Biogenesis, Cellular Localization, and Functional Activation of the Heat-Stable Enterotoxin Receptor (Guanylyl Cyclase C)†

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Received April 1, 1996; Revised Manuscript Received June 4, 1996⊗

ABSTRACT: Enterotoxigenic *Escherichia coli* elaborate a peptide called heat-stable enterotoxin (ST),[1] which binds to and activates the intestinal ST receptor (STaR). STaR, also known as guanylyl cyclase C (GC-C), is a member of the transmembrane guanylyl cyclase receptor family. The mRNA for STaR encodes an ∼120 kDa protein with the *N*-terminal ligand binding domain on the cell surface. Ligand affinity cross-linking studies have previously demonstrated several species of potential ST binding proteins, ranging in size from ∼50 to 160 kDa. Although these smaller forms of STaR (50−80 kDa) have been proposed to act *in vivo* as toxin binding proteins, their biogenesis and localization have not previously been examined. Using pulse labeling *in vivo* and synchronized translation *in vitro*, we demonstrate that these smaller forms represent incomplete translational products and are not formed through limited proteolysis of the full-length receptor, as had previously been believed. We determined, using fluorescence confocal microscopy and surface labeling, that only ∼25% of cellular receptors are expressed at the surface, while the remaining population is retained within the endoplasmic reticulum. Only full-length receptor is found at the surface of the cell, indicating this to be the biologically active form of STaR responsible for interacting with the heat-stable enterotoxin and other luminal intestinal peptides. The large intracellular receptor population, and potential for function before translocation to the cell surface, may impact on how pharmacologic modulators of this clinically important receptor are designed.

Infection with enterotoxigenic *Escherichia coli* is a leading cause of diarrheal disease in travelers and in infants in the Third World (Dupont & Ericsson, 1993). Most of these enterotoxigenic strains elaborate a peptide called heat-stable enterotoxin (ST), which binds to and activates the cell surface transmembrane ST receptor (STaR).[1] The toxin−receptor interaction produces increases in intracellular cGMP, which then stimulates the cystic fibrosis transregulator chloride channel, causing secretory diarrhea (Chao et al., 1994; Tien et al., 1994). STaR, also known as guanylyl cyclase C (GC-C), is a member of the transmembrane guanylyl cyclase receptor family (de Sauvage et al., 1991; Drewett & Garbers, 1994; Schulz et al., 1990). Other members of this diverse receptor family include the atrial natriuretic peptide receptors (GC-A and GC-B) and the retinal and ocular guanylyl cyclases (GC-D and GC-E) (Shyjan et al., 1992; Yang et al., 1995).

Most transmembrane receptors are synthesized as single polypeptide chains that are targeted to the cell surface, where their extracellular domains interact with the extracellular environment, and are expressed *in vivo* as a single protein product. The mRNA for STaR is predicted to encode a protein of ∼120 kDa with the *N*-terminal ligand binding domain on the cell surface (Schulz et al., 1990). Northern analysis of STaR demonstrates a single 3.7 kb mRNA species, without evidence for alternative splicing (Schulz et al., 1990, 1992). Surprisingly, ligand affinity cross-linking studies of STaR have demonstrated several species of ST binding proteins, ranging in size from ∼50 to 160 kDa (Cohen et al., 1993; Hakki et al., 1993; Hirayama et al., 1992; Ivens et al., 1990; Katwa et al., 1991; Vaandrager et al., 1993, 1994). Although these smaller forms have been hypothesized to act *in vivo* as toxin binding proteins, their biogenesis and potential biological role remain undefined. To better understand how the translational and posttranslational processing of STaR might affect its biological function, we examined its biogenesis and cellular compartmentalization using complementary *in vitro* and *in vivo* approaches. These findings may impact on how we design and deliver pharmacologic modulators of this clinically important receptor.

EXPERIMENTAL PROCEDURES

Plasmid Constructions. All receptor-derived constructs were derived from human STaR cDNA, which was generously provided by Dr. Fred de Sauvage (Genentech, South San Francisco). Epitope tags consisted of either the influenza hemagglutinin (HA; YPYDVPDYA) or the FLAG epitope (DYKDDDDK) (Koldodziej & Young, 1991). Epitope-tagged constructs were prepared using overlap extension PCR and standard cloning techniques. Sequences were confirmed by dideoxy sequencing. N-Terminal epitope tags were placed immediately C-terminal to the putative signal se-

† This work was supported by the Proctor & Gamble Young Investigator Award of the Infectious Diseases Society of America and a Young Investigator Award of the National Foundation for Infectious Diseases. J.S.A. has been awarded Grant 1R29 DK/GM51066-01 from the NIH.

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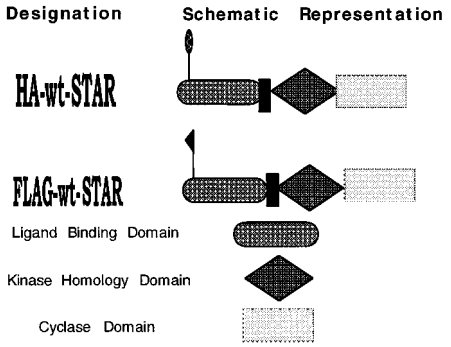
⊗ Abstract published in *Advance ACS Abstracts,* August 1, 1996.

[1] Abbreviations: ST, heat-stable enterotoxin *a*; STaR, ST receptor; GC-C, guanylyl cyclase C; Mab, monoclonal antibody; endo-H, endoglycosidase H; ER, endoplasmic reticulum.

