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EatA, an Immunogenic Protective Antigen of Enterotoxigenic Escherichia coli, Degrades Intestinal Mucin

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Enterotoxigenic Escherichia coli (ETEC) is a major cause of morbidity and mortality due to infectious diarrhea in developing countries for which there is presently no effective vaccine. A central challenge in ETEC vaccinology has been the identification of conserved surface antigens to formulate a broadly protective vaccine. Here, we demonstrate that EatA, an immunogenic secreted serine protease of ETEC, contributes to virulence by degrading MUC2, the major protein present in the small intestinal mucous layer, and that removal of this barrier in vitro accelerates toxin access to the enterocyte surface. In addition, we demonstrate that vaccination with the recombinant secreted passenger domain of EatA (rEatAp) elicits high titers of antibody and is protective against intestinal infection with ETEC. These findings may have significant implications for development of both subunit and live-attenuated vaccines against ETEC and other enteric pathogens, including Shigella flexneri, that express similar proteins.

Infectious diarrhea continues to cause substantial morbidity and remains a leading cause of death among children in areas where clean water and sanitation are limited (1, 2). Enterotoxigenic Escherichia coli (ETEC) infections range in severity from asymptomatic colonization to severe, cholera-like diarrhea (3, 4) and death that ensues from rapid dehydration. Currently, it is estimated that these infections are responsible for millions of infections and hundreds of thousands of deaths, particularly among children under the age of 2 years (5–7). While in general the death rate from diarrheal illness has declined (1, 8), these infections are still commonly associated with severe dehydrating forms of illness similar to Vibrio cholerae (9, 10). Recent data from the large Global Enteric Multicenter Study reaffirm the important role that these infections play particularly in young children (12). ETEC infections have also been shown to play a significant part in the complex association between malnutrition and repeated bouts of diarrheal illness among young children in developing countries (13, 14).

ETEC bacteria are defined by the production of heat-stable toxin (ST) and/or heat-labile toxin (LT) enterotoxins that elicit increases in cyclic nucleotides cyclic GMP (cGMP) and cyclic AMP (cAMP), respectively, in target intestinal epithelial cells. Activation of cyclic-nucleotide-dependent cellular kinases results in activation of the cystic fibrosis transmembrane regulatory (CFTR) channel leading to chloride efflux into the intestinal lumen with commensurate decreased absorption of sodium and water, ultimately resulting in profuse watery diarrhea (15).

Currently, there are no vaccines to protect against these important pathogens (16, 17). Most vaccines thus far have specifically targeted a relatively small group of antigens, namely, plasmid-encoded surface antigens known as colonization factors (CFs), or the heat-labile toxin. While prior infections with ETEC do appear to offer protection against subsequent infection (6, 18, 19), recent vaccine studies would suggest that the precise nature of this protection and the protective antigens involved are still being defined (20). Moreover, recent immunoproteomic studies (21) suggest that the immune response to ETEC infections is complex and involves recognition of multiple antigens, including both classical antigens, such as LT and the CF, and a number of novel pathotype-specific antigens.

One of the antigens identified in these studies, EatA, appears to be relatively conserved in the ETEC pathovar (22) and is represented by a geographically diverse collection of ETEC strains (23). Earlier studies demonstrated that EatA is a member of a family of molecules referred to as serine protease autotransporters of the Enterobacteriaceae (SPATE) (24). These molecules serve a variety of virulence functions in different enteric pathogens. EatA appears to modulate both adherence to epithelial cells and intestinal colonization in part by digesting EtpA, a novel exoprotein adhesin molecule that is secreted by ETEC (25).

However, a number of factors would suggest that this picture is incomplete and that EatA could have additional functions. First, while many strains that possess the eatA gene also encode the etpBAC two-partner secretion locus (26) responsible for production and secretion of EtpA, some do not. Furthermore, EatA homologues are present in other bacteria which do not make EtpA including Shigella (SepA) (27), strains of enteroaggregative E. coli (EAEC) associated with more severe diarrheal illness (28), and Shiga toxin-producing E. coli serotype O104:H4 strains that recently emerged in outbreaks of hemolytic-uremic syndrome (HUS) (29). Collectively, these data would suggest that EatA and similar molecules play an important common role in the pathogenesis of these enteric pathogens.

