Elastase in Intestinal Mucus Enhances the Cytotoxicity of Shiga Toxin Type 2d*

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John F. Kokai-Kun‡, Angela R. Melton-Celsa, and Alison D. O’Brien§

From the Department of Microbiology and Immunology, Uniformed Services University of the Health Sciences, Bethesda, Maryland 20814

The Shiga toxins (Stx) expressed by Stx-producing Escherichia coli (STECh) and Shigella dysenteriae are a family of potent cytotoxins that are comprised of a single A subunit associated with a pentamer of B subunits (see Ref. 1 for a comprehensive review of STECh). The A subunit of Stx has N-glycosidase activity that cleaves an adenine residue from the 28S ribosomal RNA within the 60S ribosome, an event that halts protein synthesis and leads to the death of the affected cell. The A subunit of Stx is susceptible to trypsin cleavage near its C terminus. Such cleavage results in the formation of an A1 subunit of 28 kDa that has the N-glycosidase activity and an A2 peptide of 4 kDa that remains associated with A1 through a single disulfide bond. The A2 peptide links A1 to the B pentamer (2). The B pentamer of most Stx family members binds preferentially to globotriaosylceramide on eukaryotic cells (3, 4).

We have previously reported (5, 6) that when a variant of Stx2, called Stx2d (formerly named SLT-IIv), is incubated with crude mucus isolated from the small intestine of mice, the cytotoxicity of the mucus-treated Stx2d increases 10–1000-fold for Vero cells. We call this enhancement of cytotoxicity “activation.” STECh strains that produce Stx2d are exquisitely virulent in a streptomycin-treated mouse oral challenge model (7). In this mouse model, pathogenicity of STECh is due primarily to toxin production, as demonstrated by the finding that mice are protected by passive immunization with anti-Stx2 antibodies (7). The low LD50 of certain STECh strains correlates with the capacity of these strains to express the activable Stx2d (6). For example, one virulent strain, B2F1, that has an oral LD50 of <10 colony forming units (8), produces two activable toxins named Stx2d1 (formerly SLT-IIvha) and Stx2d2 (formerly SLT-IIvhb) (5) to distinguish them from one another. By contrast, STECh O157:H7 strain E32511/HSC that replicates as well as B2F1 in mouse small intestinal mucus but produces nonactivable Stx2c has an oral LD50 of 1010 colony forming units (8).

Amino acid sequence comparison of the activable Stx2d and nonactivable Stx2c reveal very few sequence differences (5). Indeed, the B subunit of Stx2d2 and Stx2c are identical, an observation that suggests that the key to the activable phenotype lies within the A subunit. There are only two amino acid differences between the A subunits of Stx2d2 and Stx2c, and both of these amino acids are located in the A2 peptide (9), a fact suggesting that the effect crude intestinal mucus has on Stx2d involves the A2 peptide.

The general nature of the alterations to Stx2d that occur after incubation with mucus were examined in a previous report (5). We demonstrated that there is no apparent modification to the A1 subunit of activated Stx2d based on electrophoretic mobility; changes to the A2 subunit could not be discerned at the time of the initial report (5) because the polyclonal serum used in the Western immunoblot analysis did not detect the A2 peptide. We also showed that isolated intestinal mucus nicked the A subunit of Stx2d and Stx2 to A1 and A2 in a manner similar to trypsin treatment, but only mucus and not trypsin activates Stx2d. In support of this, we found that trypsin-nicked Stx2d that is not activated can subsequently be activated by isolated mucus (5). From these data we concluded that nicking of the Stx2d A subunit to the A1 and A2 peptides is not equivalent to activation. In this study, we sought to isolate and identify the factor(s) from mouse crude small intestinal mucus that activates Stx2d and to further address the effect of this activating factor on Stx2d.
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EXPERIMENTAL PROCEDURES

Stx2d Activation Assay—The Stx2d cytotoxicity-enhancing activity of various mucous preparations and other samples was determined as described previously (5). Briefly, 50–60 ng of Stx2d, purified as reported (10), was incubated in a total volume of 30 µl with samples of different mucous preparations (as listed in Table 1), chromatography fractions (from below), or various commercial porcine pancreatic elastase preparations (Sigma: Roche Molecular Biochemicals; Calbiochem, San Diego, CA) for 1 h at 37 °C in the appropriate buffers. The commercially available porcine pancreatic elastase preparations were of varying purity. Based on estimations of Coomassie Blue-stained SDS-PAGE gels, the elastase content of these preparations ranged from ~50% to over 90% (data not shown). The porcine pancreatic elastase (EC 3.4.21.38) from Calbiochem (the purest) had a specific activity of 313 units/mg and a chymotrypsinogen content of 0.1% (as per the manufacturer’s labeling). The commercial elastase preparations were resuspended in 140 mM NaCl, 5 mM KCl, 2.5 mM phosphate, 10 mM HEPES, pH 7.4, 2.0 mM CaCl2, and 1.3 mM MgSO4 (11). The 50% cytotoxicity doses (CD50) of the treated toxin samples were determined on Vero cells as described previously (5, 12, 13). Fold activation of Stx2d cytotoxicity by various samples was determined by measuring the increase in Stx2d (6 µg per µg of activating substance protein).