S0006-2960(96)00772-6 CCC: $12.00

Table 1: Receptor Constructs



quence. A schematic view of these constructs is shown in Table 1.

Metabolic Labeling, Immunoprecipitation, and Endo-H Digestion. COS-7 cells grown in six-well plates, 48 h after transfection with HA-tagged STaR as described previously (Rudner et al., 1995), were washed once with PBS, and starved for 2 h at 37 °C in 0.5 mL of DMEM without cysteine and methionine (Cys^-, Met^-, DMEM). Cells were metabolically labeled for 5, 15, or 30 min in the same medium containing 500 μCi/0.5 mL Expres (Dupont) [^{35}S]-protein labeling mix. Cells were rinsed once with PBS and chased in DMEM with 25 mM methionine. After designated chase times, cells were washed twice with ice-cold PBS, solubilized in RIPA buffer (250 mM NaCl, 1% NP-40, 0.5% deoxycholic acid, 0.1% SDS, 5 mM EDTA, 50 mM Tris-HCl, pH 8.0) supplemented with 35 μg/mL PMSF and 20 μg/mL aprotinin. Lysates were incubated with 40 μL of 50% protein A−Sepharose CL-4B (Pharmacia) for 1 h at 4 °C (preclearing step). Protein A−Sepharose was removed by centrifugation at 14 000 rpm for 1 min in an Eppendorf microfuge at 4 °C. The supernatant was removed and incubated with 2 μg of anti-HA (Boehringer) monoclonal antibody (Mab) for 1 h at 4 °C. Fifty microliters of 50% protein A−Sepharose CL-4B was added to the supernatant antibody mixture and incubation continued for 1 h at 4 °C. The immune complexes were recovered by centrifugation at 14 000 rpm for 30 s at 4 °C. The immune complexes were washed 2 times with 1 mL of RIPA buffer supplemented with 200 mM ammonium sulfate and once with RIPA buffer. The immune complexes were disassociated from the protein A−Sepharose by heating for 5 min at 75 °C in 30 μL of 2× SDS sample buffer with DTT. The released protein was analyzed by electrophoresis on a 7.5% SDS gel. The gel was fixed in 25% 2-propanol and 10% acetic acid for 30 min and enhanced by soaking in Amplify (Amersham, Arlington Heights, IL) for 15 min. After fixation, the gel was dried at 80 °C for 1 h and exposed overnight to BioMax MR film (Kodak). The amount of receptor precipitated was always linear with respect to the amount of receptor protein present in the preparation.

For endo-H digestion after immunoprecipitation, antigen−antibody complexes were eluted from protein A−Sepharose by incubation at 75 °C for 5 min in 20 μL of 2× SDS sample without DTT, followed by centrifugation at 14 000 rpm at 4 °C for 30 s to pellet protein A−Sepharose. The supernates were incubated with 1 μL (500 units) of endo-H (New England Biolab) for 1 h at 37 °C. Twenty microliters of 2× sample buffer containing 400 mM DTT was added to each sample, and samples were heated to 75 °C for 5 min and processed by SDS−PAGE as above.

In Vitro Transcription and Translation. The 3.4 kb cDNA for the human ST receptor (STaR) was subcloned from Bluescript SK^- (Stratagene) into the *Xho*I and *Pst*I sites of Pcite-2a, which contains a T7 promoter and a segment of the encephalomyocarditis virus (EMCV) RNA 5′ noncoding region (Novagen Corp., Madison, WI). The plasmid DNAs were transcribed with T7 RNA polymerase followed by translation in a rabbit reticulocyte lysate using Single Tube Protein System 2 (Novagen). Ten microliters of RNA transcripts from 0.5 μg of plasmid was translated in 50 μL reactions containing 1 μL of canine pancreatic microsomal membrane (Promega Corp., Madison, WI) and 40 μCi of [^{35}S]methionine. Translation reaction mixtures were incubated at 30 °C for 60 min. To perform a synchronized translation, translation reactions of desired volume were prepared as described above. The reaction was prewarmed at 30 °C for 2 min before the addition of the transcription mixture at time 0 min. To the synchronized translation, the initiation inhibitor 7-methylguanosine 5′-monophosphate (4 mM final concentration) was added at time 2 min. Twenty-five microliters of the reaction was taken at the times indicated in the figures and incubated with 0.5 μL of 10 mg/mL RNase A at room temperature for 5 min.

Translation products were diluted 20-fold with RIPA buffer for immunoprecipitation. For HA-tagged protein samples, 10 μg of anti-HA Mab was used to immunoprecipitate samples as described above. Proteins were resolved by SDS−PAGE as described above and visualized after overnight exposure to Biomax MR Kodak film.

Production of Stable Cell Lines. At 50−70% confluence, 293 cells were cotransfected with 20 μg of PME18S-STaR and 5 μg of PSV-Neo (a plasmid containing neomycin resistance) in the presence of calcium phosphate and chloroquine (Sambrook et al., 1989). Neomycin-resistant colonies were transferred to 24-well plates and propagated for cGMP assay and Western blot. Clones with cGMP production in response to ST and high-level expression of STaR were chosen for subsequent studies.

Surface Labeling. To label STaR located on the cell surface, 293 cells stably transfected with FLAG-STaR were grown in 10-cm plates 48−72 h prior to use. Cells were gently removed from the plate by spraying PBS through a pipet and resuspended in 4.5 mL of PBS. Anti-FLAG Mab was added to each cell suspension to a final concentraction of 20 μg/mL, and then cells were incubated at 4 °C for 1 h on a rotater. Cells were washed with PBS twice and lysed in 4.5 mL of RIPA buffer at 4 °C for 10 min. After removal of cell debris by centrifugation, the cell lysate was incubated with 80 μL of 50% protein A−Sepharose for 1 h at 4 °C. The immune complex was collected, and the proteins were released as described above. To label total cellular STaR, cells were lysed prior to the incubation with anti-FLAG Mab, and regular immunoprecipitation was performed as above.