Here we demonstrate that EatA degrades MUC2, the major mucin secreted by intestinal epithelium. These data potentially provide an explanation for the widespread distribution of EatA.
and its homologues in a variety of mucosal pathogens and suggest that targeting of intestinal mucins may be critical to their virulence.

**MATERIALS AND METHODS**

**Bacterial strains and growth conditions.** Bacterial strains used in these studies are outlined in Table 1. ETEC strain H10407, originally isolated from a patient with severe cholera-like diarrheal illness in Bangladesh (30), was kindly supplied from stocks of this strain maintained at the Walter Reed Army Institute of Research according to good manufacturing practices by Stephen Savarino. Maintenance and propagation of human cell lines. LS174T intestinal epithelial cells (31) were propagated in Eagle’s minimum essential medium (E-MEM) supplemented with 10% (final concentration) fetal bovine serum (FBS). Jurkat cells were propagated in RPMI 1640 medium supplemented with 10% FBS. All cells were cultivated at 37°C in a 5% CO₂ atmosphere.

**Purification of recombinant EatA passenger domain.** Recombinant EatA passenger domain (rEatAₚ) was purified from E. coli LMG194ΔfliC (pSP019) (25) culture supernatants by ultrafiltration, followed by the addition of ammonium sulfate to 80% saturation and centrifugation. Pellets containing rEatAₚ were redissolved in 50 mM sodium phosphate and 200 mM NaCl and further purified by gel filtration chromatography (HiLoad 16/60 Superdex 200 prep grade; GE Life Sciences) in the same buffer. The passenger domain of the rEatAₚH134R mutant (rEatAₚ with a passenger domain mutation within the known catalytic site at histidine residue 134 [H134R]) was purified in an identical manner from E. coli LMG194ΔfliC(pSP019). When required, additional purification was performed by anion exchange. rEatAₚ in 25 mM imidazole (pH 7.0) was applied to a 5-ml column (HiTrap Q HP), and bound protein was then eluted over a linear 0 to 1 M gradient of NaCl in the same buffer. Protein purity was assessed by SDS-PAGE, and purified protein was buffer exchanged against phosphate-buffered saline (PBS) (pH 7.4) containing 10% glycerol before storage at −80°C.

**Antibody purification.** Polyclonal rabbit antisera raised against a fragment of the EatA passenger domain (24) was cross absorbed against an immobilized E. coli lysate column (Thermo Scientific) and then affinity purified using the passenger protein immobilized on nitrocellulose as previously described (25,32).

**Isolation of mucin.** To isolate intestinal gel-forming mucin MUC2, medium supernatants from tissue culture of LS174T cells (ATCC CL-188) that produce abundant MUC2 (33, 34), were first concentrated by ultrafiltration using a 100-kDa-molecular-weight cutoff (MWCO) filter. Following buffer exchange with 10 mM Tris-HCl and 250 mM NaCl (pH 7.4), size exclusion chromatography was performed using Sepharose CL-2B (35). Fractions containing intact MUC2 as confirmed by immunoblotting...
using anti-MUC2 antibody (described below) were pooled and saved at −80°C.

**Mucin degradation.** Degradation of purified MUC2 was examined by adding various amounts of rEatAp to 0.2 μg of purified MUC2 in 20-μl final reaction mixture volume in PBS (pH 7.4) and incubating at 37°C for the indicated time. The reaction digest was resolved on 3 to 8% Tris-acetate gradient gel (NuPAGE; Invitrogen) and transferred to nitrocellulose, and MUC2 degradation products were visualized by immunoblotting using anti-MUC2 rabbit polyclonal (IgG) antibody (H-300 [catalog no. sc-15334; Santa Cruz]) (1:2,000) that recognizes an epitope corresponding to amino acids 4880 to 5179 mapping at the C terminus of mucin 2 of human origin (gene identification [ID] 4583). To inhibit serine protease activity of rEatAp, 4-amidino-phenylmethanesulfonyl fluoride hydrochloride (APMSF) was used at a final concentration of 100 μM. A previously constructed active site mutant rEatApH134R (24) was also tested for its protease activity as described above. Degradation of cell-associated MUC2 was carried out by treating confluent monolayers of LS174T cells grown in 96-well plates with rEatApH134R (50 μg/ml final concentration in MEM without FBS) for 3 h or overnight at 37°C. Cells were lyzed by boiling in SDS-PAGE sample buffer, and immunoblotting was performed as described above.

