Interaction of Guanylyl Cyclase C with SH3 Domain of Src Tyrosine Kinase

YET ANOTHER MECHANISM FOR DESSENSITIZATION*

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Protein-protein interactions mediated by the Src homology 3 (SH3) domain have been implicated in the regulation of receptor functions for subcellular localization of proteins and the reorganization of cytoskeleton. The experiments described in this article begin to identify the interaction of the SH3 domain of Src tyrosine kinase with the guanylyl cyclase C receptor after activation with Escherichia coli heat-stable enterotoxin (ST). Only one of two post-translationally modified forms of guanylyl cyclase C from T84 colonic carcinoma cells bind to GST-SH3 fusion protein of Src and Hck tyrosine kinases. Interestingly, the GST-Src-SH3 fusion protein showed 2-fold more affinity to native guanylyl cyclase C in solution than the GST-Hck-SH3 fusion protein. The affinity of the GST-Src-SH3 fusion protein to guanylyl cyclase C increased on desensitization of receptor in vivo. An in vitro cyclase assay in the presence of GST-Src-SH3 fusion protein indicated inhibition of the catalytic activity of guanylyl cyclase C. The catalytic domain recombinant protein (GST-GC) of guanylyl cyclase C could pull-down a 60-kDa protein that reacted with Src tyrosine antibody and also showed autophosphorylation. These data suggest that SH3 domain-mediated protein-protein interaction with the catalytic domain of guanylyl cyclase C inhibited the cyclase activity and that such an interaction, possibly mediated by Src tyrosine kinase or additional proteins, might be pivotal for the desensitization phenomenon of the guanylyl cyclase C receptor.

The Src homology 3 (SH3)1 domain, a small peptide motif of about 50–60 amino acid residues, brings about protein-protein interactions important for signal transduction, subcellular localization of proteins, and cytoskeletal organization in eukaryotic organisms (1–3). The SH3 domain was originally found in the amino-terminal, non-catalytic half of pp60Src, and it seems to play dual roles in signal transduction, through stabilizing the repressed form of the Src kinase and through mediating the formation of activated signaling complexes. More than 50 SH3 domains are known to date distributed in several classes of proteins, including enzymes (e.g. PLCγ, Ras-GAP, and the p85 subunit of PI3K), tyrosine phosphatases (e.g. PTP1C, Syp), adaptor proteins (Grb2, Nck and Crk), and cytoskeletal proteins (e.g. cortactin, myosin 1B, spectrin) (4–6).

Initial studies on protein-protein interactions mediated by the SH3 domain indicated lack of specificity; however, studies using peptide libraries have shown that the SH3 domain exhibits distinct binding preferences (7–9). The SH3 domain recognizes proline-rich amino acid sequences in the target protein with a minimal consensus sequence of PPGGX (3, 10).

The specificity of protein-protein interaction mediated by the SH3 domain with polyproline sequence is conferred by the amino acids adjacent to the minimal consensus sequence (3, 8, 11, 12). Although SH3 domain-mediated protein-protein interactions are well documented, we still need to understand the physiological significance of such interactions.

Receptor guanylyl cyclase C (GCC) is a member of the diverse family of transmembrane guanylyl cyclase receptors found in mammals as well as in lower eukaryotes. In mammals there are six membrane forms (GC-A–F); ligands are known for GCA, GCB (natriuretic peptides), GCC, the retinal and olfactory cyclases GCD, GCE, and GCF (13–14). Guanylin and related peptide uroguanylin are the endogenous ligands of GCC (15–16). Escherichia coli heat-stable enterotoxin ST binds to GCC and results in imbalance in the secretion of fluids and ions culminating in a form of severe secretory diarrhea (17–20). The binding of physiological ligands guanylin and uroguanylin (15–16) or ST to the extracellular domain of GCC activates the intracellular cyclase domain, which catalyzes the synthesis of the second messenger cGMP from GTP (14–15, 21). Elevated levels of cGMP in response to the ST peptide probably cross-activate cGMP-dependent protein kinase (PKG) leading to phosphorylation and subsequent opening of cystic fibrosis transmembrane conductance regulator (CFTR) (22), as well as through the cross-activation of cAMP-dependent protein kinase (PKA) (23). Upon prolonged exposure to ST, the GCC receptor expressed in T84 cells becomes refractory or desensitized to the presence of ST as implicit in the transient nature of enterotoxin ST-mediated diarrhea (24–25). The factors contributing to the induction of ST-mediated desensitization caused by the reduction in the catalytic activity of GCC are still not known. The activation of type 5 cGMP-specific phosphodiesterase has been found to regulate the levels of second messenger (cGMP) and contribute to the desensitization of the GCC receptor (24, 25). However, the homologous desensitization in vivo was observed to be different from the inactivation of the GCC receptor in vitro, and the desensitization of GCC was found to be a cell-specific phenomenon (24).