Immunostaining of Cultured Cells. 293 cells were grown on poly(L-lysine) (Sigma) coated cover slips. Cells were washed with PBS and fixed in PBS containing 4% paraformaldehyde. After fixations, cells were washed with PBS; cells were permeabilized and stained with both primary and secondary antibodies in a buffer containing PBS, 0.1% Triton X-100, and 4% fetal calf serum.

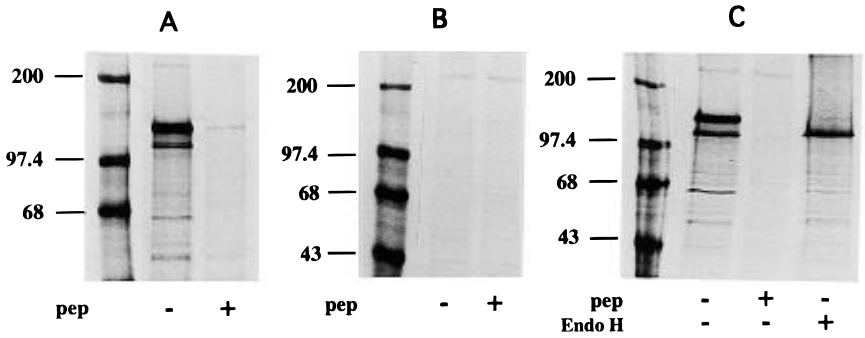


FIGURE 1: *In vivo* metabolic labeling of STaR. The left lanes (A−C) show molecular weight standards. Samples labeled pep− were immunoprecipitated with the anti-HA Mab in the absence of HA peptide. As negative controls, samples labeled were immunoprecipitated with anti-HA Mab after preincubation with an ∼50-fold excess of the HA peptide (pep+). Bands present only in the pep+ lanes are nonspecific.

RESULTS

Biogenesis of STaR in Vivo. Affinity cross-linking studies using [^{125}I]STa have revealed several species of ST binding proteins, both in crude intestinal membranes and in cultured cells expressing recombinant STaR (Cohen et al., 1993; Hakki et al., 1993; Hirayama et al., 1992; Ivens et al., 1990, Katwa et al., 1991; Vaandrager et al., 1993, 1994). These findings have led to the hypothesis that STaR undergoes limited proteolysis *in vivo* and that proteolytically generated forms might play a biologically significant role (Deshmane et al., 1995; Hirayama et al., 1992; Ivens et al., 1990; Vaandrager et al., 1994). To further examine this hypothesis, we performed pulse−chase studies in COS cells expressing HA-STaR, to establish the metabolic fate of newly synthesized receptors. After being labeled with [^{35}S]methionine and [^{35}S]cysteine, cells were washed with cold PBS, lysed in RIPA buffer; HA-STaR was then immunoprecipitated. Proteins were resolved by SDS−PAGE and analyzed by fluorography. Figure 1A depicts a 30 min pulse, revealing four major protein products: 140, 120, 66, and 50 kDa (seen in pep$^-$ lanes). Figure 1B shows the immunoprecipitation of metabolically labeled mock-transfected COS cells (negative control), demonstrating the absence of these proteins. Similar results were observed for cells expressing FLAG-STaR after immunoprecipitation with anti-FLAG Mab, further supporting that these are receptor-specific translation products (not shown). Pulse−chase studies were also performed to examine the kinetics of formation and degradation of these four species. Short pulse (5 min) labeling demonstrated that these same four proteins are all formed within this time period, indicating that they are all formed quite early in the translation process (not shown). All four proteins have a half-life of ∼7 h, suggesting that they are metabolically stable *in vivo.* Figure 1C (15 min pulse, 1 h chase) demonstrates that the 140 kDa form is converted to the 120 kDa form upon digestion with endoglycosidase H (endo-H). Since the cDNA is predicted to encode a ∼120 kDa protein, these results indicate that the core protein is modified to a higher molecular weight species consisting of high mannose oligosaccharides. The three smaller proteins are resistant to both endo-H (Figure 1C) and PNGase F (not shown), suggesting that they may not have been glycosylated. The pattern of endo-H sensitivity of the 140 kDa protein (Figure 1C) persists through a prolonged (10 h) chase (not shown). Similar results were obtained using 293 cells stably transfected with HA-STaR. In this cell line, bands ranging in size from 50 to 140 kDa were all present during the first 5−10 min of metabolic labeling, with only the full-length species being glycosylated (not shown). This suggests that the smaller forms may be generated independently of full-length STaR.

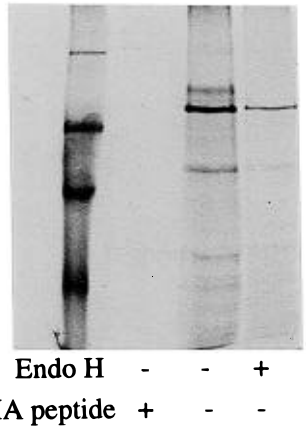


FIGURE 2: *In vitro* translation of HA-STaR and susceptibility of HA-STaR to endo-H. Molecular mass markers in the far left lane (top to bottom) are 200, 97.4, 68, and 43 kDa. Translation of HA-STaR was performed for 60 min in the presence of canine microsomal membranes, and products were immunoprecipitated with anti-HA Mab (HA peptide−) or with anti-HA Mab preincubated with HA peptide for control (HA peptide+). Samples were then treated with endo-H (endo-H+) or without endo-H for 1 h at 37 °C.