**Substrate specificity of EatA.** In order to study substrate specificity of rEatAp, we used fluorescein isothiocyanate (FITC)-labeled substrates, including casein, IgG, gelatin, and bovine submaxillary mucin (BSM) in a kinetic assay based on the release of fluorescence from quenched substrates due to proteolysis as previously described (36). We also tested other glycoproteins as potential substrates for rEatAp, including IgA, lactoferrin, and heavily glycosylated mucin-like protein CD43 expressed at the surfaces of human T lymphocytes (37). Briefly, bovine lactoferrin (Sigma) or human IgA (Sigma) was dissolved at 0.5 mg/ml in 100 μl of 100 mM morpholinepropanesulfonic acid (MOPS) and 200 mM NaCl (pH 7.3). Digests were initiated by the addition of either 5 μg rEatAp or 1 μg proteinase K and then incubated at 37°C. Samples were removed at intervals, the reaction was stopped by boiling in SDS-PAGE buffer, and the products were separated by SDS-PAGE and visualized by Coomassie blue staining.

To test CD43 degradation, 1 × 10⁶ Jurkat cells were incubated for 3 h at 37°C and 5% CO₂ in 0.2 ml of RPMI 1640 medium without FBS, a protocol similar to that outlined by Szabady et al. (38). The cells were pelleted at 400 g for 5 min, washed with PBS, and blocked with 1% BSA in PBS, and cell surface CD43 was then labeled with phycoerythrin-conjugated anti-CD43 (mouse anti-human CD43 monoclonal clone L10; Invitrogen) and analyzed by flow cytometry.

**In vitro MUC2 degradation.** LS174T cells were treated with rEatAp or rEatApH134R (at a final concentration of 50 μg/ml) or vehicle control (PBS) for 3 h and examined by immunofluorescence confocal microscopy with anti-MUC2 rabbit polyclonal antibody (IgG) (H-300 [catalog no. sc-15334; Santa Cruz]). Images were saved as z-stacks, and image data were then analyzed using Volocity three-dimensional (3D) image analysis software (version 6.2; PerkinElmer, Inc.).

**Binding of labeled cholera toxin binding to epithelial cells.** LS174T cells were treated with rEatAp (at a final concentration of 5 μg/ml) or vehicle control (PBS) for 3 h as described above and examined by immunofluorescence confocal microscopy using cholera toxin B subunit conjugated with Alexa Fluor 488 (Life Technologies). Images were saved as z-stacks, and image data were then analyzed using Volocity 3D image analysis software (version 6.2; PerkinElmer, Inc.).

**Cellular cytotoxicity assay.** To examine rEatAp–treated cells for potential cytotoxicity, LS174T cells were grown to confluent monolayers in 96-well plates. After the tissue culture medium was removed, the cells were treated with prewarmed MEM alone or supplemented with rEatAp (25 or 50 μg/ml) for 3 h at 37°C and 5% CO₂. Triton X-100 (at final concentration of 0.1%) was used as a positive control. After the cells were treated, the medium was aspirated, and the cells were treated with 0.5 mg/ml MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide) in MEM with 10% FBS at 37°C and 5% CO₂ for 30 min. The medium was aspirated immediately, and the cells were washed with 200 μl of PBS. Cells were lysed by adding 200 μl dimethyl sulfoxide (DMSO), and optical density at 630 nm (OD₆₃₀) was recorded with DMSO alone as a blank. Data represent percentage of the mock-treated cells from triplicate experiments.

