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Biochemical and Biophysical Research Communications 357 (2007) 144–149
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Structural requirements for furin-induced cleavage and activation of Shiga toxin

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Received 15 March 2007
Available online 28 March 2007

Abstract

Shiga toxin has a protease-sensitive site in the disulfide loop region of the A-chain. Cleavage of this site by furin is essential for rapid intoxication of cells by Shiga toxin. We have here investigated whether in addition to the Arg-X-X-Arg sequence, there are other structural requirements in the disulfide loop region for furin cleavage. A toxin mutant (Shiga-2D toxin) still containing the consensus motif for cleavage by furin, but lacking ten amino acids in the disulfide loop, was generated. Trypsin was able to cleave Shiga-2D toxin *in vitro*, demonstrating that the protease-sensitive region is intact. However, Shiga-2D toxin was not efficiently cleaved by furin either *in vitro* or *in vivo*. Furthermore, unless it was precleaved with trypsin, Shiga-2D toxin was much less toxic than wild type Shiga toxin in LoVo cells expressing functional furin. In contrast, LoVo/neo cells lacking functional furin were unable to activate both wild type Shiga toxin and Shiga-2D toxin. In conclusion, an extended loop structure is required for furin-induced cleavage of Shiga toxin.

Keywords: Shiga toxin; Shiga-2D toxin; Protease-sensitive site; Furin; Cleavage; Cytotoxicity

Shiga toxin (Stx) is a bacterial protein toxin produced by *Shigella dysenteriae* type 1. The toxin consists of an enzymatically active A-chain non-covalently associated to a pentamer of B-chains [1]. The toxicity of Stx is due to the ability of the A-subunit (StxA) to block protein synthesis by removing one adenine residue from the 28S ribosomal RNA of the 60S subunit [2,3]. In order to exert its toxic effect, Stx has to enter the cells and find its way to the cytosol (for review, see [4–6]). This process starts when the B-subunits bind to the glycolipid receptor Gb3 at the cell surface [7,8], and Stx is internalized. Interestingly, Stx is able to regulate its own entry into cells by activating Syk [9], and also the A-chain facilitates endocytosis of the toxin [10]. After endocytosis, Stx is transported retrogradely via endosomes and the Golgi apparatus to the endoplasmic reticulum, from where the toxin is translocated to the cytosol [4–6].

StxA contains in the C-terminal region two cysteines (Cys-242 and Cys-261) that are linked by a disulfide bond forming a loop. This loop contains a sequence (Arg-Val-Ala-Arg) that is recognized and nicked by proteases such as trypsin and furin resulting into an enzymatically active A_1 fragment (27.5 kDa) and an A_2 fragment (4.5 kDa) [11,12]. Cleavage of StxA is essential for rapid intoxication of cells [11,13]. In cells with active furin Stx processing seems to start already in endosomes. Actually, furin-

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doi:10.1016/j.bbrc.2007.03.110

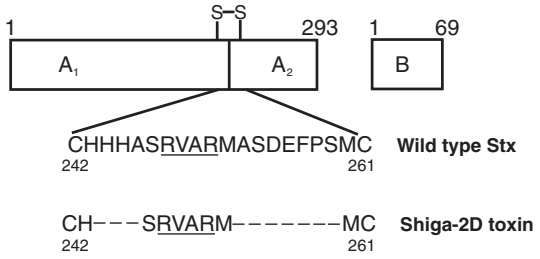

Fig. 1. Schematic representation of wild type Shiga toxin and Shiga-2D toxin. In Shiga-2D toxin ten amino acids from the disulfide loop in the A-chain have been removed. The protease-sensitive region is underlined.

induced cleavage of Stx occurs most efficiently at low pH [11]. In LoVo cells that do not produce functional furin [14], StxA is cleaved slowly and the cleavage does not take place in endosomes [11]. However, StxA was efficiently processed when these cells were transfected with the membrane-associated endoprotease furin [11]. Although furin is the protease mainly responsible for cleavage of Stx *in vivo* in most cells, other cellular proteases can cleave the toxin in cells without functional furin, although less efficiently [11]. Furin-independent cleavage seems to occur after the toxin has reached or passed through the Golgi apparatus since it is inhibited by Brefeldin A (BFA) [11]. BFA causes disassembly of the Golgi apparatus [15] and inhibits the transport of StxB to this organelle [16] and Stx cytotoxicity [17]. Moreover, calpain, a Ca^{2+}-dependent cysteine protease present in the cytosol of most animal cells [18], seems to be involved in Stx processing in cells that lack functional furin [11].