Biogenesis of STaR in Vitro. To further investigate the biogenesis of STaR, and to understand the relationship between full-length STaR and its smaller fragments, we performed *in vitro* transcription and translation studies. The translation of STaR mRNA using a rabbit reticulocyte lysate system with microsomal membranes produced radiolabeled proteins with molecular masses of 140, 120, 79, and 50 kDa (Figure 2). The predominant products were 140 and 120 kDa proteins, consistent with the glycosylated and nonglycosylated full-length receptor shown in the above *in vivo* metabolic labeling studies (Figure 1). To further characterize the nature of receptor glycosylation in this cell-free system, translated receptor was subjected to immunoprecipitation with anti-HA Mab followed by digestion with endo-H, which can cleave high mannose oligosaccharides but not complex oligosaccharides. Only the 140 kDa species is sensitive to endo-H and degraded to the 120 kDa nonglycosylated form. As seen *in vivo* (Figure 1), the smaller bands were not glycosylated, as evidenced by their resistance to endo-H (Figure 2) and to PNGase (not shown).

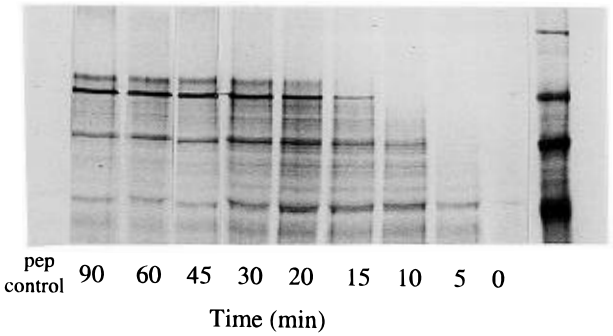


FIGURE 3: Time course in synchronized translation of HA-STaR. Molecular mass markers in the far right lane (top to bottom) are 200, 97.4, 68, and 43 kDa. Samples were taken at 0, 5, 10, 15, 20, 30, 45, 60, and 90 min of incubation, and the products were immunoprecipitated with anti-HA Mab or anti-HA Mab preincubated with 100 μg of HA peptide (pep control) and analyzed by SDS−PAGE and autoradiography. A positive control cDNA encoding β-galactosidase produced a major translation product of appropriate size but did not show the smaller size proteins seen with STaR cDNA (not shown).

To determine whether there is a precursor−product relationship between the full-length receptor and smaller fragments, we analyzed the appearance of STaR at different time points in synchronized translation *in vitro*. In this approach, protein synthesis is initiated by addition of a reticulocyte lysate to the receptor cRNA, and after 2 min, 4 mM 7-methylguanosine 5′-monophosphate (^{7}mGP), a specific inhibitor of initiation, is added to the translation mixture. The addition of ^{7}mGP prevents subsequent protein initiation but does not inhibit synthesis of nascent polypeptide chains. Because the initiation time interval (2 min) is short in comparison with the time required to complete the synthesis and translocation of STaR (20 min; data not shown), the metabolically labeled receptors are assumed to be synthesized in synchrony as a cohort population (Rothman & Lodish, 1977).

Synchronized translations were carried out in the presence of microsomal membranes, and these results are shown in Figure 3. Samples were taken at different times, and the products were immunoprecipitated and resolved by SDS−PAGE. Smaller fragments are formed within the first 10 min, while the full-length 120 kDa band is first observed at 15 min and reaches steady state at 20 min. The glycosylated form of STaR (140 kDa) appeared after 20 min. These smaller bands are seen prior to full-length forms; the relative amount of these translation products remains stable over time. This indicates that the smaller products are likely to represent premature termination products.

Subcellular Localization of STaR. Using ligand-based histochemical techniques, we previously demonstrated that STaR is localized to intestinal enterocytes, where there is a gradient of increasing expression from crypt to villus. These studies also suggested that a large population of ST receptors in rat intestinal cells are intracellular (Almenoff et al., 1993). The microscopic resolution of these ligand-based localization studies was limited, however, by the low avidity of the toxin probe. Consequently, the intracellular compartmentalization of STaR could not be precisely delineated.

Using a variety of monoclonal antibodies and receptor-expressing cell lines (Figure 4A−C, transfected 293 cells; Figure 4D,E, Caco-2 human colonic cell line), we were able to establish the cellular localization of STaR using fluorescence confocal microscopy. In Figure 4A, STaR is visualized with anti-FLAG Mab followed by FITC anti-mouse IgG. The endoplasmic reticulum (ER) is visualized using antibody to TRAP-α, a resident ER protein (Antusch et al., 1990; Hartmann & Prehn, 1994), and Texas Red anti-rabbit IgG (Figure 4B). The FITC staining pattern is consistent with localization of a limited fraction of receptors on the cell surface and a *predominant* population in the endoplasmic reticulum (ER). ER staining is shown in red and was detected using antibody to TRAP-α. Figure 4C shows that the red and green images are superimposable, indicating colocalization of STaR and TRAP-α intracellularly. Double-labeling immunofluorescence studies were also performed in the Caco-2 colonic cell line, which naturally expresses low levels of human STaR. For these experiments we used (1) 2g12, a monoclonal Ab directed to the extracellular domain of STaR with FITC anti-mouse IgG (Figure 4D), and (2) anti-TRAP-α with Texas Red anti-rabbit IgG (Figure 4E). Figure 4D demonstrates the FITC staining of STaR localized intracellularly, with limited surface staining. Figure 4E shows Texas Red staining in this same microscopic field, delineating the ER. This confirms that our findings in transfected cells (4A−C) are representative of the biology in intestinal cells that naturally express STaR. Additional double-labeling studies using the organelle-specific markers anticaveolin and transferrin (Anderson, 1993; von Zastrow & Kobilka, 1992) clearly indicated that the intracellular localization of STaR was distinct from that of intracellular caveoli and endosomes (not shown).

