pig STaRs, while Asn-348 is not. Therefore, we concluded that Asp-347 is important for STa binding.

The secondary structure prediction for the extracellular domain of pSTaR by the method of Chou and Fasman (2a) showed that the extracellular domain consists of two regions, with the first (from Ser-1 to Ala-300) consisting of a combination of α-helixes and β-sheets and the second (from Arg-333 to Ile-401) consisting of β-sheets. The positions of the mutations in M15, which showed a dramatic reduction in both STa binding by pSTaR and related GC catalytic activity, were assigned to the β-turn moiety of the sheet-turn-sheet structure in the β-sheet region (from Arg-333 to Ile-401) predicted above. In general, ligand-binding residues of receptors frequently exist in β-turn structures. These data suggest that the β-sheet region in ENTM creates the STa-binding domain to support the function of Asp-347 for STa binding. On the other hand, the site-directed mutation of NPR-A, the receptor for atrial natriuretic peptide, provided evidence that Leu-364, positioned near the transmembrane region in the extracellular domain of NPR-A, plays a critical role in both the binding with atrial natriuretic peptide and the activation of the intracellular catalytic domain (6). Leu-364 in NPR-A is located in the β-sheet just before the β-turn in a sheet-turn-sheet structure predicted by the Chou-Fasman method. Since Asp-347 and Asn-348 of the M15 site in pSTaR appear to reside at positions similar to that of Leu-364 in NPR-A, the M15 site is assumed to interact with STa in much the same fashion as Leu-364 does in NPR-A. One can therefore speculate that the GC family possesses a common structural motif for ligand binding. Indeed, Asp-347 in pSTaR is a consensus amino acid in the GC family.

The site-directed mutational analysis data with respect to STaR revealed that two residues (Arg-136 and Asp-347) played important roles in STa-pSTaR interaction. However, Arg-136 and Asp-347 are separated from one another by a considerable distance in the primary structure of pSTaR. The results reported here suggest that Arg-136 and Asp-347 should be sufficiently close to one another in the three-dimensional structure to create an STa-binding pocket. Recently, Rudner et al. (19) reported that the cytoplasmic domain of STaR plays an important role in oligomer formation, and they speculated that the weaker interactions between the extracellular domains serve to stabilize the oligomer structure or facilitate the transmembrane signalling of STaR. Since the cytoplasmic domains of pSTaR mutants prepared in this study were identical to that of the wild-type pSTaR, all of the mutants should be capable of oligomer formation. This raises the question of whether both the Arg-136 and Asp-347 residues serve to interact with STa from the same subunit or from different subunits. The stoichiometries of the intermolecular interaction between STa and STaR and the subunit interaction of STaR have not yet been determined in detail. Determination of the roles of these two amino acids in the oligomerization of pSTaR and the interaction with STa is in progress.

Photoaffinity radiolabeling with ^{125}I-ANB-STh(5-18) of recombinant pSTaR expressed in 293T cells revealed two protein bands at 145 and 130 kDa on SDS-PAGE (Figs. 2 and 4). Similar heterogeneities in molecular sizes (160 and 140 kDa) were observed in recombinant human and rat STaRs expressed in stable transformants of 293 cells (5, 25). The difference in molecular sizes between recombinant pSTaR and human or rat STaR proteins in these experiments might be a result of the experimental procedures used for detecting STaR or, alternatively, of differences in carbohydrate modifications at the N-

linked sites in pig, human, and rat STaRs. The photoaffinity radiolabeling method using ^{125}I-ANB-STh(5-18) allowed the detection of 160- and 140-kDa proteins from T84 cells as well as those reported in reference 5 (data not shown). In pSTaR, Thr replaces Asn-52, which is presumed to be an N glycosylation site in human and rat STaRs (4, 20). This would result in the absence of N glycosylation at position 52 in pSTaR (27). The lack of this N glycosylation would be expected to reduce the molecular size of recombinant pSTaR expressed in 293T cells and to possibly affect its mobility on SDS-PAGE. Thus, the 145- and 130-kDa proteins of recombinant pSTaR expressed in 293T cells originated from the inherent gene of pSTaR.

In summary, we have identified two residues (Arg-136 and Asp-347) in the extracellular domain of pSTaR which are involved in binding to STa. In addition, the region from Arg-333 to Ile-401 is required for optimal STa-binding and GC activities. The results of these experiments make it possible to clarify the reaction mechanism of STaR: binding to STa and formation (or stabilization) of an oligomer of the extracellular domain, by which STaR signals the binding of STa to the intracellular catalytic domain through the single-path transmembrane region. Detailed analysis of these regions will permit a better understanding of the relationships of ligand binding and oligomer formation followed by GC activity.

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