Some samples were preincubated with various protease inhibitors (Sigma) (see Table II and Fig. 4 for concentrations) for 30 min at 37 °C prior to the addition of Stx2d. The percentage of maximal cytotoxicity-enhancing activity following protease inhibitor treatment was determined as the fold activation in the presence of an inhibitor divided by the fold activation in the absence of the inhibitor of similarly treated samples.

Isolation of an Stx2d-activating Factor from Mouse Crude Small Intestinal Mucus (Fig. 1)—Crude mucus from the small intestine of 10–15 male CD-1 mice was isolated and pooled as described previously (5, 7). The mucus pool was diluted to a final protein concentration of 10 mg/ml with 10 mM HEPES buffer, pH 7.4, and stored at ~80 °C until further use. Samples of mucus proteins (300 µg) were precipitated by a final 60% ammonium sulfate content, pH 8.9, at 4 °C. The precipitated mucus protein was resuspended in ion exchange chromatography (IEC) buffer (50 mM diethanolamine, 0.05 M NaCl, pH 8.9). (Note, all buffers used in this and subsequent steps contained 10% glycerol and 0.1 mM EDTA, and all chromatography was carried out at 22 °C.) The resuspended sample was subjected to ion exchange chromatography on a Q Sepharose High Performance resin column (Amerham Pharmacia Biotech) in a column with a bed volume of 25 ml. Following extensive washing of the column with IEC buffer, protein bound to the ion exchange column was eluted with an increasing linear salt gradient from 0.05 M NaCl in IEC buffer. Protein elution was monitored by absorbance at 280 nm.

Ion exchange chromatography fractions that contained Stx2d cytotoxicity-enhancing activity were pooled and adjusted to 4 × NaCl and to a pH of 7.4. Hydrophobic interaction chromatography (HIC) on Phenyl Sepharose High Performance resin (Amersham Pharmacia Biotech) in a column with a bed volume of 25 ml. Following extensive washing of the column with IEC buffer, protein bound to the ion exchange column was eluted with an increasing linear salt gradient from 0 to 0.05 M NaCl and a concurrent increasing pH gradient of pH 7 to 8.9 in HIC buffer.

Hydrophobic interaction chromatography fractions that contained Stx2d cytotoxicity-enhancing activity were pooled and n-octyl β-D-glucopyranoside (Sigma) was added to a final concentration of 0.1%. The pooled fractions were filtered through a YM 100 disc ultrafilter in an Amicon pressurized, stirred cell (Millipore, Bedford, MA). The ultrafiltration flow-through material was desalted to a final NaCl concentration of less than 10 mM by repeated dilution with 0.1% octyl β-D-glucopyranoside in water and ultrafiltration using a YM 10 filter (Millipore).

The desalted sample was separated by isoelectric focusing in a mini Rotofor cell (Bio-Rad) using 2% BioLyte ampholytes, pH range 4/6 (Bio-Rad) at a constant power of 10 W. Harvested Rotofor fractions were tested for Stx2d cytotoxicity-enhancing activity, and the peak fractions were pooled. Samples of some of these pools were subjected to a second round of isoelectric focusing in the absence of additional ampholytes to determine the isoelectric point of the isolated Stx2d-activating factor.

Electrophoresis—Fractions from the various steps in the Stx2d activator isolation scheme were analyzed by SDS-PAGE (14). Samples of 250–500 µl from each fraction were precipitated with 10% cold trichlo-roacetic acid. Precipitated proteins from each sample were washed with cold acetone and then resuspended in 1 µl Tris-HCl, pH 8, and subjected to SDS-PAGE on continuous 10 or 12% gels. The polyacrylamide gels were stained with Silver Stain Plus (Bio-Rad).

Gelatin gel electrophoresis was used to identify potential Stx2d activators with proteolytic activity. The procedure was conducted as described previously (15) with some variation. Briefly, gelatin (Bio-Rad) was co-polymerized with acrylamide to a final concentration of 0.2% gelatin in an SDS-PAGE gel. Samples to be subjected to this analysis were concentrated by microcentrifugation on a Microcon YM-10 filter (Millipore), and the concentrated samples were applied directly to the gel. After separation of the proteins in the samples by electrophoresis, the gel was incubated in 2.5% Triton X-100 (Sigma) for 1 h at room temperature to remove SDS. The gel was then stained with Coomassie Blue (16).