To study the signal transduction mechanisms of GCC receptor, T84 human colonic carcinoma cell line was used as a model system (24). T84 cell line resembles mature intestinal epithe-
Briefly, bacterial cell cultures were induced with 1 mM isopropyl GST-SH3 fusion proteins were generated as previously described (30). Gentamicin was purchased from Amersham Biosciences. Src monoclonal antibody was a goat anti-rabbit horseradish peroxidase conjugate and ECL Plus kit were obtained from Amersham Biosciences. GCC-specific monoclonal antibodies were kindly provided by Dr. Ghanshyam Swarup (CCMB, Hyderabad). The cDNA clones of human GCC (Manassas, VA). ST was purified from an Escherichia coli clone. s medium/F12 and newborn calf serum were from Invitrogen. T84 cells (CCL247) were procured from ATCC and maintained in the aggregate was established at 37°C. Confluent culture of T84 cells in 6-cm dishes was treated with control and GST-SH3 (5 μg of protein) domains of Src and Hck immobilized on glutathione-Sepharose for 2 h and protein was harvested. The proteins bound to GST-SH3 were analyzed by SDS-PAGE and Western blotting with GCC-specific monoclonal antibodies (GCC:CC8). Equivalent aliquots of the solubilized receptor preparation were used for immunoprecipitation with GCC-specific polyclonal antibodies.

**Western Blot Analysis**—T84 cell membrane proteins (50 μg) and the immunoprecipitates obtained with GCC as described earlier were fractionated on 10% SDS-PAGE. The proteins were transferred onto a nitrocellulose membrane in 25 mM Tris pH 8.3, 190 mM glycine, containing 20% methanol, for 2 h at 200 mA. The nitrocellulose membrane was blocked with 5% blocking reagent for 1 h at room temperature, followed by which the membranes were washed in PBS containing 0.1% Tween-20 (PBST) and incubated for 2 h at room temperature with purified IgG (1:200) from monoclonal antibody GCC:CC8 in PBS, pH 7.2 containing 0.2% BSA and 0.1% Tween-20 and/or Src monoclonal antibody (1:1000) prepared in Tris-buffered saline (TBS), pH 7.5 containing 0.2% BSA and 0.1% Tween-20 for 1 h. The presence of bound antibody was detected by enhanced chemiluminescence (ECL) reaction using the ECL Plus kit according to the manufacturer's instructions.

**Materials**—Preparation of Native GCC with GST-SH3 Domain—T84 cells were grown to 70% confluency in a 6-cm dish and were washed twice with cytochalasin D and methionine-free Dulbecco’s modified Eagle’s medium. After preincubation in the medium for 1 h, cells were incubated in fresh medium containing 5 μCi/ml [35S]methionine for 5 h. At the end of the incubation, cells were harvested, and proteins were extracted from the membrane fraction in a buffer containing 50 mM Tris-HCl, pH 7.6, 2 mM EDTA, 5 mM MgCl2, 150 mM NaCl, 0.5% sodium deoxycholate, 2 mM PMSF, 1 μg/ml leupeptin, 1 μg/ml aprotinin, and 1% Triton X-100. Immunoprecipitation of GCC was done with GCC-specific polyclonal antibody followed by incubation in immunoprecipitation buffer containing 100 μM EDTA, 5 mM MgCl2, and 1% Triton X-100 (Sigma). The mixture was kept at 4°C for 1 h and then subjected to x-ray film.