We also examined whether exposure to ligand would alter the distribution of STaR and whether ligand−receptor interactions are altered by the anti-FLAG and 2g12 Mab's. Exposure to ligand (4 μM ST) does not interfere with binding of antibody to receptor-expressing cells. Exposure of cells to anti-FLAG and 2g12 Mab (10 μg/mL) did not interfere with the cGMP response to ST. This allows the use of antibody to localize intracellular receptors by confocal immunofluorescence microscopy, with the knowledge that they do not interfere with ligand-induced cell signaling. No difference in cellular localization was detected after 10−60 min exposure to saturating doses of ST (data not shown).

Surface Labeling. In the above studies, we demonstrated that a substantial fraction of the receptor population is retained in the endoplasmic reticulum. In order to quantitate the relative amounts of intracellular and cell surface receptor, we performed surface labeling of stably transfected 293 cells using the anti-FLAG Mab. This antibody recognizes the FLAG epitope, located in the N-terminal extracellular domain of FLAG-STaR. Figure 5 shows the quantity of both total cellular receptor and surface receptor in $\sim 10^7$ cells. On the basis of densitometry scanning, the surface-labeled material comprised ~25% of the total cellular receptor population. This distribution is consistent with the confocal immunofluoresence staining shown above (Figure 4). Only the glycosylated 140 kDa protein was found at the cell surface. The lower molecular weight bands represent a small fraction of the total cellular receptor and were not detected at the cell surface.

DISCUSSION

The cellular localization and posttranslational processing of signal transduction proteins have a major impact on their

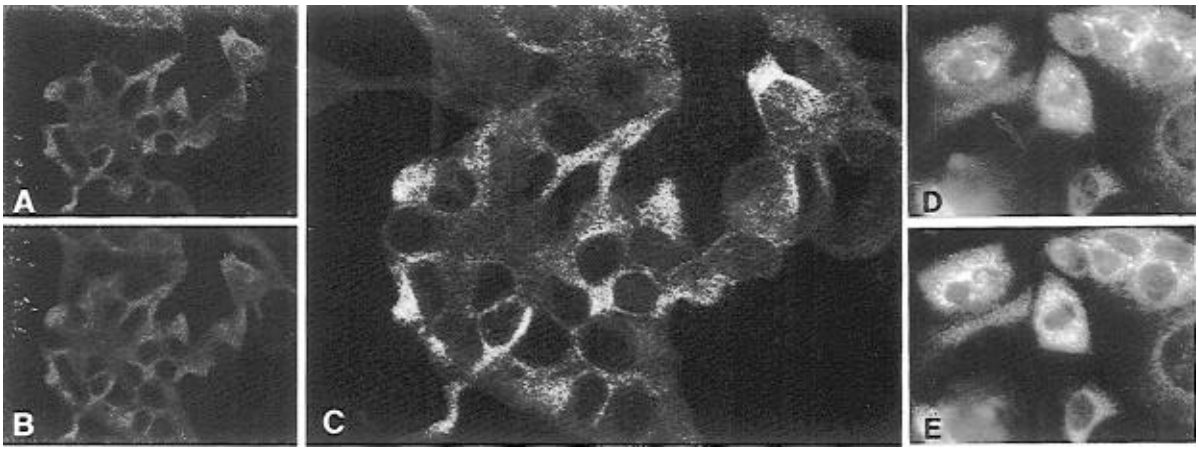


FIGURE 4: Subcellular localization of STaR using double labeling immunofluorescence. Stably transfected 293 cells expressing FLAG-STaR were grown on cover slips and then fixed and permeabilized. (A) The images in green depict the cellular localization of FLAG-STaR. Cover slips were incubated with FLAG-Mab (1.8 μg) followed by FITC anti-mouse IgG (secondary Ab). (B) The images in red delineate the ER. TRAP-α, a resident ER protein, was detected using a specific polyclonal Ab antibody (from Dr. C. Nicchitta, Department of Cell Biology, Duke University). This was followed by incubation with Texas Red anti-rabbit IgG (secondary Ab). (C) An RGB image superimposing red and green channels is shown. Yellow areas represent regions of overlap. Magnification is 630×. No bleed-through was detected at either wavelength. No staining with FLAG-Mab and FITC secondary Ab was observed in nontransfected controls. (D and E) Double labeling of Caco-2, a human intestinal cell line, stained with Mab 2g12, which is specific for the extracellular domain of STaR, and also with TRAP-α, followed by Texas Red-conjugated anti-rabbit and FITC-conjugated anti-mouse secondary antibodies (Jackson Laboratories). Fluorescence microscopy was performed using standard filter sets for Texas Red and FITC. (D) This figure shows FITC staining of Caco-2 cells, reflecting the cellular localization of STaR. (E) Texas Red emission is shown here, demonstrating the distribution of TRAP-α, a resident ER protein. No bleed-through was detected in parallel controls stained separately with either 2g12/anti-mouse FITC or TRAP-α/anti-rabbit Texas Red.

