INFECTION AND IMMUNITY, June 1990, p. 1817–1820
0019-9567/90/061817-04$02.00/0

Vol. 58, No. 6

Heterogeneity of Intestinal Receptors for *Escherichia coli* Heat-Stable Enterotoxin

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The structure of rat intestinal cell receptors for *Escherichia coli* heat-stable enterotoxin (ST) was investigated by affinity cross-linking to ^{125}I-ST and analysis by denaturing gel electrophoresis. Cross-linking of labeled toxin to intestinal membranes and analysis by nonreducing sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) revealed five specifically labeled proteins with molecular masses of 160, 136, 78, 71, and 56 (kilodaltons) kDa. Exhaustive reduction of these samples resulted in a similar pattern of labeling. Affinity-labeled proteins were further analyzed by nonreducing SDS-PAGE, reduction of the resulting separated proteins, and further separation by SDS-PAGE in the presence of β-mercaptoethanol. Thus, the 160-kDa band on nonreducing gels consisted of two different receptors: a 160-kDa polypeptide not further reducible and one composed of at least two subunits, one of which was the 78-kDa subunit. Similarly, the 136-kDa band on nonreducing gels consisted of a 136-kDa polypeptide not further reducible and one composed of at least two subunits, one of which was the 71-kDa subunit. The 78-, 71-, and 56-kDa subunits were not further reducible. These data suggest heterogeneity of the ST receptor subunit structure and organization in rat intestinal epithelia.

Escherichia coli heat-stable enterotoxin (ST) produces various diarrheal diseases, including traveler's diarrhea and epidemic diarrhea in newborns (13, 16). ST binds to specific receptors on intestinal epithelial cell surfaces, activating particulate guanylate cyclase and elevating intracellular concentrations of cyclic GMP, which alters fluid and electrolyte transport, producing secretory diarrhea (4, 9, 10, 17). The ST receptor is central to this cascade, yet little is known concerning its structure and function. The native ST receptor migrates as a single species with a molecular mass of 160 kilodaltons (kDa), as determined by classical separation techniques (11). Studies of affinity cross-linking of labeled ST to the receptor demonstrated three labeled proteins with estimated molecular masses of 80, 68, and 60 kDa on sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) in the presence of reducing agents (6, 11). However, the relationship of these labeled proteins to the native ST receptor remains unknown. This report demonstrates multiple proteins in crude intestinal membranes which specifically bind ST and suggest heterogeneity of the receptor subunit structure and organization. Putative ST receptor species composed of single polypeptide chains with molecular masses of 160 and 136 kDa are described. Additionally, putative receptors composed of two or more subunits and demonstrating a precursor-product relationship between the 160- and 78-kDa and 136- and 71-kDa subunits are described. Possible relationships of the various ligand-binding proteins to native receptors are discussed.

MATERIALS AND METHODS

Membrane preparation. Intestinal mucosa from Sprague-Dawley rats was homogenized in buffer containing 50 mM Tris hydrochloride (pH 7.6), 1 mM EDTA, 1 mM dithiothreitol, 250 mM sucrose, and 0.1 mM phenylmethylsulfonyl fluoride. All procedures were performed at 4°C. Homogenates were centrifuged at 105,000 × *g* for 60 min, supernatants were discarded, and pellets were washed three times with ice-cold 20 mM phosphate-buffered saline (pH 7.0) containing 0.1% (wt/vol) bacitracin (buffer A). The final pellet was resuspended in buffer A, its protein content was quantified (14), and it was diluted with buffer A to 2 mg of protein per ml. Aliquots were stored at −70°C until used, and no sample was frozen and thawed more than one time.

Receptor-ligand cross-linking. Intestinal membranes (40 μg of protein) were incubated with 10^6 cpm of ^{125}I-ST (40 nM) in phosphate-buffered saline for 15 min at 37°C. Some incubations contained excess (500 nM) unlabeled ST. Ligand-receptor complexes were cross-linked by adding disuccinimidyl suberate dissolved in dimethyl sulfoxide to a final concentration of 1 mM and incubating the mixture for 15 min at 22°C. Cross-linking was terminated by heating each sample for 5 min at 95°C in an equal volume of a buffer containing 0.125 M Tris hydrochloride (pH 6.8), 20% glycerol, 6% SDS, and 0.004% bromophenol blue (SDS sample buffer) in the presence or absence of 10% β-mercaptoethanol.