In order to investigate the importance of the structure of the disulfide loop in StxA for furin cleavage and activation of Stx, we have generated a mutant toxin, Shiga-2D, in which several amino acids surrounding the furin-recognition site have been deleted. In particular, the Shiga-2D toxin has two deletions in the C-terminal part of the A-chain: from His-244 to Ala-246 (three amino acid residues) and from Ala-253 to Ser-259 (seven amino acid residues) (Fig. 1). Thus, Shiga-2D toxin contains the protease-sensitive recognition site, but has a considerably shortened disulfide loop.

Importantly, our experiments show that not only the sequence known to be a minimal furin-recognition site, but also the structure around this site are important for furin processing of StxA and for rapid intoxication.

Materials and methods

Materials. Brefeldin A was purchased from Epicentre Technologies (Madison, WI, USA). Calpain inhibitor I was purchased from Roche (Basel, Switzerland). Trypsin was obtained from Sigma (St. Louis, MO, USA). $Na^{125}I$ and [^{3}H]leucine were purchased from Perkin-Elmer (Waltham, MA, USA). Furin was a gift from Dr. Gary Thomas (Vollum Institute, Oregon Health Sciences University, Portland, OR). Shiga toxin was purified as described previously [13] and ^{125}I-labeled to a specific activity of 30,000–40,000 cpm/ng as described by Fraker and Speck [19].

Oligonucleotide-directed mutagenesis. (A) Mutations in the A-subunit of Stx were introduced by means of Altered Sites System (Promega). Oligonucleotides used to generate mutations were synthesized based on the toxin sequence reported earlier [20]. Mutations were confirmed by DNA sequencing. Oligonucleotide sequences for mutagenesis were as follows 1. Deletions 244–246 and 253–259 in the A-subunit: 5′-TCCATCTGCCGGACACATCATTCTGGCAACTCGCGA-3′ and 5′-TCTGGCAACTCGCCAATGACAATTGAGTAT-3′. Recombinant vector pALTER was generated by means of restriction with the *Eco*R1 and *Bam*HI restriction endonucleases and subsequent ligation with the *Eco*R1–*Bam*HI restriction fragment produced from the initial plasmid pSHT23. Annealing was carried out in a total volume of 20 μl, containing 0.05 pmol of single-stranded DNA of recombinant pALTER, 0.25 pmol of oligonucleotide restoring the resistance to ampicillin, and 1.25 pmol of mutagenic primer in the annealing buffer (20 mM Tris–HCl, pH 7.7, 10 mM $MgCl_2$, and 50 mM NaCl). The mixture was heated to 70 °C for 5 min and then slowly cooled to room temperature for 30 min. Synthesis and ligation of the mutant DNA strand was carried out in a total volume of 30 μl. A total of 10 U of T4 DNA polymerase and 2 U of T4 DNA ligase were added to the annealing mixture, and the incubation was performed in a buffer (10 mM Tris–HCl, pH 7.5, 0.5 mM dNTP, 1 mM ATP, and 2 mM DTT) for 90 min at 37 °C. After the incubation, the mixture was used for transformation of the competent cells BMH 71–18 mut S. After the transformation, the cells were not seeded on plates, but continued growing in 4 ml of liquid LB medium in the presence of 125 μg/ml ampicillin overnight. Night culture of BMH 71–18 mut S was used for the isolation of plasmid DNA, with the help of which transformation of the JM-109 competent cells was performed. After transformation, the cells were seeded on LB agar containing 125 μg/ml ampicillin, and analyzed by use of restriction, hybridization with the mutagenic primer, or direct sequencing.