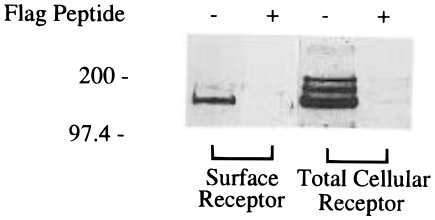


FIGURE 5: Surface labeling. 293 cells stably expressing FLAG-STaR were incubated with anti-FLAG Mab, followed by immunoprecipitation, and Western blot as descrbed in Experimental Procedures. FLAG peptide+ represents controls in which anti-FLAG Mab was preincubated with 200 μg/mL FLAG peptide for 1 h at 4 °C prior to the addition of STaR-expressing cells or cell lysate.

function and regulation. These factors also play a key role in the design and targeting of drugs to modulate signal transduction. In this report, we describe the biogenesis, posttranslational processing, and subcellular localization of STaR. These studies demonstrate an unusual pattern of biogenesis and cellular localization for this transmembrane ligand receptor and elucidate the biological relevance of previous biochemical findings.

Ligand affinity cross-linking studies done by many laboratories have demonstrated several species of ST binding proteins ranging in size from ~50 to 160 kDa both in the intestine and in cells expressing recombinant STaR (Cohen et al., 1993; Hakki et al., 1993; Hirayama et al., 1992; Ivens et al., 1990; Katwa et al., 1991; Vaandrager et al., 1993, 1994). Northern analysis has demonstrated that STaR is a single 3.7 kb mRNA; hence these smaller forms (<120 kDa) do not arise from alternative splicing. Some investigators have hypothesized that these truncated forms play a physiologic role (Deshmane et al., 1995; Hirayama et al., 1992; Ivens et al., 1990; Vaandrager et al., 1994), and others have suggested that they represent proteolytic degradation products (Cohen et al., 1993; de Sauvage et al., 1992; Vaandrager et al., 1993). To understand the biogenesis of these smaller forms, we performed pulse−chase studies to establish the metabolic fate of newly synthesized receptors. These studies demonstrated that the four proteins are all formed quite early in the translation process. These proteins consist of the full-length glycosylated receptor (140 kDa), a nonglycosylated form of the full-length receptor (120 kDa), and two minor protein bands of 50 and 60 kDa, neither of which is glycosylated. All four proteins have a half-life of ~7 h, and their relative amounts remain constant over time, suggesting that they are all metabolically stable. The data suggest that smaller forms of STaR are generated independently of the full-length receptor and that, in contrast to previous hypotheses (de Sauvage et al., 1992; Vaandrager et al., 1993), they are not generated by proteolysis of the full-length protein *in vivo*. Instead, they may reflect the presence of either premature termination or translational pause sites (Wolin & Walter, 1988).

The above findings are further supported by *in vitro* translation studies. The translation of STaR mRNA in the presence of microsomal membranes using a rabbit reticulocyte lysate system produces radiolabeled proteins with molecular masses of 140, 120, 79, and 50 kDa (Figure 2), consistent with the affinity labeling studies of other laboratories (Cohen et al., 1993; Hakki et al., 1993; Hirayama et al., 1992; Ivens et al., 1990; Katwa et al., 1991; Vaandrager et al., 1993, 1994). The predominant products were 140 and 120 kDa proteins, with glycosylation profiles that were identical to the above *in vivo* metabolic labeling studies (Figure 1). In addition, synchronized translation was performed to determine whether there is a product−precursor relationship between the full-length receptor and smaller fragments. In these experiments, 4 mM 7-methylguanosine 5′-phosphate (^{7}mGP), a specific inhibitor of initiation, was added to the translation mixture, and the metabolically labeled receptors were synthesized as a synchronized cohort

population (Rothman & Lodish, 1977). The 50 and 79 kDa protein products were formed within the first 10 min of the translation, while the full-length receptor was first detected at 15 min, and full-length STaR production reached steady state at 20 min. Only the full-length receptor is glycosylated in the presence of microsomes. The smaller bands appear before the full-length receptor is synthesized, and the relative amount of these translation products remained stable over time. This indicates that the smaller products are likely to represent premature termination products that are not transported to the cell surface and that full-length STaR is not cleaved to form these smaller products.

Other workers have hypothesized a biological role for these N-terminal fragments of STaR. The current studies demonstrate using both *in vitro* and *in vivo* approaches that the smaller forms of STaR that we and others observe represent premature termination products. These products are neither glycosylated nor transported to the cell surface but are instead retained in the cell. Vaandrager et al. (1994) have previously proposed that full-length STaR and N-terminal receptor fragments associate at the cell surface to form a functional heterooligomer as described for the TGF-β receptor subtypes (Henis et al., 1994). This hypothesis was based on indirect evidence including ultracentrifugation and radiation inactivation techniques. The current studies take a more direct approach, using metabolic and cell surface labeling, and reveal that only the full-length receptor is glycosylated and translocated to the surface. Recent work suggests that the binding of ST and of guanylin (Deshmane et al., 1995; Forte et al., 1993) to GC-C has multiple affinity states and has led to the speculation that truncated forms at the cell surface could account for these findings. More likely, these multiple affinities result from ligand-induced allosteric mechanisms influencing the conformational state of the full-length receptor (DeMeyts et al., 1976; Jewett et al., 1993;, Milligan & Koshland, 1991), rather than receptor heterogeneity at the cell surface. Recently, we have developed dominant inhibitory mutants of STaR, which indicate that oligomerization of full-length STaR is necessary for activation (Rudner et al., 1995). The findings presented here are consistent with this paradigm, because activation requires homooligomerization of full-length receptors but does not require association with N-terminal fragments.

