Activation of Src Family Kinase Yes Induced by Shiga Toxin Binding to Globotriaosyl Ceramide (Gb3/CD77) in Low Density, Detergent-insoluble Microdomains*

Yohko U. Katagiri, Tetsuya Mori, Hideki Nakajima, Chihiro Katagiri, Tomoko Taguchi, Tae Takeda, Nobutaka Kiyokawa, and Junichiro Fujimoto

Shiga toxin (Stx) is an enterotoxin produced by Shigella dysenteriae serotype 1 and enterohemorrhagic Escherichia coli, which binds specifically to globotriaosylceramide, Gb3, on the cell surface and causes cell death. We previously demonstrated that Stx induced apoptosis in human renal tubular cell line ACHN cells (Taguchi, T., Uchida, H., Kiyokawa, N., Mori, T., Sato, N., Itorie, H., Takeda, T. and Fujimoto, J. (1999) Kidney Int. 53, 1681-1685). To study the early signal transduction after Stx addition, Gb3-enriched microdomains were prepared from ACHN cells by sucrose density gradient centrifugation of Triton X-100 lysate as buoyant, detergent-insoluble microdomains (DIM). Gb3 was only recovered in DIM and was associated with Src family kinase Yes. Phosphorylation of tyrosine residues of proteins in the DIM fraction increased by 10 min and returned to the resting level by 30 min after the addition of Stx. Since the kinase activity of Yes changed with the same kinetics, it was thought to be responsible for the hyperphosphorylation observed in DIM proteins. Unexpectedly, however, all of the Yes kinase activity was obtained in the high density, detergent-soluble fraction. Yes was found to be activated and show increased Triton X-100 solubility in the early phase of retrograde endocytosis of Stx-Gb3 complex. Since Yes activation by the Stx addition was suppressed by filipin pretreatment, Gb3-enriched microdomains containing cholesterol were deeply involved in Stx signal transduction.

Shiga toxin (Stx)1 of Shigella dysenteriae serotype 1 and enterohemorrhagic Escherichia coli is one of the major cause of hemolytic uremic syndrome (HUS). Stx consists of an A subunit of 32 kDa associated with five B subunits of 7.5 kDa each. The A subunits act to remove the adenine base at position 4324 of 28 S rRNA and are responsible for inactivation of protein synthesis and toxicity (2). The A subunits lacking B subunits, however, do not show any toxicity because of their inability to bind to the cell surface receptor. The B subunits bind specifically to cell surface glycosphingolipid (GLS) receptors-Gb3,2 also known as CD77 or blood group Pk (3). Once Stx is internalized, protein synthesis is suppressed, leading to cell death.

Cell death is widely known to take place through two distinct processes, necrosis or apoptosis. In contradiction to Williams report (4), a number of recent studies have clearly demonstrated that Stx induces apoptosis in several different cell types, including Burkitt’s lymphoma cells (5), Vero cells (6), human renal tubular derived ACHN cells (1), and normal human renal tubular epithelial cells (7, 8). Especially, the later two studies indicate the importance of apoptotic cell death as one mechanism of damage to renal epithelium in the pathogenesis of HUS. Although the B subunit has no inhibitory effect on protein synthesis, a series of studies indicates that it alone participate to transduce cell signaling and can induce apoptosis in some instances such as Burkitt’s lymphoma (5, 9). These reports encourage us to determine how and where Gb3 delivers the signal.

In recent years, our knowledge of the characteristics and cellular function of low density, detergent-insoluble microdomains (DIM) rich in GSL, cholesterol, sphingomyelin, glycosphingosylphosphatidylinositol (GPI)-anchored protein, and lipid-modified protein, namely caveolae or raft, has considerably increased. Originally, caveolae were thought to function only in receptor-mediated potocytosis (10); however, speculation into their biological role has since expanded to include such diverse functions as endocytosis independent of the coated pit pathway, sorting and internalization of GPI-anchored proteins, and signal transduction. Many studies have examined the receptor molecules and the signal transducers in DIM. Some gangliosides in DIM were reported to be associated with transducer molecules that are activated by stimulation of ganglioside (11, 12). By analogy to this model, the Stx receptor Gb3 is expected to reside in DIM and to be involved in signal transduction.