Stx2d (0.6–6 µg as indicated in figure legends) or Stx2d (5 µg) were treated with either mucus, trypsin (Promega, Madison, WI) or various samples from the Stx2d activator isolation scheme (as indicated in Figs. 5 and 6) and then analyzed by continuous 10% SDS-PAGE, two-dimen-sional electrophoresis, or 4–12% NuPAGE Bis-Tris gels (as per the manufacturer’s instructions; Novex, San Diego, CA). Two-dimensional electrophoresis was done by the method of O’Farrell (17) with a Bio-Rad Protein II xi 2-D Cell. Proteins were separated in the first dimension by isoelectric focusing in a combination of (11%) and 4/6 amphoteries (4.1 ratio, respectively). The second dimension separated proteins based on size on continuous 15% SDS-PAGE. Separated proteins from these three gel systems were transferred to BAS-NC nitrocellulose (Schleicher & Schuell) by electroblotting (18). Western immunoblot analysis of the nitrocellulose filter was conducted as described previously (5) with a 1:10000 dilution of either polyclonal rabbit anti-Stx2 or affinity-purified anti-Stx2d Asp (preparation of both described below) as the primary antibody.

Production of a Rabbit Anti-Stx2 Polyclonal Serum and Affinity Purified Rabbit Anti-Stx2d Asp Antibodies—Antiserum against Stx2d was prepared by inactivating Stx2d, purified as previously reported (10), with a 0.1% final concentration of formaldehyde at pH 7.5 for 6 weeks at ambient temperature. Next, Green and White rabbits (15) were given intraperitoneal injections of this Stx2d toxoid in Titermax (as per the manufacturer’s instructions, CytRx, Norcross, GA) every 2 weeks on the following schedule: injections 1–3, 25 µg of Stx2d toxoid; injection 4, 25 µg of Stx2d toxoid plus 100 ng of native Stx2d; injection 5, 25 µg of Stx2d toxoid plus 5 µg of native Stx2d; and injections 6–9, 25 µg of Stx2d toxoid plus 25 µg of native Stx2d. Sera collected from test bleeds were inactivated at 55 °C for 30 min and tested for Stx2d neutralization activity on Vero cells and for specificity by Western immunoblot analysis (data not shown).

Antiserum specific for the A5 fragment of Stx2d was generated at Genemed Biotechnologies Inc. (San Francisco, CA) by injecting rabbits with a synthetic peptide, NEESQFECQITGDRP, conjugated to keyhole limpet hemocyanin. The antiserum was purified by affinity chromatography with the peptide as the absorbent. The specificity of the purified antiserum was tested by immunoblot analysis with native or denatured toxin preparations (data not shown).

Amino Acid Sequencing—To identify the isolated intestinal mucus factor with Stx2d activating activity, amino acid sequence information was obtained. The eluant with Stx2d cytotoxicity-enhancing activity from hydrophobic interaction chromatography column (total volume, 10 ml) was concentrated to 1 ml by Amicon ultrafiltration with a YM 10 filter. This sample was further concentrated by precipitation with 20% trichloroacetic acid and subjected to SDS-PAGE electrophoresis as described above. The separated proteins were stained by coloidal Coomasie Blue, and the stained gel was sent to the W. M. Keck Biomedical Mass Spectrometry Laboratory at the University of Virginia for sequencing of the activator protein by capillary liquid chromatography-mass spectrometry and capillary liquid chromatography-tandem mass spectrometry. Peptide sequences obtained by these methods were compared with the nonredundant data base of GenBank mouse EST entries by BLAST (19) and Sequest algorithm searches.

Protein Concentration Determination—Protein concentrations were measured with a bicinchoninic acid protein assay kit (Pierce) with bovine serum albumin as a standard.

RESULTS

Survey of Cytotoxicity-enhancing Activity by Crude Intestinal Mucus from Various Sources—Previous work (5) showed that...
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TABLE I
Activation of Stx2d by crude intestinal mucus from various sources

| Mucus sourcea | CD-1 mice | Balb/c mice | SCID miceb | Gnotobiotic mice | CD/CD pigletc | CD/CD piglet colon | Weaned pigletd | Piglet protein-depletede | Ferret colonf |
|---------------|-----------|-------------|------------|----------------|---------------|-------------------|---------------|-----------------------|-------------|
| Fold Stx2d activation/μg protein | 0.5′ (0.2–0.7)g | 0.7 (0.2–1.0) | 0.9 (0.5–2.0) | 0.2 (0.1–0.3) | 0.03 (0.02–0.05) | 0.08 (0.05–0.1) | 0.1 (0.08–0.2) | Noneb | 0.08 (0.05–0.2) | Nonec |

a All crude mucus is from the small intestine unless otherwise stated.
b SCID, severe combined immunodeficient mice.
c CD/CD, cesarean-derived, colostrum-deprived (20).
d Isolated as described in Ref. 20.
e Used at maximum volume allowed by assay.
f All numbers represent geometric means.
g Range.
h No Stx2d activation activity detected.
i No activation activity detected in 55 μg of protein.
j No activation activity detected in 200 μg of protein.