We were surprised to observe persistent endo-H sensitivity of STaR, since endo-H resistance is usually acquired after terminal processing in the trans-Golgi stacks. The extracellular domain of StaR has several potential N-linked glycosylation sites; it is possible that not all of them are terminally modified en route to the cell surface. Persistent endo-H sensitivity has previously been described for the α chain of MHC class ll, which has two N-linked carbohydrate units, one of which is terminally processed to the complex (endo-H resistant) form while the other retains high mannose properties (i.e., endo-H sensitivity) (Alting-Mees & Barber, 1986). In contrast, the smaller forms of STaR were not glycosylated either *in vitro* or *in vivo*. This further supports the view that these smaller forms represent incompletely processed translation products.

Confocal immunofluorescence microscopy was used to determine the precise localization of STaR. We established that a large population of STaR was located intracellularly in the ER of several different cell types including the Caco-2 intestinal cell line. In these figures (4A−E), the surface staining is faint compared to the intense intracellular signal. These observations were corroborated by surface labeling (Figure 5), which revealed that only ~25% of the receptor population was at the cell surface. This is in contrast to other transmembrane receptors, including the tyrosine kinase and G-protein-coupled receptors, where the predominant pool resides at the cell surface (Barak et al., 1994; Heldin, 1995; von Zastrow & Kobilka, 1992). Our surface labeling studies did not identify ligand-mediated changes in the density of surface receptors. Interestingly, recent studies by Urbanski et al. suggest that STaR is rapidly internalized and recycled back to the cell surface after binding ST. This finding is consistent with our observations that ligand stimulation does not lead to recruitment of additional receptor to the cell surface, as descibed for other intracellular receptors including thrombin, the α-adrenergic receptor, and the glut-4 glucose transporter (Hein et al., 1994; James & Piper, 1994; von Zastrow & Kobilka, 1992).

Defining the biological role of this intracellular receptor population will be an interesting area for future investigation. Signal transduction by intracellular receptor subpopulations has been demonstrated for the angiotensin II receptor (De Mello, 1994) and also for the PDGF receptors (Keating & Williams, 1988). Guanylin is the only putative ligand that has been identified for STaR; however, its cellular distribution differs from that of STaR (Currie et al., 1992; Giannella, 1995; Schulz et al., 1992). Recent studies by Urbanski et al. (1995) have demonstrated that ST is internalized by T84 cells and thus raise the possibility that endogenous ligand(s) for STaR may also be internalized by enterocytes.

Another question that arises from this work involves the mechanism by which the intracellular receptor population is retained within the cell. Specific intracellular retention sequences have been identified for other transmembrane proteins including the erythropoetin receptor protein and the immunoglobulins. These retention signals are believed to provide posttranslational control of protein expression. Alternatively, this large intracellular population may represent selective retention by intracellular chaperones of misfolded receptor molecules (Doms et al., 1988; Hammond & Helenius, 1994; Watowich et al., 1994). We have previously shown that STaR spontaneously forms homooligomers (Rudner et al., 1995); thus it is possible that some of these oligomers might be improperly folded or assembled, resulting in ER retention. Furthermore, we observe that the surface receptor is glycosylated. Therefore, the nonglycosylated or improperly folded forms might be retained in the ER. Retention of proteins in the ER is thought to be a mechanism for quality control, as has been described for several other membrane proteins (de Silva et al., 1990; Hurtley & Helenius, 1989).

In summary, using complementary *in vitro* and *in vivo* approaches, we have demonstrated that only full-length STaR is translocated to the cell surface and is thus the only species available to interact directly with the extracellular environment. In contrast to previous hypotheses, we have demonstrated that the smaller forms represent incompletely processed forms of STaR that are not transported to the cell surface. We have identified a large intracellular population that may undergo spontaneous oligomerization and may have the capacity to be activated in the intracellular milieu. Further understanding of how guanylin and other potential endogenous ligand(s) interact with STaR *in vivo* will enable

us to use this new information for the design of novel agents to regulate intestinal secretion.

ACKNOWLEDGMENT

We thank Ms. Kathryn Mandal for expert technical assistance.