**Immunoprecipitation of GCC from T84 Membrane Extracts—**Immunoprecipitation of GCC from T84 membrane extracts was performed with control and GST-SH3 (5 μg of protein) domains of Src and Hck immobilized on glutathione-Sepharose for 2 h and protein was harvested. The proteins bound to GST-SH3 were analyzed by SDS-PAGE and Western blotting with GCC-specific monoclonal antibodies (GCC:CC8). Equivalent aliquots of the solubilized receptor preparation were used for immunoprecipitation with GCC-specific polyclonal antibodies.
Fig. 1. Primary amino acid sequence analysis for the presence of proline-rich sequences in guanylyl cyclase C. A, schematic representation of various proline-rich sequences of GCC. B, consensus proline-rich sequences for binding to SH3 domains.

Fig. 2. GST-SH3 domain specifically associates with one of the two post-translationally modified forms of guanylyl cyclase C. A, GST-SH3 fusion proteins of Hck and Src tyrosine kinases. The GST-SH3 fusion proteins expressed in E. coli were affinity-purified from bacterial lysates by incubation with glutathione-Sepharose. The glutathione-Sepharose was then washed, and the bound proteins were eluted with 20 mM free glutathione. The purified GST and GST-SH3 fusion proteins were resolved by 12% SDS-PAGE and visualized by Coomassie Blue staining. B, co-precipitation of GCC with GST-SH3 domain of Hck and Src tyrosine kinase. T84 cell membrane fraction was prepared and solubilized in interaction buffer as described under “Experimental Procedures.” The solubilized receptor preparation was incubated with either GST as a control or GST-SH3 domain of Src and Hck bound to glutathione-Sepharose for 2 h at 25°C on a shaker. The solubilized receptor preparation was also subjected to immunoprecipitation with polyclonal antibody to GCC. GCC immunoprecipitate and the proteins bound to SH3 fusion proteins were eluted with SDS-sample buffer, fractionated on a 10% SDS-PAGE, transferred onto nitrocellulose, and probed with a monoclonal antibody (GCC:C8) against GCC as described under “Experimental Procedures.”

~12,000 cpm 32P-labeled cGMP added per tube in 50 mM sodium acetate buffer pH 4.75 containing 5 mg/ml BSA. cGMP antiserum at a dilution of 1:5000–1:10,000 was incubated with known and unknown concentrations of cGMP for 12–16 h at 4°C in a total volume of 300 μl. Standard cGMP concentration was taken over a range of 3 fmol to 5 pmol/tube. Antibody bound to cGMP was separated from free cGMP by the addition of 0.2% activated charcoal in 50 mM potassium phosphate buffer, pH 6.3 containing 1 mg/ml BSA. Tubes were centrifuged, and the charcoal-bound radioactivity monitored in a γ counter. Data were analyzed by Graph Pad Prism. A standard curve of specific binding versus cGMP concentration was plotted from which the unknown cGMP concentration was determined.

Data Analysis—Data shown were analyzed on Prism (GraphPad) and expressed as a mean ± S.E., and the Student’s t test was used to test statistical significance.

RESULTS

Presence of Proline-rich Sequences in GCC—To understand the molecular mechanisms that are involved downstream of the activated GCC receptor, the protein sequence of GCC was analyzed for the presence of any known motif involved in pro-
tein–protein interactions. Interestingly, five proline-rich sequences were identified in the intracellular domain of GCC some of which appeared to be similar to the consensus sequences for the binding of the SH3 domain of the Src family of kinases like Src and Hck (Fig. 1).