TABLE II
The effect of various protease inhibitors on the Stx2d cytotoxicity-enhancing activity of mouse crude small intestinal mucus and the partially purified Stx2d activating factor

| Protease inhibitora | Final concentration of inhibitorb | Percentage of maximal activation by mucus | Percentage of maximal activation by partially purified activator |
|---------------------|----------------------------------|------------------------------------------|-------------------------------------------------------------|
| Sigma mix           | 1 μl/d                           | 8                                        | 11                                                          |
| Aprotinin           | 2 μg/ml                           | 100                                      | 48                                                          |
| Antipain            | 50 μg/ml                          | 100                                      | 28                                                          |
| α-antitrypsin       | 280 μg/ml                         | 1.4                                      | 0                                                           |
| Chymostatin         | 60 μg/ml                          | 100                                      | 8                                                           |
| 3,4 DCI             | 50 μg/ml                          | 0                                        | 0                                                           |
| E.64                | 10 μg/ml                          | 48                                       | 22                                                          |
| EDTA                | 0.5 μg/ml                         | 100                                      | 100                                                         |
| Leupeptin           | 0.5 μg/ml                         | 77                                       | 28                                                          |
| α-macroglobulin     | 333 μg/ml                         | 22                                       | 4                                                           |
| PMSF                | 170 μg/ml                         | 15                                       | 2.4                                                         |
| Trypsin Inhibitor (Soy Bean) | 100 μg/ml | 5                                       | 8                                                           |
| TLCKc               | 50 μg/ml                          | 100                                      | 50                                                          |
| TPCKe               | 100 μg/ml                         | 83                                       | 40                                                          |

a All inhibitors were purchased from Sigma.
b Manufacturers suggested maximum concentrations.
c Represents results of two repetitions.
d In a 30 μl total volume reaction.
e 3,4 DCI, 3,4-dichloroisocoumarin.
f PMSF, phenylmethylsulfonyl fluoride.
g TLCK, Nα-p-tosyl-l-lysine chloromethyl ketone.
h TPCK, Nα-tosyl-l-phenylalanine chloromethyl ketone.

Crude mucus from the small intestine and colon of mice and the colon of humans activates (increases) the cytotoxicity of Stx2d for Vero cells. To begin to identify the Stx2d-activating factor in mucus, the cytotoxicity-enhancing activity of crude intestinal mucus preparations from several readily available sources was investigated. Mouse crude mucus preparations from all sources examined had activating activity similar to CD-1 mouse small intestinal mucus and the partially purified Stx2d activating factor from mouse crude small intestinal mucus for isolation of the Stx2d-activating factor based on specific activity and availability.

Some Serine Protease Inhibitors Blocked the Stx2d Cytotoxicity-enhancing Activity of CD-1 Mouse Crude Small Intestinal Mucus—Mouse small intestinal mucus contains proteolytic activity as evidenced by the capacity of crude mucus to nick the Stx2d A subunit to the A1 and A2 peptides in a manner similar to purified trypsin (5). This nicking activity alone is not equivalent to activation (5), however. In this study we found that a general protease inhibitor mix blocked the Stx2d cytotoxicity-enhancing activity of crude mucus (Table II). This observation taken with our findings that protein-depleted mucus lost activity (Table I) and that the cytotoxicity-enhancing activity in

FIG. 1: A flow diagram of the steps used to isolate the Stx2d-activating factor from mouse crude small intestinal mucus.
mucus was sensitive to boiling and freeze-thawing (data not shown) suggested that this activator was a protein with proteolytic activity. To determine the kind of protease the activator might represent, more specific protease inhibitors were tested for the capacity to prevent Stx2d activation (Table II). Some protease inhibitors specific for serine proteases, e.g. soybean trypsin inhibitor, blocked the cytotoxicity-enhancing activity of mouse crude small intestinal mucus, whereas others did not. The pattern of the inhibition of Stx2d cytotoxicity-enhancing activity of crude mucus by various serine protease inhibitors suggested that the Stx2d activator had serine protease activity (Table II) but did not immediately suggest the identity of the activator.

**Isolation of a Factor from Mouse Crude Small Intestinal Mucus That Activated Stx2d Activity**—The Stx2d activator isolation scheme presented in Fig. 1 and detailed under “Experimental Procedures” was developed empirically. Stx2d cytotoxicity-enhancing activity generally eluted at 0.3 M NaCl from the Q Sepharose column and 1 M NaCl from the Phenyl Sepharose HP column (data not shown). Table III shows the increase in specific cytotoxicity-enhancing activity achieved at each step in the isolation. Each step of the isolation procedure also reduced the number of protein bands in the fractions that contained Stx2d cytotoxicity-enhancing activity (Fig. 2). Following the final isolation step, a single dominant 32-kDa protein consistently remained in the Rotofor fractions that had Stx2d cytotoxicity-enhancing activity (Fig. 2). This protein was found to have an isoelectric point of ~5.2 (data not shown). This 32-kDa protein also had proteolytic activity when examined on gelatin-containing SDS-PAGE (Fig. 2).