SDS-PAGE and autoradiography. Cross-linked samples were analyzed by SDS-PAGE with 6% acrylamide as described previously (11). β-Mercaptoethanol (0.8 M) was incorporated into the sample buffer and polyacrylamide gel system when needed. Higher concentrations of reducing agent did not alter the recovery of radioactivity or the pattern of incorporation into proteins. Molecular mass standards were myosin (200 kDa), β-galactosidase from *E. coli* (116 kDa), phosphorylase *b* (97 kDa), bovine serum albumin (66 kDa), and ovalbumin (43 kDa). Gels were stained and dried, and autoradiograms were prepared as described previously (11). In some experiments, cross-linked samples were subjected to SDS-PAGE in the absence of β-mercaptoethanol as described above, wet gels were recovered, and autoradiograms were prepared at −20°C. Labeled proteins

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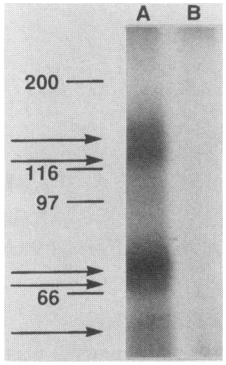

FIG. 1. Autoradiography of affinity-labeled ST receptor on SDS-PAGE. Cross-linking of labeled ST to membranes, electrophoresis on 6% polyacrylamide gels in the absence of reducing agents, and autoradiography were performed as described in Materials and Methods. Lane A shows labeling in the absence of excess unlabeled ST. Lane B shows nonspecific labeling in the presence of 0.5 μM unlabeled ST. Molecular mass markers (kilodaltons) included myosin (200), β-galactosidase (116), phosphorylase *b* (97), bovine serum albumin (66), and ovalbumin (43). Arrows indicate the positions of affinity cross-linked receptor subunits.

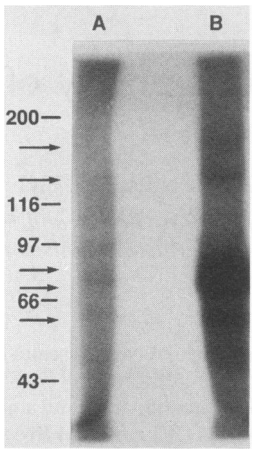

FIG. 2. Autoradiography of affinity-labeled, reduced ST receptor on SDS-PAGE. Cross-linking, electrophoresis, and autoradiography were performed as outlined in the legend to Fig. 1, except that the sample was prepared and electrophoresis was performed in the presence of 0.8 M β-mercaptoethanol. Similar amounts of labeled membranes were placed in the wells in these experiments and those depicted in Fig. 1. However, autoradiograms were exposed twice as long in this experiment. Lane A shows nonspecific labeling in the presence of 0.5 μM unlabeled ST. Lane B shows labeling in the absence of cold ST. Standards and arrows are the same as those in Fig. 1.

visualized by autoradiography were excised from these gels and homogenized in SDS sample buffer containing 0.8 M β-mercaptoethanol, and supernatants were separated from homogenized gels by centrifugation. Higher concentrations of reducing agent did not alter the recovery of radioactivity or the pattern of incorporation into proteins. Supernatants were removed, and the residual gel was subjected to two further extractions as described above. With this technique, at least 80% of the protein-bound radioactivity was recovered from the gel slices. Gel extracts were heated at 95°C for 10 min and subjected to SDS-PAGE on linear gradient gels of 5 to 12% polyacrylamide. Longer incubations did not alter the pattern of incorporation of label into protein. These gels were stained, dried, and exposed for autoradiography as described above.

Miscellaneous methods. ST receptor binding was quantified as described previously (11). ST was iodinated to a specific activity of 300 Ci/mmol by the lactoperoxidase method (Enzymobeads) as described previously (11).

Materials. ST was purchased from Penninsula Labs, Belmont, Calif.; Enzymobeads and Biogel P2 were purchased from Bio-Rad Laboratories, Richmond, Calif.; $Na^{125}I$ was purchased from Amersham Corp., Arlington Heights, Ill.; β-D-glucose and bacitracin were purchased from Sigma Chemical Co., St. Louis, Mo.; and discuccinimidyl suberate was purchased from Pierce Chemical Co., Rockford, Ill. All other reagents were of analytical grade and were obtained as previously described (11).