Interaction of GST-Hck-SH3 and GST-Src-SH3 Fusion Proteins with GCC from T84 Colonic Carcinoma Cell Line—To establish the above observation, the interaction of native GCC extracted from the solubilized membrane fraction of T84 cells was checked with GST-SH3 fusion proteins of the Src and Hck tyrosine kinases (Fig. 2). The solubilized receptor was treated with GST as a control or Src and Hck GST-SH3 domains (Fig. 2A) bound to glutathione-Sepharose for 2 h at 25 °C on a shaker. Similar aliquots of the solubilized receptor preparation were used for immunoprecipitation with GCC-specific polyclonal antibody. Bound proteins and the immunoprecipitate were analyzed by Western blotting using a monoclonal antibody (GCC:C8) to GCC. GCC associated with GST-Src-SH3 and GST-Hck-SH3 fusion proteins in solution but not with GST alone (Fig. 2B). It was interesting to find that the Src SH3 domain bound 2-fold more receptor than the Hck SH3 domain and only one (140 kDa) out of the two differentially posttranslationally modified forms of GCC from T84 cells interacted with Src and Hck SH3 domains. This observation indicated a difference in the affinity of Src and Hck SH3 domains for GCC receptor as well as showed the specificity of interaction; therefore further studies on SH3 domain–mediated interactions with GCC were performed with the SH3 SH3 domain.

Physiological Significance of Protein–Protein Interaction Mediated by SH3 Domain—To check the physiological significance of the interaction between GCC and SH3 domain, the solubilized GCC receptor preparations from T84 cells after treatment with ST for 18 h were incubated with GST and GST-Src-SH3 immobilized on glutathione-Sepharose. The proteins that co-precipitated with GST–SH3 and GST–Src–SH3 fusion proteins in solution but not with GST alone (Fig. 2B). It was interesting to find that the Src SH3 domain bound 2-fold more receptor than the Hck SH3 domain and only one (140 kDa) out of the two differentially posttranslationally modified forms of GCC from T84 cells interacted with Src and Hck SH3 domains. This observation indicated a difference in the affinity of Src and Hck SH3 domains for GCC receptor as well as showed the specificity of interaction; therefore further studies on SH3 domain–mediated interactions with GCC were performed with the SH3 SH3 domain.

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incubation with ST (3 × 10⁻⁷ M) for 18 h than the control and activated receptor (treatment with ST for 15 min). The equivalent amount of GST-Src-SH3 bound at least 3-fold more receptor from cells treated with ST for 18 h than from control and cells stimulated for 15 min with ST in vivo (Fig. 3).

Inhibition of cGMP Production in the Presence of GST-Src-SH3 Fusion Protein in an in Vitro Guanylyl Cyclase Assay—To further examine the importance of such an interaction, it was intriguing to check the effect of the presence of the Src SH3 domain on the production of cGMP in an in vitro guanylyl cyclase assay. T84 cell membrane proteins were preincubated with GST and GST-Src-SH3 followed by incubation with ST (1 × 10⁻⁷ M). The samples obtained after the assay were subjected to radioimmunoassay to check the levels of cGMP produced. As shown in Fig. 4, the basal levels of production of cGMP did not change significantly in the presence of GST and GST-Src-SH3. However, there was a significant (p < 0.05) decrease in ST-stimulated production of cGMP in the presence of GST-Src-SH3 indicating that binding of the Src SH3 domain to the proline-rich sites in the catalytic domain of GCC could modulate the ligand-stimulated production of cGMP.

Differential Profiling of Proteins Interacting with GCC in T84 Cells—To investigate the cellular proteins that might be interacting with GCC under different physiological conditions, the proteins which co-immunoprecipitated with native GCC from T84 cells were studied by different approaches. In one of the approaches, GCC was immunoprecipitated with a specific antibody, and the immunoprecipitate was subjected to autophosphorylation in the presence of [γ⁻³²P]ATP followed by SDS-PAGE and autoradiography as shown in Fig. 5A. In another experiment T84 cells were labeled with [³⁵S]methionine followed by immunoprecipitation of GCC from solubilized membrane proteins. The proteins co-immunoprecipitated with GCC were detected by SDS-PAGE and autoradiography as shown in Fig. 5B. Fig. 5C shows the co-immunoprecipitated proteins from control T84 cells and after the treatment with ST (3 × 10⁻⁷ M) for 18 h in vivo. Immunoprecipitates were subjected to phosphorylation in the presence of protein kinase A and [γ⁻³²P]ATP. The proteins were visualized as described for earlier two experiments. The interesting observation from all the three experiments was that a 60-kDa protein got co-immunoprecipitated with GCC and there was 3-fold increase in the serine/threonine phosphorylation of 60-kDa protein after desensitization on prolonged exposure of T84 cells to ST.