**Various Serine Protease Inhibitors Block the Stx2d Cytotoxicity-enhancing Activity in HIC Fractions**—The ~32-kDa protein isolated as described above and suspected of having Stx2d cytotoxicity-enhancing activity frequently stuck to the filtration membranes used in the later steps of the activator isolation scheme (data not shown). Because of this loss of material in the ultrafiltration and desalting steps, hydrophobic interaction chromatography eluant fractions that contained Stx2d cytotoxicity-enhancing activity were used to conduct some further functional assays. HCI eluant was incubated with a series of protease inhibitors prior to incubation with Stx2d. Many of these inhibitors eliminated or reduced the Stx2d cytotoxicity-enhancing activity of HCI eluant (Table II). Although the pattern of activator inhibition still did not definitively identify the Stx2d activator, the results supported the theory that the activator was a serine protease.

**Identification of the Isolated Mucus Protein with Stx2d Cytotoxicity-enhancing Activity**—To determine the identity of the dominant ~32-kDa protein in isolated fractions that contained Stx2d cytotoxicity-enhancing activity, some amino acid sequence data were determined. Material eluted from the HCI column was separated by SDS-PAGE, and the ~32-kDa protein believed to be the Stx2d-activating factor was eluted from the gel, digested with trypsin, and subjected to mass spectrometry.

![Fig. 2. A composite of SDS-PAGE of pooled peak Stx2d cytotoxicity-enhancing activity fractions from various steps in the isolation scheme of the Stx2d-activating factor and a lane from a gelatin gel on which the isolated activator was tested for proteolytic activity. Samples from the various steps were concentrated by precipitation with trichloroacetic acid where appropriate and subjected to electrophoresis on gelatin-containing SDS-PAGE.](http://www.jbc.org/)

### Table III

| Procedure                  | Total protein | Total activity | Recovery | Specific activity | Purification |
|----------------------------|---------------|----------------|----------|------------------|--------------|
| Crude mucus                | 375,000       | 245,578        | 100      | 0.65             | 0.97         |
| (NH₄)₂SO₄ precipitation    | 375,000       | 236,250        | 96       | 0.63             |              |
| Q Sepharose                | 962.5         | 28,875         | 11.7     | 30               | 46           |
| Phenyl-Sepharose HP        | 50            | 8050           | 3.3      | 161              | 247          |
| Ultrafiltration            | 25            | 7550           | 3        | 302              | 464          |
| Rotofor*                   | 10            | 3250           | 1.3      | 325              | 500          |

*The presence of ampholytes in Rotofor samples interferes with protein concentration assays, so the concentration of Stx2d activator in Rotofor peak samples was estimated based densitometric comparisons of silver-stained SDS-PAGE (data not shown).*
Alignment of the human and mouse translated elastase IIIB genes with peptides reported from sequencing of the isolated 33-kDa protein.

**Peptide 5**

| **Mouse** | **Human** |
|----------|-----------|
| VYRGSAAPVSHMMPGCYI | VYRGSAAPVSHMMPGCYI |

**Peptide 3**

| **Mouse** | **Human** |
|----------|-----------|
| CISTBYTQVGLGSHRENYGQERQYQTVIHMADLFYVPKNHMCYS | CISTBYTQVGLGSHRENYGQERQYQTVIHMADLFYVPKNHMCYS |

**Peptide 4**

| **Mouse** | **Human** |
|----------|-----------|
| CAVDAXAYV | CAVDAXAYV |

**Peptide 2**

| **Mouse** | **Human** |
|----------|-----------|
| SGNALGALQQLLNNVVDY | YTHCGLDQGAQDSVVPRPEEEEISSSGSKSTTMWACGIDSE |

**Peptide 1**

| **Mouse** | **Human** |
|----------|-----------|
| GCHDOSGPGLNPEGQSVQGVSFTAPQGNCW3REKVPVTTSYA | GCHDOSGPGLNPEGQSVQGVSFTAPQGNCW3REKVPVTTSYA |

**Fig. 3.** Alignment of peptide sequences obtained from mass spectrometry-based sequencing of the 32-kDa protein with the translated amino acid sequence of a mouse gene predicted to be homologous to the human elastase IIIB gene (22). Alignment of peptide sequences 3, 4, and 5 with the predicted amino acid sequence of *Mus musculus* clone 1001269 (gb:AA771563) (Mouse) and the amino acid sequence of human elastase IIIB precursor (gb:M18692) (Human). Sequences shown are the mature elastase IIIB proteins. X in peptide 4 indicates that the corresponding amino acid was not determined. The lowercase letters in the mouse sequence represent amino acids homologous to the human elastase IIIB that are reported to be in a different reading frame than the rest of the protein (i.e. frameshifts caused by sequencing errors). The asterisk indicates the predicted stop codon in the main open reading frame of the mouse gene.

Five peptide sequences of various lengths were obtained from this procedure. Three of these peptide sequences were found by a Sequest search of the EST data base (data not shown) to have homology with various elastases (21). A BLAST search of the nonredundant data base of the GenBank mouse EST entries revealed that the amino acid sequence of these three peptides closely matched the translated amino acid sequence of a predicted mouse gene (gb:AA771563) that has homology to the human elastase IIIB gene (gb:M18692; Ref. 22 and Fig. 3). The other two peptide sequences (LASPVTLNAR and ATITLTTSAQGK) had homology to mouse trypsin (23) and an *E. coli* outer membrane protein (24), respectively. Based on previous data that showed that trypsin does not activate Stx2d cytotoxicity (5) and the current finding that mucus from gnotobiotic animals contained cytotoxicity-enhancing activity (Table I), we concluded that the Stx2d activator was most likely a mouse elastase.