RESULTS

Binding of iodinated ST to receptors localized on intact intestinal membranes was concentration dependent and saturable (Fig. 1), as described previously (2, 3, 5, 7, 8). Analysis of these data by the method of Scatchard revealed a single class of binding sites with a K_d of 5×10^{-9} M. Labeled ST could be displaced from receptors in a quantitative fashion by unlabeled ST. Indeed, analysis of these data by the method of Cheng and Prussoff (1) revealed a K_i of 4×10^{-9} M. Thus, close agreement between the K_d and K_i demonstrated that iodination does not alter the affinity for the receptor or the binding characteristics of the toxin.

When receptors on intact intestinal membranes were cross-linked to ^{125}I-ST and subjected to SDS-PAGE under nonreducing conditions, proteins with molecular masses (average mass ± standard deviation of five experiments) of 155 ± 4, 136 ± 4, 78 ± 5, 71 ± 5, and 56 ± 1 kDA were visualized by autoradiography (Fig. 1, lane A). To establish the specificity of protein labeling, we cross-linked ^{125}I-ST to intestinal membranes in the presence of excess (0.5 μM) unlabeled ST. Unlabeled ST completely displaced ^{125}I-ST from all five proteins, since labeled bands were not detected in these experiments (Fig. 1, lane B).

^{125}I-ST was cross-linked to intestinal membranes as described above, and samples were reduced with β-mercaptoethanol (0.8 M) and analyzed by SDS-PAGE. Under reducing conditions, the same five proteins with molecular masses of 165 ± 3, 136 ± 2, 78 ± 3, 71 ± 2, and 59 ± 1 kDa were labeled (Fig. 2, lane B). Again, labeling was specific, since radioactivity was completely displaced from these proteins by unlabeled ST (Fig. 2, lane A). Although this pattern was qualitatively similar to that observed under nonreducing conditions, there was a quantitative difference in the distribution of radioactivity among the labeled proteins in reduced samples (Fig. 3). Indeed, the relative intensities of the labeled bands were dramatically different, with less radioactivity in the 160- and 136 kDa proteins and more in the 78- and 71-kDa proteins.

To elucidate the relationship between these proteins, we cross-linked intestinal membranes to ^{125}I-ST and subjected them to SDS-PAGE under nonreducing conditions. Labeled proteins were localized on wet, unstained gels by autoradiography, excised, exhaustively reduced in SDS sample buffer containing β-mercaptoethanol, and again subjected to SDS-PAGE (Fig. 3). Autoradiography revealed that the 165-kDA band in nonreducing gels was composed of two different ligand-binding proteins: a single polypeptide of 165 kDa and a multisubunit protein, one subunit of which was 78 kDa (Fig. 3, lane A). Similarly, the 136-kDA band in nonre-