Here we show that Gb3 is only found in DIM and is associated...
ated with the Src family tyrosine kinase Yes. We further demonstrate that Stx binding to Gb3 causes temporal activation of Yes, suggesting that DIM is deeply involved in Gb3-mediated signal transduction. The activation-related topology of Yes and the recruitment of Yes after Stx addition are discussed.

**EXPERIMENTAL PROCEDURES**

**Antibodies, GSL, and Other Reagents**—The rat IgM anti-Gb3 mAb 38.13 was purchased from Coulter/Immunotec, Inc. (Westbrook, MA). The mouse IgM anti-Gb3 mAb 1A4 was a generous gift from Dr. Hakkaku of the University of Washington (Seattle, WA). Anti-Yes mAb, anti-Lyn mAb, and rabbit anti-caveolin polyclonal antibody were purchased from Transduction Laboratories Inc. (Lexington, KY). Anti-phosphotyrosine mAb 4G10 was obtained from Upstate Biotechnology Inc. (Lake Placid, NY). Anti-CD24 mAb OKB2 was purchased from Ortho Diagnostic System Inc. (Raritan, NJ). Antibodies for Stx-1 used were 13C4 mAb from ATCC CRL 1794 (13) and rabbit polyclonal Ab (14). Gb3 from bovine erythrocytes and filipin complex were purchased from Sigma. Stx-1 was prepared as described previously (14).

**Cells**—The human renal tubular derived ACHN cell line was obtained from the American Type Culture Collection (ATCC CRL 1611) and maintained in Eagle's minimum essential medium supplemented with 10% fetal bovine serum and nonessential amino acids solution (Life Technologies, Inc.). Cell surface proteins were labeled with sulfosuccinimidyl-6-(biotinamido)hexanoate (Pierce) according to the method of Lisanti (15). For the binding assay of Stx, 5 × 10^6 cells were incubated with Stx-1 at 100 ng/ml in the culture medium on ice for 30 min.

**DIM Preparation**—DIM were prepared as described by Sargiacomo et al. (16) with slight modification. Briefly, cells in a 15-cm culture plate were lysed and homogenized using a Teflon glass homogenizer in 1.5 ml of PIPES-buffered saline (25 mM PIPES, pH 6.5, 0.15 mM NaCl) containing 1% Triton X-100, 1 mM phenylmethylsulfonyl fluoride, 1 mM Na3VO4, and aprotinin (10 trypsin inhibitory units/ml). Cell extracts were adjusted to 40% sucrose in PIPES-buffered saline. A linear gradient (5–30% sucrose in PIPES-buffered saline) was formed above the lysate and centrifuged at 39,000 rpm for 18 h at 4 °C in a Beckman SW40 Ti rotor. A light scattering band was recovered as DIM. Detergent-free purification of DIM under hypertonic conditions (0.5 mM Na2CO3, pH 11) (17) was also performed.

**Lipid Analysis**—According to Iwabuchi's description (12), the gradi- ent fractions were dialyzed against distilled water and lyophilized. The residues were extracted with chloroform/methanol (2:1, v/v) and separated by high performance thin layer chromatography (TLC) plate (Merck, Darmstadt, Germany) in a solvent system of chloroform/methanol/0.2% CaCl2 (60:35:8, v/v/v).

**Co-immunoprecipitation of Gb3 and Transducer Molecules in DIM**—DIM prepared from biotinylated cells were washed with Tris-buffered saline (25 mM Tris-Cl, pH 7.5, 0.15 mM NaCl) containing 1% Triton X-100 and suspended in the same buffer, or further solubilized in Triton X-100/HTG lysis buffer (Tris-buffered saline containing 1% Triton X-100, 60 mM n-heptyl-β-D-thioglycoside (HTG, Dojin Chem. Co., Kumamoto, Japan), 1 mM phenylmethylsulfonyl fluoride, 1 mM Na3VO4, and aprotinin), followed by centrifugation at 15,000 rpm at 4 °C for 30 min in a microcentrifuge. DIM or its lysate thus obtained were mixed with protein A-agarose (Roche Molecular Biochemicals) bound to anti-CD77 antibody (19) or rabbit anti-caveolin polyclonal antibody (14) and incubated for 1 h at room temperature. The immune complexes were separated by SDS-PAGE in 10% acrylamide gel and transferred to nitrocellulose sheets (Hybond-C; Amersham Pharmacia Biotech). After transfer, the blots were treated with peroxidase-conjugated streptavidin (b), anti-Yes (c), anti-Lyn (d), anti-CD24 (e), and anti-caveolin (f). Panel e was obtained by reprobing of blot d after quenching with 0.1% NaN3 in phosphate-buffered saline overnight. The fractions prepared from Stx-bound cells were dot-blotted and stained with rabbit anti-Stx-1 polyclonal antibody (g). Peroxidase-conjugated secondary antibodies or streptavidin bound to membranes was detected by enhanced chemiluminescence (ECL Western blotting system; Amersham Pharmacia Biotech).