**Elastatinal Inhibited the Cytotoxicity-enhancing Activity of Crude Mucus and Isolated Stx2d Activator**—To provide further evidence in support of elastase as the Stx2d-activating factor found in crude mucus, the elastase-specific inhibitor elastatinal (25) was preincubated with either crude mucus from the small intestine, HIC eluant, or the isolated 32-kDa protein from the Rotofor fractions. As shown in Fig. 4, elastatinal inhibited the cytotoxicity-enhancing activity of all three samples, a finding that substantiates our hypothesis that the Stx2d activator is an elastase.

**Commerically Available Porcine Pancreatic Elastase Preparations Activated Stx2d Cytotoxicity**—To test whether elastase isolated from other animal sources can activate the cytotoxicity of Stx2d, various preparations of porcine pancreatic elastase from several suppliers were tested. All of these elastase prepar-
rations ranged from 0.8- to 4.7-fold activation/μg protein, whereas the isolated mouse elastase had an average specific activity of 170-fold activation/μg protein.

Elastase Can Nick the Stx2d A Subunit to the A1 and A2 Peptides in a Trypsin-like Manner—When Stx2d was treated with isolated mouse elastase (from Rotofor fractions, Fig. 5) or commercially available porcine pancreatic elastase (data not shown), activation occurred and the Stx2d A subunit was nicked to A1 and A2. The nicking of the Stx2d A subunit to A1 by elastase was inhibited by elastatinal, whereas nicking by trypsin was not (Fig. 5). Conversely, trypsin nicking of the Stx2d A subunit was inhibited by soybean trypsin inhibitor, whereas elastase nicking of the A subunit to A1 and A2 was not (Fig. 5). These findings, taken with the activation studies above, suggest that elastase has Stx2d nicking as well Stx2d cytotoxicity-enhancing activity. The nicking of the Stx2d A subunit to A1 and A2 by elastase appeared to be a separate event from the enhancement of Stx2d cytotoxicity by elastase because complete nicking occurred at concentrations of elastase below the minimum required to activate Stx2d cytotoxicity (Figs. 4B and 5). Furthermore, Stx2d was completely nicked by concentrations of porcine pancreatic elastase too low to activate the toxin, and the porcine elastase-nicked Stx2d could be activated by the addition of higher concentrations of elastase (data not shown).

Crude Mucus from Mouse Small Intestine and Isolated Mouse Elastase Affected the Mobility and Isoelectric Point of the Stx2d A2 Peptide—Crude small intestinal mucus from mice has no apparent effect on the size of the A1 peptide (5). To assess whether such crude mucus alters the size of the A2 peptide, we generated an antiserum specific for the Stx2d A2 peptide and used gel systems capable of resolving small differences between the molecular weights and changes in the isoelectric point of peptides. These analyses determined that there was a slight but reproducible difference in the mobility of the A2 peptide of Stx2d treated with trypsin alone versus Stx2d treated with trypsin plus crude mucus or isolated mouse elastase (Fig. 6). The increase in mobility of the A2 peptide was only seen in activated Stx2d samples and was completely inhibited by the addition of elastatinal (Fig. 6).

To further assess possible changes in elastase-treated Stx2d, two-dimensional electrophoresis was conducted. This analysis revealed that there was a change in the isoelectric point of the A2 peptide of elastase-treated Stx2d (activated) versus buffer-treated Stx2d (nonactivated). The A2 peptide from elastase-treated Stx2d migrated to a more basic pH, 82 mm from the cathode end of the gel in the first dimension of the electrophoresis, whereas the A2 peptide from buffer-treated Stx2d migrated to a more acidic pH, 91 mm from the cathode end of the gel (Fig. 7). There was no apparent change in the isoelectric points of the A1 subunits of elastase-treated versus buffer-treated Stx2d (data not shown). Elastase treatment also did not enhance the cytotoxicity of Stx2 for Vero cells (data not shown).

DISCUSSION

We have identified an Stx2d-cytotoxicity enhancing factor from mouse crude small intestinal mucus as an elastase. Al-
Elastatinal Enhances Stx2d Cytotoxicity