**EatA induction studies.** In order to test whether intestinal mucins can induce secretion of EatA by ETEC, we grew overnight cultures of ETEC H10407 in M9 medium containing 0.2% glucose for 3 h, and then the medium was supplemented with purified MUC2 (final concentration of 2 μg/ml). Samples of culture supernatant were collected at indicated time points, precipitated with trichloroacetic acid (TCA), and used to detect secreted EatA by immunoblotting using affinity-purified EatA antibody.
Immunization and intestinal colonization of mice. Groups of 10 CD-1 mice were immunized intranasally with either PBS, 1/100 g of heat-labile toxin (LT), or 1/100 g of LT plus 30/100 g of rEatA on days 0, 14, and 28. On day 42, mice were pretreated with streptomycin; the mice were given streptomycin (5 g per liter) in drinking water for 24 h, followed by drinking water alone for 12 h. Fecal samples (6 pellets/mouse) were collected on day 42, resuspended in buffer (10 mM Tris, 100 mM NaCl, 0.05% Tween 20, 5 mM sodium azide [pH 7.4]) overnight at 4°C, and then centrifuged the next day to pellet insoluble material and recover supernatant to test fecal antibody response (see below). After administration of famotidine to reduce gastric acidity, mice were challenged with ~10⁵ CFU of the lac-ZYA::Km' j876 (39) strain by oral gavage. Twenty-four hours after infection, the mice were sacrificed, their sera were collected, and dilutions of saponin intestinal lysates (40) were plated onto Luria agar plates containing kanamycin (25 μg/ml).

Immune responses to EatA and LT were determined using previously described kinetic enzyme-linked immunosorbent assay (ELISA) (41, 42). Briefly, ELISA wells were coated with 1 μg/ml of rEatA or LT in 0.1 M NaHCO₃ buffer (pH 8.6) overnight at 4°C. The next day, the wells were washed three times with Tris-buffered saline containing 0.005% Tween 20 (TBS-T), blocked with 1% bovine serum albumin (BSA) in TBS-T for 1 h at 37°C, and 100 μl of fecal suspensions (undiluted) or sera (diluted 1:100 in TBS-T with 1% BSA) was added per ELISA well and incubated at 37°C for 1 h. Horseradish peroxidase-conjugated secondary antibodies were used, and signal was detected with TMB (3,3',5,5'-tetramethylbenzidine)-peroxidase substrate (KPL) substrate.

To examine any differences in clearance of wild-type (WT) and eatA mutant strains, we monitored fecal shedding of bacteria by infected mice. Briefly, mice were challenged with either the lacZYA::Km' strain or eatA::Cm' mutant bacteria at 10⁵ CFU/dose, and stools (6 fecal pellets/mouse) were collected at days 1, 2, 3, 4, and 5 postinfection, resuspended in phosphate-buffered saline, and plated onto agar containing kanamycin (25 μg/ml) or chloramphenicol (15 μg/ml).

RESULTS

EatA preferentially degrades MUC2 intestinal mucin. Previous studies of EatA demonstrated that this protease was associated with enhanced intestinal fluid accumulation and epithelial cell...
One possible explanation for these findings is that EatA could enhance access to enterocytes by degradation of the mucin barrier in the small intestine. To test this hypothesis, we first examined the ability of purified rEatAp (Fig. 1a) to degrade the major mucin present in the small intestinal lumen, MUC2. Treatment of LS174T cell monolayers with rEatAp significantly reduced the amount of MUC2 associated with these cells (Fig. 1b to d) as detected by immunoblotting (Fig. 1b) and by confocal microscopy (Fig. 1c and d).

Deletion of eatA in ETEC H10407 resulted in significant loss of its MUC2-degrading activity. Concentrated culture supernatants from the H10407 strain degraded MUC2, while those obtained from the eatA mutant grown under identical conditions had no appreciable activity (Fig. 2a). Likewise, eatA mutant complementation with pSP014 expressing rEatAp regained the ability to degrade MUC2, while complementation with the plasmid (pSP019) expressing mutant rEatApH134R with a passenger domain mutation within the known catalytic site at histidine residue 134 (H134R) or the vector alone (pSB001) failed to rescue this phenotype. Similarly, purified rEatAp from a recombinant E. coli LMG194ΔflfC strain expressing pSP014 showed activity against mucin, while rEatApH134R purified from LMG194ΔflfC(pSP019) displayed no activity (Fig. 2b).