Interaction of Src Tyrosine Kinase with GCC in Solution—To investigate whether Src tyrosine kinase interacts with GCC, the proteins co-immunoprecipitated with GCC-specific antibody (GCC:C8) from T84 membrane protein extract and autophosphorylated in the presence of [γ⁻³²P]ATP were subjected to SDS-PAGE and Western blotting with Src tyrosine kinase and GCC-specific antibodies. The same blot was subjected to immunoprecipitation with Src-specific antibody and analysis was done as for the other samples followed by immunoblotting with Src tyrosine kinase antibody. The result shown in Fig. 6, clearly indicate that Src tyrosine kinase associated with GCC in solution. There was an increase in the Src tyrosine kinase (60-kDa band) per se (Fig. 6B.1)
and/or in the phosphorylated form after desensitization of GCC receptor (Fig. 6A,2). These observations along with interaction studies with the GST-Src-SH3 domain indicated the interaction of Src tyrosine kinase with GCC, and this interaction changed when receptor was stimulated with ST and on desensitization. It was also interesting to note that there was decrease in the post-translationally modified form of GCC (160 kDa) after ST stimulation and after desensitization of GCC receptor at 18 h of ST treatment (Fig. 6A,1) and more of it was dephosphorylated (Fig. 6A,2).

**Co-precipitation of Src Tyrosine Kinase with the Catalytic Domain of GCC**—To check whether the interaction of Src tyrosine kinase with GCC involved the catalytic domain of the receptor, catalytic domain fusion protein (GST-GCD) bound to glutathione-Sepharose matrix was incubated with solubilized T84 cell membrane proteins. The proteins, which associated with GST-GCD, were autophosphorylated in the presence of [γ-32P]ATP. The samples were subjected to SDS-PAGE, Western blotting with Src tyrosine kinase-specific antibody, and autoradiography. The catalytic domain fusion protein (GST-GCD) pulled down a 60-kDa protein (Fig. 7), which was recognized by Src tyrosine kinase antibody (Fig. 7A) and it was also autophosphorylated (Fig. 7B). Similar experiment with GST did not show such an interaction with a 60-kDa protein (Fig. 7). Binding of Src tyrosine kinase to GST-GCD was interfered by the simultaneous presence of GST-SH3 (Fig. 7, A and B) giving preliminary evidence that the interaction of GCC with Src tyrosine kinase might be through the SH3 domain.

**FIG. 7.** Interaction of recombinant catalytic domain-GST fusion protein (GST-GCD) of guanylyl cyclase C with Src tyrosine kinase. The proteins extracted from T84 membrane were incubated with GST and GST-SH3 followed by binding to GST-GCD on glutathione-Sepharose. The proteins bound to GST-GCD were eluted with glutathione (20 m units 50 mM Tris buffer, pH 8.0), dialyzed and subjected to autophosphorylation in the presence of [γ-32P]ATP. The proteins were analyzed by SDS-PAGE and Western blotting, with Src-specific antibody and autoradiography. A, autoradiogram showing the proteins auto-phosphorylated in the presence of [γ-32P]ATP. B, immunoblotting of the same membrane with Src tyrosine kinase-specific antibody.

**FIG. 8.** A schematic diagram of the regulation of GCC-mediated signal transduction. The recruitment of SH3 domain-containing proteins to GCC on prolonged exposure to ST leads to the inhibition of catalytic activity of the receptor. The regulation of catalytic activity of GCC by SH3 domain-mediated interactions has added to the understanding of the mechanism for the desensitization phenomenon of GCC receptor from T84 colon carcinoma cell line. GCC-mediated signaling is regulated by cross-talk with other signaling pathways that activate cGMP-phosphodiesterases, which downregulate the levels of cGMP and/or activate protein kinase C, which potentiates ST-stimulated guanylyl cyclase activity. Additional signaling pathways linking to GCC may involve phosphorylated tyrosine or other protein-protein interactions. Identification of proteins interacting with GCC would provide insights into the greater understanding of the physiological role of GCC.