**RESULTS**

**Distribution of Cellular Protein after Sucrose Density Gradient Centrifugation**—Most cellular proteins were recovered in fractions 10–12 (Fig. 1a). Many parts of biotinylated proteins were liberated with 1% Triton X-100 from plasma membrane and found in fractions 10–12 as high density DS (Fig. 1b). However, some were not solubilized from plasma membrane and thus were recovered in buoyant, low density fractions, fractions 5–7, as DIM. A total of 6.29 mg and 5.93 μg of protein was recovered from confluent ACHN cells (1 × 10^7) in a 15-cm plate in DS and DIM, respectively. In contrast, about 30–50% of the Src family tyrosine kinase, Yes (Fig. 1c) and Lyn (Fig. 1d) and all GPI-anchored CD24 protein, a marker of distal tubules in kidney (Fig. 1e) were found in DIM. Caveolin, a structural protein rich in caveolae, separated similarly to the Src family tyrosine kinases and was found in both DIM and DS (Fig. 1f). Stx bound to ACHN cells was certainly detected in DIM (Fig. 1g). Detergent-free preparation under hypertonic conditions gave similar distribution pattern except that a little more protein was recovered in DIM than under detergent-containing conditions (data not shown).
Bovine Gb3 (100 ng) was used as a standard. Chl blotted to polyvinylidene difluoride membrane (Millipore Corp., Bedford, MA) and immunostained with 1A4 (b) orcinol-sulfuric reagent (b) or Dragendorf-Ditmmer reagent (35280). Lac-cer sphingomyelin; DIM, a biotinylated protein with an apparent molecular mass in DIM—In anti-Gb3 mAb 38.13 immunoprecipitates from plasma membrane. results indicate that DIM thus obtained is a typical low density, glycolipid-enriched microdomain distinct from other plasma membrane.

Association of Gb3 with Yes and a 27.2-kDa Membrane Protein in DIM—in anti-Gb3 mAb 38.13 immunoprecipitates from DIM, a biotinylated protein with an apparent molecular mass of 27.2 kDa was always found (Fig. 3a, lane 1, arrow). In contrast to the nonspecific binding proteins that migrate around 62 and 50 kDa, this 27.2-kDa protein band was not detected in normal rat IgM immunoprecipitates (lane 2). When this blot was reprobed with anti-Yes mAb or anti-caveolin antibody, Yes was clearly detected (Fig. 3b, upper panel), but caveolin was not identified (Fig. 3b, lower panel). Although another Src-family kinase Lyn was found in DIM, Lyn was not detected in mAb 38.13 immunoprecipitates (data not shown). Immunoprecipitation with another anti-Gb3 mAb, 1A4, gave similar results (data not shown).