(B) Hybridization with mutagenic primer. This method of selection was used for the production of mutant toxin carrying the deletion of ten amino acid residues. This substantial difference resulted in low mutagenesis efficiency (8–10%). Mutagenic primer was phosphorylated using [^{32}P]γATP. Individual colonies were seeded in a certain order onto nitrocellulose membrane drawn into sections. The membrane was placed on LB agar in a Petri dish and incubated overnight at 37 °C. The following solutions were prepared: SSC (20×) (3 M NaCl, 400 mM Na citrate, pH 7.0), SSPE (20×) (3.6 M NaCl, 200 mM NaH_2PO_4, and 20 mM EDTA, pH 7.4), Denhardt's solution (50×) (5 g Ficoll, 5 g polyvinylpyrrolidone, 5 g bovine serum albumin, and water to 500 ml). Membrane was placed in 0.5 M NaOH for 3 min, then washed in 1 M Tris–HCl two times for 1 min and dried at 80 °C for 2 h between two sheets of Whatman 3M paper. After that, membrane was placed in 20 ml of prehybridization solution (6× SSC, 10× Denhardt's solution, and 0.2% SDS), incubated for 1 h at 67 °C, and then washed in 100 ml of 6× SSC. Hybridization was performed in the buffer containing 6× SSC, 10× Denhardt's solution, and 8 pmol of labeled primer in the same conditions. After the hybridization, membrane was washed three times in 6× SSC for 5 min at 22 °C, wrapped in Saran Wrap film, and exposed with the X-ray film for 1 h at 22 °C. During the following washes the temperature of washing solution was raised up to 50, 60, and 70 °C. The colonies, the DNA of which most steadily hybridized to mutagenic primer, were selected and analyzed by sequencing.

Cell culture. LoVo (from human colon carcinoma) cells transfected with mouse furin (LoVo/fur) and control vector (LoVo/neo) [21] were a gift from Dr. E. Mekada (Kurume University, Kurume, Fukuoka, Japan). LoVo cells were grown in Ham's F-12 supplemented with 10% serum, 100 U/ml penicillin, and 100 μg/ml streptomycin at 37 °C in an atmosphere of 5% CO_2/95% air. HEp-2 cells (from epidermoid carcinoma) were obtained from ATCC (Rockville, MD) and grown in Dulbecco's modified Eagle's medium supplemented with 5% serum, 100 U/ml penicillin, and 100 μg/ml streptomycin at 37 °C in an atmosphere of 5% CO_2/95% air.

Trypsin sensitivity of Shiga toxin and Shiga-2D toxin. ^{125}I-labeled toxins at a concentration of 1 μg/ml in Hepes medium were treated with trypsin (2.5 μg/ml) for 4 min at room temperature. The reaction was stopped by addition of sample buffer containing 2-mercaptoethanol and

PMSF. The samples were immediately boiled and then subjected to SDS–PAGE and autoradiography.

In vitro cleavage of Shiga toxin and Shiga-2D toxin by purified furin. The cleavage was performed in a reaction volume of 25 μl containing 5 mM $CaCl_2$, 1 mM 2-mercaptoethanol, 100 mM buffer (sodium acetate, pH 5.0; MES, pH 5.5–7.5), 10 ng of ^{125}I-Shiga toxin, and 3 ng of purified furin. The reaction mixture was incubated for 3 h at 37 °C, and the reaction was stopped by adding SDS sample buffer with 2-mercaptoethanol. The samples were boiled and subjected to SDS–PAGE.

Cleavage of Shiga toxin and Shiga-2D toxin by cultured cells. The cells were washed in Hepes medium and incubated with ^{125}I-labeled toxin (100 ng/ml) for 1 or 5 h in Hepes-buffered medium at 37 °C. Then the cells were washed three times with phosphate-buffered saline and lysed in 1% Triton (1% Triton X-100, 20 mM Hepes, 140 mM NaCl, and 1 mM PMSF, pH 7.4) on ice for 20 min. The cell lysate was transferred to Eppendorf tubes, nuclei were removed by centrifugation, and proteins were precipitated for 30 min on ice in the presence of 5% trichloroacetic acid. After centrifugation the pellet was washed in ether, dissolved in sample buffer, and then subjected to SDS–PAGE.

Cytotoxicity assay. Cells were transferred to 24-well plates at a density of 3×10^4 cells/well 2 or 3 days prior to the experiments. The cells were incubated with increasing amounts of toxin for 3 h and then with 1 μCi/ml [^{3}H]leucine in Hepes medium without leucine for 10 min. The medium was removed and the cells were washed twice in 5% trichloroacetic acid. Finally, acid precipitable proteins were solubilized in 0.1 M KOH, and the radioactivity was measured. The results are expressed in percentage of [^{3}H]leucine incorporated into cells incubated without toxin. Deviations between duplicates varied by less than 10%.

SDS–PAGE and autoradiography. Electrophoresis was carried out as described by Laemmli [22]. Then, the gels were fixed for 30 min in 4% acetic acid and 27% methanol. For autoradiography, Kodak XAR films were exposed to dried gels at −80 °C. The bands were quantified by densitometry.