**DISCUSSION**

This report demonstrates the interaction of SH3 domains with the GCC receptor and its role in the ST-induced desensitization and establishes the interaction of the SH3 domain of Src tyrosine kinase with catalytic domain of GCC receptor in T84 colon carcinoma cell line. It is interesting to find that only one out of the two differentially post-translationally modified forms of GCC interacts with the SH3 domain. The affinity of GCC for the Src SH3 domain increases after desensitization of the receptor. Binding of the SH3 domain of Src tyrosine kinase to the proline-rich site in the catalytic domain of GCC and the resulting decrease in cyclase activity most likely contribute to the mechanism of ST-induced receptor desensitization.

Co-immunoprecipitation experiments with antibody specific to GCC indicate that Src tyrosine kinase associates with GCC receptor. The protein kinase A-catalyzed phosphorylation of proteins co-immunoprecipitated with control and desensitized GCC indicate that there is either an increase in the protein per se or there is more of the dephosphorylated form of 60-kDa protein associated with desensitized receptor. However, it does indicate that ST-induced protein-protein interactions with accessory proteins might be important for the desensitization process of GCC. The analysis of autophosphorylated and [35S]methionine-labeled proteins co-immunoprecipitated with GCC suggest that there are other proteins (40, 55, and 80 kDa) that interact with GCC, and these proteins could also be the...
It is also to be noted that there is turnover of the GCC receptor as shown by [35S]methionine incorporation within 5 h of incubation.

It is observed that there is change in the phosphorylation status of the receptor on desensitization. There is significant decrease in the amount as well as phosphorylation of the 160-kDa form of GCC after 18 h of exposure of T84 cells to ST. Interestingly, it is only the 140-kDa form of GCC that interacted with the SH3 domain, and the affinity of such an interaction increased on desensitization of the receptor.

It has been demonstrated that the transitory nature of ST-mediated diarrhea is due to the ligand-mediated loss of responsiveness or desensitization of the GCC receptor. It has been shown that there is no significant degree of internalization of GCC receptor in T84 cells, which may be due to efficient recycling of the receptor in T84 cells without degradation of either the receptor or the peptide (35). Their studies have indicated that the desensitization observed in vivo could be due to changes in the catalytic activity of GCC that could be regulated by altered interactions of GCC with other cellular proteins when activated with its ligands. Bakre and Visweswariah (25) have shown the role of cGMP-phosphodiesterase in inducing ST-mediated desensitization of T84 cells by altering the levels of second messenger (cGMP). The regulation of guanylyl cyclases by additional proteins has been suggested earlier for solubility of second messenger (cGMP). The regulation of guanylyl cyclase-mediated desensitization of T84 cells by altering the levels of the SH3 domain present in the intestinal cells that might be important insights; however, lacunae still exist in the understanding of the mechanism of desensitization of GCC receptors.

The induction of cellular refractoriness by increased activity of cGMP-binding, cGMP-specific phosphodiesterase (PDE5) in a ligand-dependent manner suggests the possibility of a feedback loop that regulates cGMP accumulation in human colonic cells (46). Binding of cGMP to the non-catalytic sites of PDE5 is essential for its further phosphorylation by cGMP- or cAMP-dependent kinases; however, the contribution of this phosphorylation to the enhancement of catalytic activity of PDE5 is not known (47). Tyrosine phosphorylation of intracellular domain of GCC has been demonstrated, but its role in the regulation of receptor activity has not been reported (48). Protein kinase C (PKC)-mediated phosphorylation of Ser-1029 of GCC activates the cyclase, especially in synergy with ST (49). Consequently, ligand-dependent increases in cGMP concentration activate the cyclic nucleotide-dependent protein kinases that phosphorylate cystic fibrosis transmembrane conductance regulator (CFTR) leading to change in water and ion balance of the cells.

Therefore, it is important to delineate the molecular interactions of GCC with other proteins that might be important for the regulation of GCC activation and desensitization. The identification of proteins interacting with GCC might provide insights into strategies to control excessive GCC activation by ST. The interaction of the SH3 domain with GCC might be a novel approach to design an anti-secretory strategy by interruption of transmembrane signaling by high affinity analogues of the SH3 domain. However, the ability of the SH3 domain analogue to disrupt GCC signaling and its potential as anti-secretory therapy remains untested.

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