Fig. 6. Crude mucus from mouse small intestine and isolated mouse elastase alter the mobility of the A2 peptide in activated samples of Stx2d on SDS-PAGE. Stx2d (5.6 μg) was incubated for 2 h at 37 °C with trypsin (1.25 μg in all samples) plus buffer (Control), plus mucus (Mucus, 200 μg), or plus Rotofor peak fractions that contained mouse elastase (Activator, 3 μg, as estimated by densitometry). This incubation followed a preincubation at 37 °C for 30 min in the absence or presence of elastatinal (+Elastatinal, 40 μg). The samples were concentrated by microcin purification and resolved on a 4–12% NuPAGE Bis-Tris gel (Novex) and then electrotransferred to nitrocellulose. The Western immunoblot was developed with affinity-purified anti-Stx2d A2 antiserum incubated overnight at 1:1000 followed by a conjugated secondary antibody and chemoluminescence-based detection. The arrows at the left indicate the mobility of the activated (bottom arrow) versus the nonactivated (top arrow) Stx2d A2 peptides. Note that elastatinal alone did not alter the mobility of the Stx2d A2 subunit (data not shown). The cytotoxicity-enhancing activity for each sample was determined by testing a portion of each sample on Vero cells. The fold activation was determined by dividing the CD50 of each sample by the CD50 for the buffer-treated Stx2d. Fold activation results less than or equal to 1 were considered 0-fold activation.

though elastases have not previously been associated with activation or enhancement of toxic activity of bacterial toxins, a number of such toxins are expressed as inactive protoxins that require activation by proteolytic digestion to become toxic. This type of activation can occur either extracellularly (e.g. Clostridium septicum α toxin (26)) or intracellularly (e.g. Pseudomonas exotoxin (27), anthrax toxin protective antigen (28), and others (29)). A second kind of toxin activation is more unusual and involves enhancement of toxicity of an already active toxin by exogenous proteases (e.g. Clostridium perfringens enterotoxin (30–32)). Stx2d appears to fall into both of these categories. To become fully cytotoxic (33), Stx2d is nicked by proteases in or near a loop containing two Cys residues to separate the A subunit into the A1 and A2 peptides (2, 29), and then, as we report here, this fully cytotoxic Stx2d peptide appears to undergo an additional proteolytic cleavage event that enhances its cytotoxicity. The proteases that activate many toxins are often trypsin or chymotrypsin, in the case of extracellular activation (28, 30, 31), or furin, in the case of some intracellular activation (28). This is the first report of which we are aware that links elastase to toxin activation.

Protein sequencing based on mass spectrometry yielded five peptide sequences for the isolated activator. Three of these peptide sequences matched the predicted amino acid sequence of a translated mouse gene identified in the nonredundant database of GenBank™ mouse EST entries (Fig. 3). This gene is similar to the human elastase IIIB gene (22). The other two peptides had homology to mouse trypsin (24) and E. coli outer membrane protein 1 (24), respectively. Possible explanations for the presence of these two peptides in the sequencing reaction include: (i) the peptides were artifacts of mass spectrometry-based sequencing; (ii) one or both of these peptides originated from proteins that corepuriﬁed with the mouse elastase, e.g. mouse trypsin has a similar molecular weight and isoelectric point to mouse elastase (23); and (iii) one or both of these peptides may actually be part of the mouse elastase IIIB protein. The translated amino acid sequence for the mouse elastase IIIB gene (as presented in GenBank™) appears to be shorter than would be predicted from comparison with the human elastase IIIB protein sequence (Fig. 3). Therefore, the two unidentiﬁed peptides may have originated from the putative missing C-terminal amino acid sequence of this mouse elastase.

Elastatinal is a peptide derived from Actinomyces species that speciﬁcally inhibits elastase activity (25). This peptide completely inhibited the cytotoxicity-enhancing activity in both HIC peak fractions (Fig. 4) and the isolated 32-kDa protein in Rotofor fractions (Figs. 4 and 6). Higher concentrations of elastatinal also inhibited most, if not all, of the Stx2d cytotoxicity-enhancing activity of crude intestinal mucus (Figs. 4 and 6). These results are consistent with our conclusion that the 32-kDa protein is a mouse elastase with Stx2d cytotoxicity-enhancing activity.

Examination of Table II reveals some differences between the effect of various protease inhibitors on the cytotoxicity-enhancing activity of mouse crude small intestinal mucus versus partially puriﬁed Stx2d activating factor. Notably, although aprotinin, antipain, chymostatin and N-α-p-tosyl-L-lysine chloromethyl ketone did not have any effect on the cytotoxicity-enhancing activity of crude mucus when these inhibitors were used at the maximum recommended concentrations (see Table II for concentrations), these protease inhibitors blocked some of the cytotoxicity-enhancing activity of the partially puriﬁed activator. Two possible explanations for these apparently contradictory observations are as follows. First, the possibility exists that there are proteases other than elastase in intestinal mucus that can enhance the cytotoxicity of Stx2d, and these proteases are not inhibited by any of these four protease inhibitors. Second, and an explanation we consider more likely, is that the maximum recommended concentration for these protease inhibitors is insufficient to block the cytotoxicity-enhancing activity of elastase in crude mucus because elastase is only one of many serine proteases found in intestinal mucus. The many serine proteases in crude mucus may compete for inhibitors that are not speciﬁc or as efﬁcient for elastase and overwhelm their capacity to block elastase-mediated enhancement of Stx2d. This theory is supported by examination of the inhibition activities of elastatinal and 3,4-dichloroisocoumarin. Elastatinal is an elastase-speciﬁc inhibitor and almost completely blocked the cytotoxicity-enhancing activity of crude mucus (Fig. 4), and 3,4-dichloroisocoumarin is described by the manufacturer as “inhibiting serine proteases like elastase” and completely blocked the cytotoxicity-enhancing activity of crude mucus (Table II).