Results and discussion

Furin is unable to cleave Shiga-2D toxin in vitro

It has previously been shown that the subtilisin-like endoprotease furin [23] cleaves and activates StxA [11]. Furin recognizes the sequence Arg-Val-Ala-Arg located in the disulfide loop near the C-terminal end of StxA and cleaves StxA in two fragments, A_1 (27.5 kDa) and A_2 (4.5 kDa). However, cleavage by furin could also be dependent on protein conformation. In order to investigate structural requirements in the disulfide loop for furin cleavage of StxA, a mutant Shiga toxin in which ten amino acid residues surrounding the furin-recognition site have been deleted, Shiga-2D toxin, was generated (Fig. 1). The pH optimum for furin proteolytic activity varies depending on the substrate [24]. Therefore, the ability of furin to cleave ^{125}I-labeled toxin *in vitro* was measured at different pH values. As indicated by the absence of the A_1 fragment in Fig. 2A (the A_2 fragment migrates with the front of the gel and it is not shown in the figure), furin is not able to cleave Shiga-2D toxin in the pH range 5–7.5. In contrast, as shown in Fig. 2B, Shiga-2D toxin was cleaved by the protease trypsin (2.5 μg/ml) *in vitro* demonstrating that the protease-cleavage site in Shiga-2D toxin is intact, and that the structural requirements for furin and trypsin cleavage of Stx are different. Finally, and in agreement with previous results [11], control experiments indicated that wild type Stx was efficiently cleaved by furin (Fig. 2A) and by trypsin (Fig. 2B) *in vitro*.

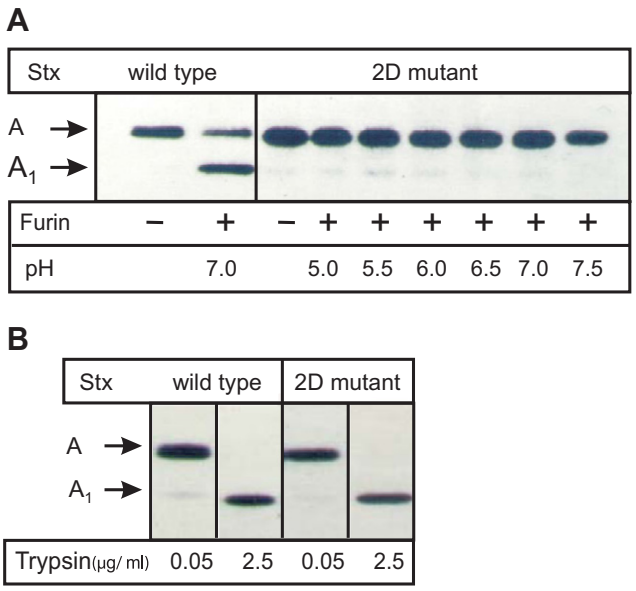

Fig. 2. *In vitro* cleavage of wild type Shiga toxin and Shiga-2D toxin by furin and trypsin. ^{125}I-labeled toxins were incubated with a soluble form of furin for 3 h at 30 °C at several pH values (A), or with trypsin for 4 min at room temperature (B). The reaction products were analyzed by SDS–PAGE and autoradiography. The gel shows intact A-chain and the A_1 fragment (the A_2 fragment migrated with the front of the gel and it is not shown). The figure shows a representative experiment.

Furin is unable to cleave Shiga-2D toxin in vivo

Furin is expressed in a wide variety of cell lines, and it is the cellular protease mainly responsible for the cleavage and activation of Stx in cells [13]. Furin is mainly located in the trans-Golgi network, but cycles between this cellular compartment, the endosomes, and the plasma membrane [25,26]. Cleavage of Stx by furin is likely to take place mainly in endosomes since it occurs optimally at low pH [11]. This idea is in agreement with the finding that the cleavage is not affected by BFA [11], a drug that disperses the Golgi apparatus [15] and that inhibits the transport of StxB to the Golgi apparatus [16]. To investigate whether Shiga-2D toxin can be cleaved *in vivo*, HEp-2 cells and stably transfected LoVo cells expressing either furin (LoVo/fur) or the vector alone (LoVo/neo) were used. Both HEp-2 cells and LoVo/fur cells contain active furin, whereas LoVo/neo cells do not [14]. The cells were incubated with ^{125}I-labeled toxin for 1 or 5 h, and then lysed and subjected to SDS–PAGE and autoradiography to study the formation of the A_1 fragment. As shown in Fig. 3A and B, Shiga-2D toxin was processed much more slowly than wild type Stx in HEp-2 cells. After 1 h incubation cleavage of Shiga-2D toxin was not observed, but approx. 25% of the cell-associated Shiga-2D toxin was cleaved after a 5 h-long incubation (Fig. 3B). However, furin does not seem to be responsible for the cleavage of