Increased Tyrosine Phosphorylation and the Amount of Yes Protein in DIM with the Addition of Stx—to determine whether tyrosine phosphorylation of DIM protein is affected by Stx, DIM was prepared from cells cultured in the presence of Stx for various times, and the tyrosine phosphorylation was analyzed by immunoblotting with 4G10 mAb. The amount of protein recovered in DIM was not changed by Stx treatment (3.93 ± 0.23 μg/1 × 10^6 cells, n = 5). DIM prepared in hyper tonic buffer was examined by immunoblotting, because hypertonic buffer was found to retain more substrate proteins for tyrosine kinase than 1% Triton X-100 buffer. Although several proteins were spontaneously tyrosine-phosphorylated without Stx treatment, phosphorylation of tyrosine residues increased to a maximum at 10 min after Stx addition and then decreased to resting level at 60 min (Fig. 4a). Thus, we concluded that Stx specifically stimulates tyrosine kinases, which temporally induce hyperphosphorylation of DIM proteins. The amount of Yes protein present in DIM (Fig. 4b) as well as associated with Gb3 (Fig. 4c) increased to a maximum level at 10 min after the Stx addition and then decreased. This indicates that Stx stimulation recruited Yes into the DIM, resulting in an increase in Yes association with Gb3. One may argue whether Stx binding to Gb3 interferes with the formation of an immune complex between Gb3 and anti-Gb3 mAb; however, flow cytometric analysis showed that Stx-bound ACHN cells were stained by 1A4 with the same mean fluorescence intensity as unbound cells (data not shown). This means that anti-Gb3 mAb can react with Gb3 to which Stx has already bound.

Augmentation of Yes Activity by Stx—to confirm that Yes activity is mediated by Stx signal transduction, Yes was partially purified by immunoprecipitation from whole cell lysate of Stx-treated cells, and the enzyme kinetics of the Yes protein was analyzed. As shown in Fig. 5a, there is no significant variation in immunoprecipitated Yes protein in each preparation. Stx treatment resulted in a 2-fold increase in Yes activity on enolase between 3 and 10 min, which returned to the resting level at 30–60 min (Fig. 5, b (left) and c (closed triangle)). Interestingly, autophosphorylation of Yes also occurred in a similar time course fashion following Stx treatment (Fig. 5, b (left) and c (closed triangle)). On the other hand, Yes activity of the untreated cells almost remained at the resting level (Fig. 5, b (right) and c (open symbols)). Stx solution preadsorbed with immobilized anti-Stx-1 mAb 13C4, which inhibits Stx binding to Gb3 on the cell surface, did not show this augmentation, whereas one preadsorbed with irrelevant mAb showed the augmentation similar to unadsorbed Stx (data not shown), indicating that Stx specifically induced Yes kinase activation.

Augmentation of Yes Activity in DS but Not in DIM after Stx Addition—Since Gb3, to which Stx binds, was localized in DIM and since Yes was associated with Gb3, we predicted the increase in Yes activity shown above occurring in DIM. However, we failed to detect any increase in Yes activity in the DIM fraction (Fig. 6). In contrast, Yes kinase activity in DS showed the same kinetics as that of whole cell lysate, indicating that the activated Yes was mostly located in the DS. That Yes activity in DIM was lost or inactivated in the experimental procedure was unlikely, because Yes in DS prepared simultaneously exhibited enough activity as shown above. Based on all

**FIG. 2.** TLC analysis of lipid in fractions separated by sucrose density gradient centrifugation. The plate was sprayed with orcinol-sulfuric reagent (a) or Dragendorf-Ditmmer reagent (c) to visualize glycolipids and phospholipids, respectively. Lipids on a TLC plate were fractionated by sucrose density gradient centrifugation. The plate was sprayed with orcinol-sulfuric reagent (a) or Dragendorf-Ditmmer reagent (c) to visualize glycolipids and phospholipids, respectively. Lipids on a TLC plate were fractionated by sucrose density gradient centrifugation.**

**FIG. 3.** Association of CD77 with the biotinylated membrane protein and Yes in DIM. The blot containing immune complex with 38.13 (lane 1) or normal rat IgM (lane 2) and an aliquot of total lysate (lane 3) from biotinylated cells was probed with peroxidase-conjugated streptavidin (a). Then the blot was reprobed with anti-Yes mAb (b, upper sheet) or anti-caveolin antibody (b, lower sheet) after quenching as described in the Fig. 1 legend.
of these results, we hypothesized that Yes becomes detergent-soluble, in other words loosely anchored to DIM, when it is activated by Stx stimulation through Gb3.

Suppression of Yes Retention in DIM and Stx-induced Yes Activation by Filipin—Filipin is known to disrupt the structure and function of DIM by binding to cholesterol a constituent of DIM (18). Pretreatment of cells with filipin dose-dependently reduced the amount of Yes protein, but not caveolin, recovered in DIM (Fig. 7a). The aliquots of DIM lysates (b) or the immune complex with I44 (c) were separated by SDS-PAGE and transferred to a nitrocellulose sheet. The blot was probed with anti-Yes mAb.