Purified rEatAp degraded MUC2 in a dose-dependent fashion (Fig. 2c). Mucin degradation by rEatAp was inhibited by the addition of the serine protease inhibitor APMSF (Fig. 2d). Consistent with the previously described proteolytic activity of EatA, rEatApH134R was completely deficient in the ability to degrade MUC2 over time (Fig. 2e and f).

Recombinant rEatAp passenger domain exhibited its greatest proteolytic activity against MUC2 at neutral to slightly acidic pH (Fig. 3), consistent with the environment of the small intestine (43). However, with this pH range, we were not able to demonstrate activity against other substrates, including IgA, IgG, casein, gelatin, lactoferrin, mucin-like glycoprotein CD43, or commercially available bovine submaxillary mucin (Fig. 4), indicating a high specificity of rEatAp for human intestinal mucin MUC2.

We also examined the effect of mucin on growth of wild-type and eatA mutant strains of ETEC. We saw no evidence that supplementation of media with purified MUC2 enhanced growth of either strain (data not shown). Likewise, the addition of MUC2 to growth media failed to stimulate the secretion or production of EatA by ETEC (data not shown).
EatA enhances toxin access to epithelial cells. Both heat-labile toxin (LT) and the closely related cholera toxin (CT) must engage GM1 ganglioside receptors on the surfaces of intestinal epithelial cells for their cellular activity. We reasoned that degradation of MUC2 on the surfaces of epithelial cells would unmask these receptors and promote enterotoxin binding. Indeed, treatment of epithelial cells with rEatAp significantly enhanced access of labeled cholera toxin B subunit to cognate receptors on the cell surface (Fig. 5a and b). In contrast, as might be predicted from its lack of homology to known cytotoxins in this class of proteins (44), rEatAp had no effect on the integrity of target epithelial cells, suggesting that it lacks any direct cytopathic effect (Fig. 5c).

EatA is highly immunogenic and a protective antigen. ETEC virulence is thought to require bacterial transit to the mucosal surface where these organisms directly engage enterocytes to effectively deliver LT and ST to receptors on the surfaces of these cells. In theory, the ability to degrade mucin would accelerate this process by promoting direct access of the bacteria to the enterocyte surface. Theoretically, bacteria that are incapable of degrading mucin would be quickly eliminated from the intestine as they are propelled along the intestinal lumen by peristaltic flow (45). However, we found that in a murine ETEC infection model, the eatA mutant appeared in only slightly greater numbers in stool (Fig. 6a) and while the mutant was eliminated somewhat faster than the wild-type ETEC parent (Fig. 6b), these differences in fecal shedding were not statistically different.

Nevertheless, we also questioned whether neutralization of EatA activity could serve as an effective complement to existing vaccine strategies, which so far have primarily targeted either the toxins themselves or fimbrial adhesin molecules (16, 17). Because we have recently shown that EatA is recognized by antibody present in convalescent-phase sera from patients following ETEC infection (21), we examined whether vaccination with rEatAp would offer protection against ETEC infection of the small intestinal mucosa in the murine model.

As predicted by earlier studies, immunization with rEatAp generated robust serum and mucosal antibody responses (Fig. 7a to d). More importantly, we found that mice vaccinated with rEatAp demonstrated significantly reduced small intestinal colonization following challenge with ETEC (Fig. 7e). Collectively, these data provide additional evidence suggesting that EatA plays a significant role in the pathogenesis of ETEC and potentially other important enteric pathogens that make very similar molecules.

DISCUSSION
Intestinal mucin represents a major host defense mechanism that significantly limits interaction of both commensal and pathogenic organisms with enterocytes to maintain mucosal integrity (46, 47). MUC2, the predominant gel-forming mucin present in the lumen of the small intestine (46, 48, 49) forms a complex multimeric glycoprotein network that bacterial pathogens must navigate if they are to effectively engage epithelial cells. Enteric pathogens have evolved a number of mechanisms to counter this barrier (46). These mechanisms include glycosidases to degrade oligosaccharide residues and peptidases that degrade the peptide backbone of these complex glycoproteins.