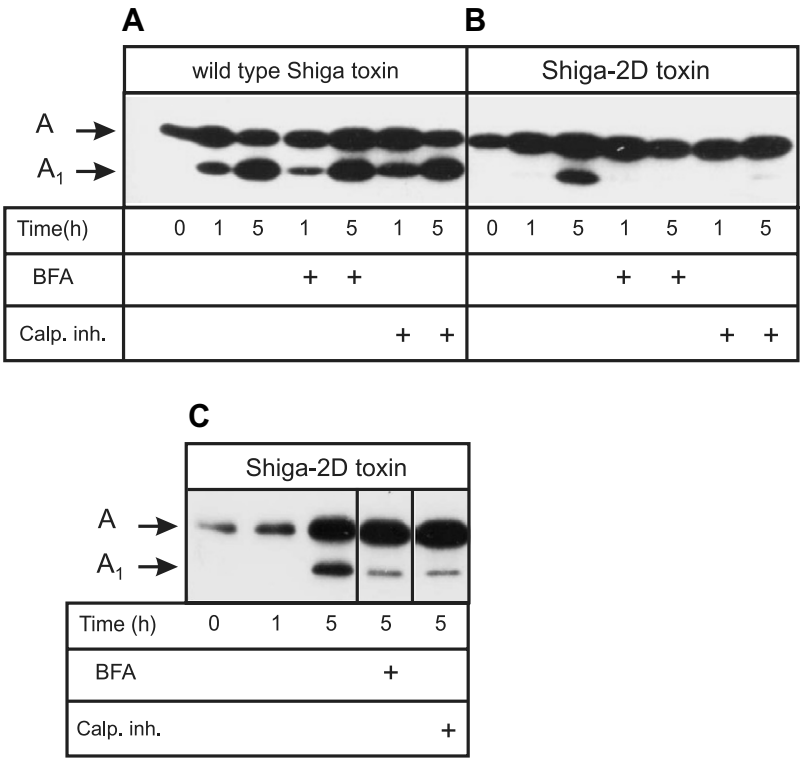

Fig. 3. Processing of wild type Shiga toxin and Shiga-2D toxin. HEp-2 cells (A, B) or LoVo/neo cells (C) were preincubated with or without BFA (2 μg/ml) or calpain inhibitor (Calp. inh.) (100 μg/ml) for 20 min at 37 °C. ^{125}I-labeled wild type Shiga toxin (A) or Shiga-2D toxin (B,C) were added and the incubation was continued for 1 or 5 h. Then, the cells were lysed and the proteins were precipitated and dissolved in sample buffer to be analyzed by SDS–PAGE and autoradiography. The first lane in A, B, and C (0 h) shows ^{125}I-labeled toxins that had not been incubated with cells. The figure shows a representative experiment.

Shiga-2D toxin after long incubation times since the cleavage was strongly inhibited by BFA (Fig. 3B), thus indicating that it occurs after Shiga-2D toxin has reached or passed through the Golgi apparatus. Furthermore, calpain is a cytosolic protease that has previously been implicated in the cleavage of Stx in cells lacking functional furin [11], and of Stx with mutations in the protease-cleavage sequence (Arg-X-X-Arg) [13]. As previously observed for Shiga toxin mutants where the two Arg have been substituted by His thereby preventing cleavage by furin [13], the processing of Shiga-2D toxin that occurs after long incubation times was blocked by a membrane permeable inhibitor of this protease (Fig. 3B). This result suggests that Shiga-2D toxin is able to reach the cytosol and then it might be cleaved by calpain. Moreover, in agreement with the idea that furin is responsible for the rapid cleavage of Stx in HEp-2 cells, the processing of the wild type Shiga toxin was not significantly affected either by BFA or by a calpain inhibitor (Fig. 3A). Finally, the processing of Shiga-2D toxin was also investigated in LoVo/neo cells (Fig. 3C) and in LoVo/fur cells (data not shown). Shiga-2D toxin was processed very slowly in LoVo/neo cells, but was cleaved to some extent during a 5 h-long incubation (Fig. 3C). Also in this case the cleavage of Shiga-2D toxin was reduced by BFA and by a calpain inhibitor, thus suggesting that furin is not responsible for this cleavage. In conclusion, these experiments indicate that Shiga-2D toxin cannot be processed by furin *in vivo*, but the mutant can be cleaved to some extent by other cellular proteases after long incubation times.