**DISCUSSION**

In the present study, we demonstrated that Gb3 associates with Yes in DIM on the cell surface of human renal tubular epithelium-derived cells and that Stx binding to Gb3 temporarily causes activation of Yes. Specific interactions between GSL and receptor molecules such as GM3 and epidermal growth factor receptor (19), and GM1 and Trk, a high affinity receptor for nerve growth factor (20), suggest that GSL participates in signal transduction by interacting with membrane receptor molecules. In contrast, Gb3 is itself a receptor for Stx. Lingwood’s group (21, 22) reported the role of Gb3 as a signal transducer in CD19 and interferon-α. Although some glycolipids are also known to be receptors for bacteria toxins (23), they

**FIG. 4.** Time course change of tyrosine phosphorylation in DIM and the amount of Yes protein in DIM and in Gb3-linked form. DIM was prepared from the culture at the indicated time after the Stx addition (400 pg/ml) under hypertonic conditions and was separated by SDS-PAGE and transferred to a nitrocellulose sheet. The blot was probed with 4G10 to analyze changes in tyrosine phosphorylation (a). DIM prepared from each culture was further solubilized with Triton X-100/HTG lysis buffer. The aliquots of DIM lysates (b) or the immune complex with I44 (c) were separated by SDS-PAGE and transferred to a nitrocellulose sheet. The blot was probed with anti-Yes mAb.

**FIG. 5.** Kinetic analysis of Yes activity in ACHN cells treated with Stx. The culture in the presence (closed) or absence (open) of Stx-1 (400 pg/ml) was stopped at the indicated time by the addition of Triton X-100/HTG lysis buffer. Yes was purified from each lysate by immunoprecipitation with anti-Yes mAb and subjected to in vitro kinase assay (see “Experimental Procedures”). Yes protein in the precipitates was detected by immunoblotting with anti-Yes mAb (a). 32PPO4-incorporated Yes and enolase were separated by SDS-PAGE and exposed to x-ray film (b). The kinase activity detected by incorporation of [γ-32P]ATP into enolase (circle) and Yes (triangle) was expressed as photostimulated luminescence (PSL) counted by BAS 2000 (c).

**FIG. 6.** Kinetic analysis of Yes activity in DIM and DS of ACHN cells treated with Stx. DIM (fractions 5–7) and DS (fractions 10–12) were separated from cells treated with Stx by sucrose density gradient centrifugation and dialyzed against Tris-buffered saline, followed by concentration with polyethylene glycol 20,000 in a dialysis bag. DIM was further solubilized in Triton X-100/HTG lysis buffer for 1 h on ice. Immunoprecipitates from both lysates with anti-Yes mAb were subjected to in vitro kinase assay. a and b, see Fig. 5 legend. The kinase activity of DIM (○) and DS (●) detected by incorporation of [γ-32P]ATP into enolase is shown in c as in Fig. 5.

**FIG. 7.** Effect of filipin on retardation of Yes in DIM and Stx-induced Yes activation. DIM of filipin-treated cells was separated by SDS-PAGE, followed by transfer to a nitrocellulose membrane. The blot was stained with anti-Yes or anti-caveolin (a). The Yes activity of filipin-treated cells 10 min after the Stx addition was assayed as described in the Fig. 5 legend.

Further supports the idea that cholesterol-enriched DIM is indeed involved in Stx-mediating signal transduction.
Activation of Src Family Kinase Yes by Stx Binding to Gb3

are not known to stimulate Src family kinases like Gb3 as shown in this study. This is the first example that shows a link between Yes and the GSL Gb3 localized in DIM in a special type of cells.

Src family members are anchored to the plasma membrane via myristylation and/or palmitoylation at the N-terminal region, and clusters of basic residues near the anchoring site promote membrane binding (24). Since Gb3 is only in the external leaflet of the membrane bilayer, there can be other membrane-spanning molecules that link Yes and Gb3. The biotinylated protein with an apparent molecular mass of 27.2 kDa communoprecipitated with anti-Gb3 mAb is a possible candidate that ligates Gb3 with Yes in ACHN cells. The precise characterization of this 27.2-kDa protein is now under way.