REFERENCES

Almenoff, J. S., Williams, S. I., Scheving, L. A., Judd, A. K., & Schoolnik, G. K. (1993) *Mol. Microbiol. 8*, 865−873.
Alting-Mees, M., & Barber, B. H. (1986) *Mol. Immunol. 23*, 847−861.
Anderson, R. G. W. (1993) *Proc. Natl. Acad. Sci U.S.A. 90*, 10909−10913.
Antusch, D., Bonifacino, J. S., Burgess, W. H., & Klausner, R. D. (1990) *J. Immunol. 145*, 885−890.
Barak, L. S., Tiberi, M., Freedman, N. J., Kwatra, M. M., Lefkowitz, R. J., & Caron, M. G. (1994) *J. Biol. Chem. 269*, 2690−2695.
Chao, A. C., de Sauvage, F. J., Dong, Y. J., Wagner, J. A., Goeddel, D. V., & Gardner, P. (1994) *EMBO J. 13*, 1065−1072.
Cohen, M. B., Jensen, N. J., Hawkins, J. A., Mann, E. A., Thompson, M. R., Lentze, M. J., & Giannella, R. A. (1993) *J. Cell. Physiol. 156*, 138−144.
Currie, M. G., Fok, K. F., Kato, J., Moore, R. J., Hamra, F. K., Duffin, K. L., & Smith, C. E. (1992) *Proc. Natl. Acad. Sci. U.S.A.. 89*, 947−951.
De Mello, W. C. (1994) *J. Cardiovasc. Pharmacol. 23*, 640−646.
DeMeyts, P., Bainco, A. R., & Roth, J. (1976) *J. Biol. Chem. 251*, 1877−1888.
de Sauvage, F. J., Camerato, T. R., & Goeddel, D. V. (1991) *J. Biol. Chem. 266*, 17912−17918.
de Sauvage, F. J., Horuk, R., Bennett, G., Quan, C., Burnier, J. P., & Goeddel, D. V. (1992) *J. Biol. Chem. 267*, 6479−6482.
Deshmane, S. P., Carrithers, S. L., Parkinson, S. J., Crupper, S. S., Robertson, D. C., & Waldman, S. A. (1995) *Biochemistry 34*, 9095−9102.
de Silva, A. M., Balch, W. E., & Helenius, A. (1990) *J. Cell Biol. 111*, 857−866.
Doms, R. W., Ruusala, A., Machamer, J., Helenius, A., Helenius, J., & Rose, J. K. (1988) *J. Cell Biol. 105*, 1957−1969.
Drewett, J. G., & Garbers, D. L. (1994) *Mol. Endocrinol. 15*, 135−152.
Dupont, H. L., & Ericsson, C. D. (1993) *N. Engl. J. Med. 328*, 1821−1827.
Forte, L. R., Eber, S. L., Turner, J. T., Freeman, R. H., Fok, K. F., & Currie, M. G. (1993) *J. Clin. Invest. 91*, 2423−2428.
Giannella, R. A. (1995) *J. Lab. Clin. Med. 125*, 173−181.
Hakki, S., Robertson, D. C., & Waldman, S. A. (1993) *Biochim. Biophys. Acta 1152*, 1−8.
Hammond, C., & Helenius, A. (1994) *J. Cell Biol. 126*, 41−52.
Hartmann, E., & Prehn, S. (1994) *FEBS Lett. 349*, 324−326.
Hein, L., Ishii, K., Coughlin, S. R., & Kobilka, B. K. (1994) *J. Biol. Chem. 269*, 27719−27726.
Heldin, C.-H. (1995) *Cell 80*, 213−223.
Henis, Y. I., Moustakas, A., Lin, H. Y., & Lodish, H. F. (1994) *J. Cell Biol. 126*, 139−154.
Hirayama, T., Wada, A., Iwata, N., Takasaki, S., Shimonishi, Y., & Takeda, Y. (1992) *Infect. Immun. 60*, 4213−4220.
Hurtley, S. M., & Helenius, A. (1989) *Annu. Rev. Cell Biol. 5*, 277−307.
Ivens, K., Gazzano, H., O'Hanley, P., & Waldman, S. (1990) *Infect. Immun. 58*, 1817−1820.
James, D. E., & Piper, R. C. (1994) *J. Cell Biol. 126*, 1123−1126.
Jewett, J. R. S., Koller, K. J., Goeddel, D. V., & Lowe, D. G. (1993) *EMBO J. 12*, 769−777.
Katwa, L. C., Parker, C. D., Dybing, J. K., & White, A. A. (1991) *Arch. Biochem. Biophys. 200*, 397−406.
Keating, M. T., & Williams, L. T. (1988) *Science 239*, 914−916.
Koldodziej, P. A., & Young, R. A. (1991) in *Methods in Enzymology*, pp 508−519, Academic Press, New York.
Milligan, D. L., & Koshland, D. E., Jr. (1991) *J. Biol. Chem. 268*, 1991−1997.
Rothman, J. E., & Lodish, H. F. (1977) *Nature 269*, 775−780.
Rudner, X. L., Mandal, K. K., de Sauvage, F. J., Kindman, L. A., & Almenoff, J. S. (1995) *Proc. Natl. Acad. Sci. U.S.A. 92*, 5169−5173.
Sambrook, J., Fritsch, E. F., & Maniatis, T. (1989) in *Molecular Cloning: A Laboratory Manual* (Sambrook, J., Fritsch, E. F., & Maniatis, T., Eds.) pp 16.32−16.39, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
Schulz, S., Green, C. K. K., Yuen, P. S. T., & Garbers, D. L. (1990) *Cell 63*, 941−948.
Schulz, S., Chrisman, T. D., & Garbers, D. L. (1992) *J. Biol. Chem. 267*, 16019−16021.
Shyjan, A. W., de Sauvage, F. J., Gillett, N. A., Goeddel, D. V., & Lowe, D. G. (1992) *Neuron 9*, 727−737.
Tien, X. Y., Brasitus, T. A., Kaetzel, M. A., Dedman, J. R., & Nelson, D. J. (1994) *J. Biol. Chem. 269*, 51−54.
Urbanski, R., Carrithers, S. L., & Waldman, S. A. (1995) *Biochim. Biophys. Acta 1245*, 29−36.
Vaandrager, A. B., Schulz, S., De Jonge, H. R., & Garbers, D. L. (1993) *J. Biol. Chem. 268*, 2174−2179.
Vaandrager, A. B., van der Wiel, E., Hom, M., Luthjens, L. H., & de Jonge, H. R. (1994) *J. Biol. Chem. 269*, 16409−16415.
von Zastrow, M., & Kobilka, B. K. (1992) *J. Biol. Chem. 267*, 3530−3538.
Watowich, S. S., Hilton, D. J., & Lodish, H. F. (1994) *Mol. Cell. Biol. 14*, 3535−3549.
Wolin, S. L., & Walter, P. (1988) *EMBO J. 7*, 3559−3569.
Yang, R.-B., Foster, D. C., Garbers, D. L., & Fulle, H.-J. (1995) *Proc. Natl. Acad. Sci. U.S.A. 92*, 602−606.

BI9607721