Although commercially available porcine pancreatic elastase preparations activated Stx2d cytotoxicity, the specific activity of these preparations was signiﬁcantly lower than that of isolated mouse elastase (see text). Although Tani et al. (22) have demonstrated that a human elastase IIIA (an elastase very similar to human elastase IIIB) cDNA probe hybridizes to RNA prepared from porcine pancreas, it is possible that this elastase type is not found in commercial porcine pancreatic elastase preparations. This possibility may explain the lower speciﬁc activity of the porcine pancreatic elastase preparations.

Human pancreatic elastase IIIB is similar (22) to another human elastase, cholesterol-binding pancreatic protease (34) (also called human pancreatic elastase I (35)). The preferred sites for cholesterol-binding pancreatic protease cleavage have...
Elastase Enhances Stx2d Cytotoxicity

Fig. 7. Isolated mouse elastase treatment alters the isoelectric point of the Stx2d A1 peptide. Stx2d (5 μg) was pretreated with 1.25 μg of trypsin for 15 min at 37 °C and then incubated for 2 h at 37 °C with 200 μl of HIC eluant (containing Stx2d activating activity) or buffer alone. The samples were concentrated by microspin concentration and separated by two-dimensional electrophoresis. In the first dimension, the proteins were separated based on isoelectric point in a pH range 3/10 and 4/6 ampholyte mixture. In the second dimension, proteins were separated on 15% continuous SDS-PAGE. The separated proteins were electrotransferred to nitrocellulose, and the Western immunoblot was developed with affinity-purified anti-Stx2d A1 antiserum and anti-Stx2 antisem incubated in combination overnight at 1:1000 followed by a conjugated secondary antibody and chemoluminesence based detection. A, buffer-treated Stx2d. B, HIC eluant-treated Stx2d. C, same as B superimposed over A. The single arrow at the left indicates the electrophoretic mobility of the Stx2d A1 peptide in the second dimension, whereas the double arrow indicates the electrophoretic mobility of the Stx2d A1 peptide in the second dimension. The buffer-treated Stx2d A1 peptide focused 91 mm from the cathode end of the isoelectric focusing gel at a pH of ~4.78, whereas the HIC eluant-treated Stx2d A1 peptide focused 82 mm from the cathode end of the isoelectric focusing gel at a pH of ~5.15. The numbers at the left of A and the corresponding marks on each panel indicate the position of molecular mass markers in kilodaltons for the second dimension. The cytotoxicity of the sample in B was enhanced 17-fold for Vero cells versus the cytotoxicity of the sample in A.

been determined to be the peptide bonds at the carboxyl ends of Ala, Val, Leu, Ser, His, and Thr (34). This observation is relevant to our studies because one of the two differences between the A2 subunit of activatable Stx2d2 and nonactivatable Stx2c is a Ser at position 291 in Stx2d2 in the place of a Phe in Stx2c (5). This Ser in Stx2d2 lies on the N-terminal side of a Leu found in both activatable and nonactivatable variants of Stx. We speculate that perhaps this leucine-serine bond is the site of the elastase cleavage that results in the activation of Stx2d2. Consistent with this speculation, the Stx2d A2 peptide demonstrates a slight increase in electrophoretic mobility following elastase treatment (Fig. 6). Elastase treatment of Stx2d2 also increased the isoelectric point of the A2 peptide, a finding that is consistent with the removal of a negatively charged amino acid like the glutamate at the C terminus of the Stx2d A2 (5). Identification of the precise site at which the Stx2d A2 is clipped by elastase and how this effect serves to increase the cytotoxicity of Stx2d2 is the subject of a separate ongoing study.2

Elastase cleaved Stx2d at two sites: between the A1 and A2 peptides (Fig. 5) and within the A2 peptide (Figs. 6 and 7). We were unable to determine whether the clipping of Stx2d at the first site (either by elastase or another protease) was required prior to enhancement of cytotoxicity by elastase. We were also unable to define the precise location at which the Stx2d A2 was clipped by elastase and how this effect serves to increase the cytotoxicity of Stx2d2. The amino acid residue at the activation site may not.

In this study we showed that the cytotoxic activity of Stx2d is increased by various elastases of mouse and porcine origin. These proteolytic enzymes are found in the intestinal mucus content of many species including humans (21, 22, 35, 36), where they would presumably be available for the activation of Stx2d produced by infecting E. coli. We have shown that strains of E. coli that express activable Stx2d are exquisitely virulent in a streptomycin-treated mouse oral challenge model (8), and we now hypothesize that in that model mouse pancreatic elastase can activate Stx2d in vivo and increase the virulence of the infecting strain (5, 6). Elastatin is nontoxic to mice (25), and it may be possible to decrease the virulence of strains of STEC that express Stx2d through the use of orally administered elastatin.

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