While the total amount of Yes per cell did not change during the course of the process, the amount of Yes in DIM increased after Stx treatment. Thus, we speculate, as a consequence of Stx stimulation, the entry of inactive Yes, previously present in DS, into DIM, resulting in the association with Gb3.

Stx was reported to be internalized after binding to Gb3 into the Golgi apparatus via retrograde endocytosis as a Stx-Gb3 complex (25). If this is also the case in ACHN, Yes is supposed to move into the intracellular space with the Stx-Gb3 complex. However, we confirmed that all Yes protein in ACHN cells was recovered in membrane fractions and was completely solubilized with detergent containing both 60 mM HTG and 1% Triton X-100, suggesting that, once bound to Gb3, inactive Yes may become active either by dephosphorylation with protein phosphatase or by another mechanism, resulting in the increased solubility in Triton X-100, i.e. recovered in DS, and may be easily liberated from DIM. Since activation of Src family kinases does not affect the Golgi apparatus via retrograde endocytosis as a Stx-Gb3 complex.

Interestingly, we observed that the entire Yes kinase activity was detected not in DIM but in DS. This indicated the increased solubility of active Yes in Triton X-100. Thus, it is probable that, once bound to Gb3, inactive Yes may become active either by dephosphorylation with protein phosphatase or by another mechanism, resulting in the increased solubility in Triton X-100, i.e. recovered in DS, and may be easily liberated from DIM. Since activation of Src family kinases does not affect the sequence of motif anchoring to membrane, the conformational change caused by the activation may be responsible for being loosely anchored to plasma membrane. This may explain why Yes in DIM being activated with Stx was easily solubilized in Triton X-100 and did not move into the intracellular space with the Stx-Gb3 complex.

Iwabuchi et al. (26) separated the detergent-insoluble, GM3-enriched, cholesterol-free microdomains involved in cell adhesion of B16 melanoma cells from the other microdomains containing caveolin and termed them the "glycosignaling domain." They showed that adhesion of B16 cells to Gg3 was not affected by filipin treatment. Since disruption of DIM integrity of ACHN cells with filipin dramatically suppressed the Yes activation with Stx, Gb3-enriched microdomains containing cholesterol may collectively act in transducing Stx signal as one mass of glycosignaling domain.

It is not clear yet that activation of Yes by Stx binding to Gb3 in ACHN cells proceeds to the final process of cell death. A precise study on the downstream events following Stx-mediated activation of Yes is particularly important not only to understand the role of Gb3 in cellular function but also to develop a therapeutic approach to prevent Stx-mediated renal dysfunction in HUS.

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Activation of Src family kinase Yes induced by Shiga toxin binding to globotriaosyl ceramide (Gb3/CD77) in low density, detergent-insoluble microdomains.

Yohko U. Katagiri, Tetsuya Mori, Tomoko Taguchi, Tae Takeda, Nobutaka Kiyokawa, and Junichiro Fujimoto

Page 35282: In our paper we stated, “Iwabuchi et al. (26) separated the detergent-insoluble, GM3-enriched, cholesterol-free microdomains involved in cell adhesion of B16 melanoma cells from the other microdomains...”

The term “cholesterol-free” is incorrect here. In fact, in the abstract of the cited paper (Iwabuchi, K., Handa, K., and Hakomori, S. (1998) J. Biol. Chem. 273, 33766–33773), it is stated: “The GM3-enriched subfraction, separated by anti-GM3 monoclonal antibody DH2, contains sphingomyelin, cholesterol, c-Src, and Rho A but not caveolin.”

This correction does not alter the conclusions of our own study in any way.

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Interaction between the conserved region in the C-terminal domain of GRK2 and rhodopsin is necessary for GRK2 to catalyze receptor phosphorylation.

Xiao-qing Gan, Ji-yong Wang, Qi-heng Yang, Zhong Li, Feng Liu, Gang Pei, and Lin Li

Page 8471, Fig. 2: The symbols at the top and bottom of the figure were incorrect. The figure with the correct symbols appears below.

![Corrected Figure 2](image)

A: 𝑛𝑉 − + − +

B: 2^32_P Incorporation (%)

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