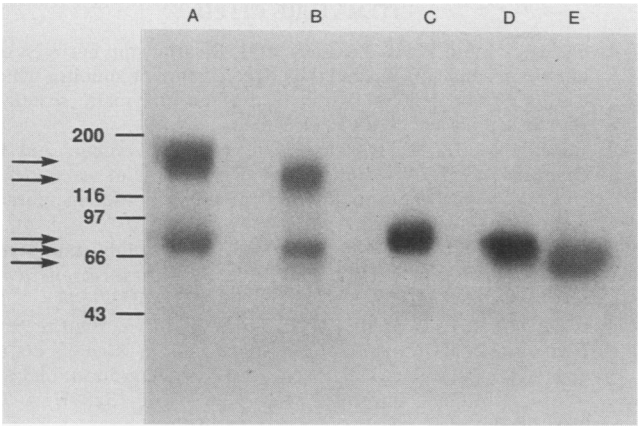

FIG. 3. Autoradiography of affinity-labeled ST-binding subunits separated by nondenaturing SDS-PAGE followed by analysis of individual bands by SDS-PAGE in the presence of β-mercaptoethanol. Cross-linking of labeled ST was performed as described in the legend to Fig. 1. Samples were analyzed by SDS-PAGE in the absence of reducing agents. The resulting wet gel was exposed for autoradiography. Labeled proteins identified in this fashion were sliced from the nonreducing gel, extracted with SDS sample buffer containing 0.8 M β-mercaptoethanol, and subjected to SDS-PAGE on a 5 to 12% gradient polyacrylamide gel as described in Materials and Methods. Binding proteins from the first nonreducing gel placed in each lane of the polyacrylamide gel were as follows: lane A, 164 kDa; lane B, 136 kDa; lane C, 80 kDa; lane D, 71 kDa; lane E, 59 kDa. Arrows represent the positions of the 164-, 136-, 80-, 71-, and 59-kDa subunits, from top to bottom, respectively.

ducing gels was composed of two different receptors: a single polypeptide of 136 kDa and a multisubunit protein, one subunit of which was 71 kDa (Fig. 3, lane B). The 78-, 71-, and 56-kDa proteins were not altered by reduction with β-mercaptoethanol (Fig. 3, lanes C, D, and E).

DISCUSSION

The present study is the first report of multiple populations of receptors for ST which differ in subunit structure. Included are receptors composed of a single polypeptide chain of about 160 or 136 kDa. These forms were resistant to reduction in SDS sample buffer with β-mercaptoethanol (Fig. 2 and Fig. 3, lanes A and B). Also receptors with a native molecular mass of about 160 or 136 kDa but composed of multiple subunits, one of which was 78 or 71 kDa, respectively, were observed. These multisubunit receptors could be further classified on the basis of the forces stabilizing their quaternary structure. For example, there were 160- and 136-kDa receptors which were reduced in SDS sample buffer to their component 78- and 71-kDa subunits, respectively (Fig. 2, lane A). It is likely that the native structure of these receptors is stabilized by noncovalent forces such as hydrophobic and hydrophilic interactions, since their subunits were easily dissociated by SDS (Fig. 2, lane A). In contrast, there were 160- and 136-kDa receptors which were dissociated into those subunits only upon reduction with β-mercaptoethanol (Fig. 2 and Fig. 3, lanes A and B). The native structure of these receptors appears to be stabilized by intrachain disulfide bridges, which require reduction to separate the respective subunits.

Thus, six populations of ST receptor differing in subunit composition or the forces stabilizing the subunits have been demonstrated. A previous study demonstrated that the native ST receptor migrates as a single molecular species of about 160 kDa when subjected to classical separation techniques such as gel filtration chromatography or sucrose density gradient centrifugation (11). These data define a single form of the native ST holoreceptor in rat intestinal membranes. In light of the present observations, these data suggest that the single species of the native ST receptor may have a polymorphic subunit structure. Furthermore, the separation techniques used in the previous study may not have been sufficiently sensitive to discriminate between species differing by 25 kDa (11). Consequently, that study could not have demonstrated separate populations of receptors with molecular masses of 136 and 160 kDa. Thus, although previous studies have demonstrated a single native form of the ST receptor by less sensitive separation techniques, there may be more than one native population of this receptor with heterogeneous subunit compositions.

As discussed above, binding subunits with molecular masses of 78, 71, and 56 kDa were observed in the absence of reducing agents (Fig. 2). However, when crude detergent extracts of intestinal mucosa membranes were subjected to sucrose density gradient centrifugation or gel filtration chromatography, ST receptor-binding activity migrated as a single symmetrical peak of 160 kDa (11). There were no shoulders or secondary peaks of activity noted at lower molecular masses in that study (11). These data suggest that labeled proteins of 78, 71, and 56 kDa do not represent populations of low-molecular-mass holoreceptors. Rather, the 78- and 71-kDa proteins appear to be subunits of larger holoreceptors with molecular masses of 160 and 136 kDa, respectively (Fig. 3, lanes A and B). The origin of the 56-kDa labeled protein and its relationship to the native ST receptor remain unclear. Alternatively, there may be native holoreceptors for ST which have these low molecular masses but which represent only a small proportion of the total receptor present in the cell membrane. In this case, a minor fraction of low-molecular-mass ST receptors might have yielded a peak of binding activity which could not be resolved by the separation techniques previously used (11).