Reduced cytotoxicity of Shiga-2D toxin

Cleavage of Stx by furin is important for intoxication of cells [11]. In order to investigate the cytotoxicity of Shiga-2D toxin, protein synthesis was measured after 3 h incubation with the toxin. As shown in Fig. 4, Shiga-2D toxin prenicked with trypsin was able to inhibit protein synthesis to the same extent as prenicked wild type Stx both in LoVo/fur cells (Fig. 4A) and LoVo/neo cells (Fig. 4B), thus indicating that the deletion of ten amino acids residues does not change the routing or the stability of Shiga-2D toxin. However, LoVo/fur cells (Fig. 4A) and HEp-2 cells (data not shown) were less sensitive to uncleaved Shiga-2D toxin than to wild type Stx, in agreement with the results showing that furin can cleave and thereby activate Stx but not Shiga-2D toxin (Fig. 3). Furthermore, in LoVo/neo cells that lack functional furin both uncleaved wild type Stx and Shiga-2D mutant were less toxic than if precleaved toxins were added to the cells (Fig. 4B), thus indicating that the reduced toxicity of the Shiga-2D toxin is due to the fact that it cannot be cleaved by furin. As previously reported [11] and as shown in Fig. 4, wild type Stx and precleaved wild type Stx were similarly toxic in LoVo/fur cells, but

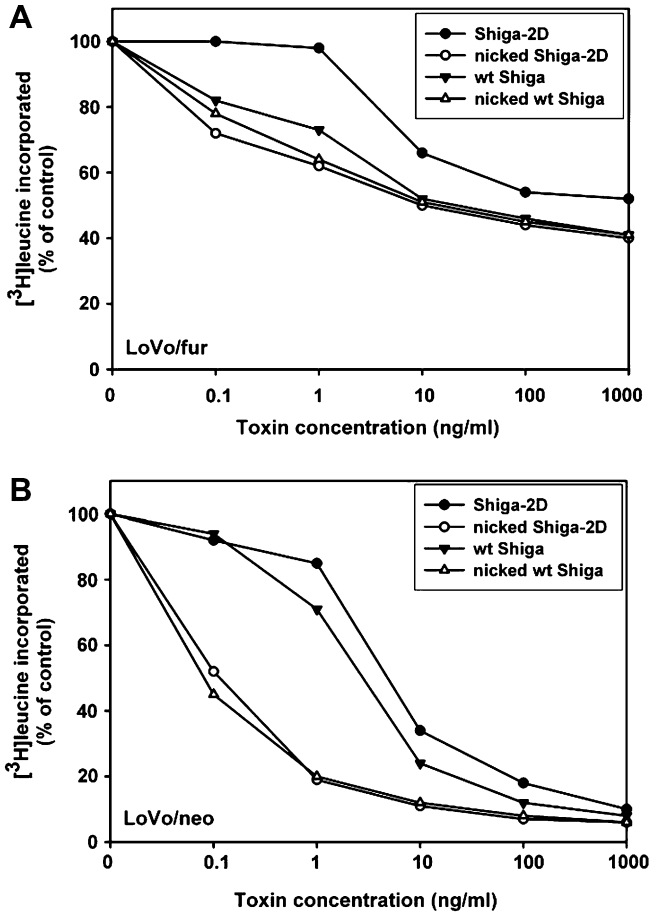

Fig. 4. Ability of intact and protease-nicked wild type Shiga toxin and Shiga-2D toxin to inhibit protein synthesis in LoVo/fur and LoVo/neo cells. LoVo/fur (A) and LoVo/neo (B) cells were incubated with increasing concentrations of nicked or unnicked toxins as indicated. The ability of the cells to incorporate [^{3}H]leucine was measured after 3 h at 37 °C. The figure shows a representative experiment.

not in LoVo/neo cells that lack functional furin and cannot efficiently process wild type Stx.

In conclusion, these experiments clearly show the importance of the disulfide loop in the A-chain of Stx for furin-induced cellular cleavage and activation.

Acknowledgments

The present study was supported by The Norwegian Cancer Society, The Research Council of Norway, The Functional Genomics (FUGE) programme from The Research Council of Norway, The Jahre foundation, Jeanette and Søren Bothner's legacy. J.V. Kozlov was supported by the Russian Foundation for Basic Research (Project No. 04-04-49854).

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