Scatchard analysis of ST binding demonstrates a single population of ligand-binding sites which do not interact in a cooperative fashion (2, 3, 5, 7, 8, 11). However, multiple populations of ST receptors which differ in their subunit organization were demonstrated in the present study. These data suggest that different populations of ST receptors share similar ligand-binding affinities, although they differ significantly in their peptide-binding subunits. Alternatively, there may be differences in the binding affinities of the various receptor populations which are not apparent by classical Scatchard analysis because of the relative abundance or paucity of each type of receptor. Indeed, a similar phenomenon has been observed with the receptor for atrial natriuretic peptide. This ligand has at least three different receptors which are distinguishable on the basis of their molecular masses under reducing and nonreducing conditions (17, 18). All share a similar affinity for the native peptide (12, 17, 18). However, the different receptors can easily be distinguished when a truncated 21-amino-acid analog is used for binding (12, 17, 18). Although the various forms of ST receptor cannot be distinguished with respect to ligand binding of native toxin, these receptors may be distinguishable with the appropriate toxin analog. Studies in this laboratory are focusing on the development of ST analogs which demonstrate differential receptor affinities.

There are a variety of explanations for the existence of a single population of native ST receptors with apparent

heterogeneity of subunit structure. For example, the various populations of receptors may represent different gene products with a convergent function. This seems unlikely in light of the similarity of the receptor subunits (e.g., the 160-kDa receptor with one or more subunits) and of their ligand-binding affinities. Alternatively, these receptors may be the product of a single gene which undergoes divergent posttranscriptional or posttranslational modifications resulting in heterogeneity of subunit organization. Also, the receptors may have identical subunit structures but differ in their glycosylation. Indeed, glycoprotein migration on SDS-PAGE varies as a function of size and degree of glycosylation (15). Another possibility is that the various populations of receptors result from differential proteolysis during membrane preparation. Indeed, it may be that the 136-kDa receptor is derived from the 160-kDa receptor while the 71-kDa subunit is derived from the 78-kDa subunit by limited proteolysis. Furthermore, the 56-kDa subunit may be related to a larger subunit by proteolysis. However, in our preliminary experiments, when membranes were heated at 37°C for increasing amounts of time, there were no alterations in the relative proportions of labeled proteins when these preparations were cross-linked to labeled ST and subjected to SDS-PAGE. Similarly, repeated freezing-thawing did not alter the relative proportions of subunits in these experiments. Therefore, the role of proteolysis in the generation of various ST-binding subunits is unclear. These relationships will be clarified once the native ST receptor has been purified and the gene(s) encoding its structure has been analyzed.

The structure of the ST receptor has been previously analyzed by cross-linking to labeled toxin (6, 11). However, in those studies, only subunits with molecular masses of 78, 71, and 56 kDa were observed (6, 11). It is not clear why those studies did not visualize the higher-molecular-mass subunits. In one study in which a photoaffinity probe for ST was used to label the receptor, a 15% SDS gel was used to analyze labeled proteins (6). Thus, higher-molecular-mass subunits may not have been visualized because of poor penetration into the separating polyacrylamide gel (6). Indeed, the gel used in that study demonstrated a high-molecular-mass band near the top of the gel which may have been the 136- and/or 160-kDa proteins (6). Similarly, the other study done with affinity cross-linking for ST receptor subunit analysis used a 5 to 15% gradient polyacrylamide gel for separating subunits; this gel may not have permitted adequate separation of the higher-molecular-mass receptor species (11). Also, that study used a different protocol for preparing intestinal membranes: hypotonic $MgCl_2$ precipitation was used to purify brush border membranes (11). It may be that differences in membrane preparation or in brush border versus crude intestinal membranes could explain differences in receptor subunit composition in that study as compared with the present study (11).

In summary, the ST receptor demonstrates considerable heterogeneity of subunit structure and organization. Receptors composed of single polypeptide chains with molecular masses of 160 and 136 kDa were observed. Also, receptors with molecular masses of 136 and 160 kDa and composed of subunits with molecular masses of 71 and 78 kDa, respectively, were observed. Some of these multisubunit receptors were stabilized by noncovalent interactions such as ionic and hydrophobic forces. In addition, some were stabilized by intrachain disulfide bridges. The relationship of these different receptor populations to each other or to a common precursor awaits purification of the receptor to homogeneity.

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