MUC2 is the major gel-forming mucin in both the small and large intestine. The core region of this molecule is heavily glycosylated and very resistant to the action of proteases, while interactions of the C-terminal and N-terminal ends contribute to the
colonization by degrading the EtpA adhesin (25) would suggest ability of EatA to modulate adhesion, toxin delivery, and intestinal neutralization of EatA enzymatic activity. Earlier studies of the domain of EatA, which possesses MUC2-degrading activity, afforded significant protection against infection of the small intestinal mucosa in the murine model. However, we should point out that it is not clear from these studies that this relates directly to neutralization of EatA enzymatic activity. Earlier studies of the ability of EatA to modulate adhesion, toxin delivery, and intestinal colonization by degrading the EtpA adhesin (25) would suggest that this enzyme potentially plays a complex role in the pathogenesis of ETEC. Moreover, despite the robust activity of EatA in degradation of human MUC2 in vitro, the anticipated impact on fecal shedding in mice proved to be modest, potentially relating to structural differences in the major gel-forming mucins of mice and humans or differential enzymatic activity in the complex environment of the intestine. Additional effort will likely be required to address the impact of EatA and related proteases on these very complex phenotypes.

EatA is a member of the serine protease autotransporters of the Enterobacteriaceae (SPATE) family of proteins. Another SPATE protein that is shared by both enteroaggregative E. coli and Shigella flexneri, Pic, has been shown to degrade a number of substrates, including mucins (50). However, unlike Pic, we were unable to demonstrate that EatA had any activity against bovine submaxillary mucin. These results are consistent with earlier studies of the EatA homologue SepA (44). Although several SPATE molecules, including Pic, have been shown to target a number of glycoproteins rich in O-linked glycans involved in the leukocyte trafficking and inflammation (37), this property is not shared by SepA, suggesting that these proteins may target a different class of molecules. Indeed, EatA was incapable of degrading the mucin-like CD43 molecule in our studies. Collectively, these findings suggest that enteric pathogens have evolved divergent mechanisms to address the barrier imposed by intestinal mucins.

EatA shares approximately 80% homology to SepA, a serine protease of Shigella flexneri. Similar to EatA of ETEC, the SepA Shigella homologue appeared to accelerate fluid accumulation in the ileal loop model of infection (24, 27). Although the exact function of SepA in Shigella pathogenesis is still unknown, our studies could suggest that SepA may be involved in degradation of MUC2 covering the colonic mucosa, the preferential site for Shigella colonization.

Additional molecular epidemiology studies may attest to the importance of EatA as a virulence determinant in ETEC and other enteric pathogens. In otherwise heterogeneous collections of ETEC from Chile (23) and Guinea Bissau (22), eatA genes were present in more than 70% of ETEC strains. Recently, EatA homologues have been identified in enteroaggregative E. coli associated with more-severe infections (28) and in serotype O104 Shiga toxin-producing E. coli that emerged suddenly in food-borne outbreaks in Europe (29). These organisms likely share in their need to penetrate mucin to cause severe infections. While ETEC infections may range from mild clinical illness to severe cholera-like diarrhea (3) associated with rapid dehydration, the contribution of eatA to the more severe manifestations is not clear at present. Nevertheless, the clear association of these organisms with death due to severe diarrheal illnesses in young children (12) provides a strong impetus to understand which virulence factors might contribute to these more life-threatening phenotypes.

As noted in our accompanying paper (53), ETEC strains also encode the type II secretion system effector protein VgHl, a metalloprotease which also degrades intestinal mucins. Collectively, these studies provide further corroborative evidence that the ability to breach the physical barrier imposed by mucins is an important virulence attribute for enteric pathogens, including enterotoxigenic E. coli.

Four decades after the discovery of ETEC (51), a broadly protective vaccine for ETEC has yet to be developed (16). The identification of novel immunogens and elucidation of the contribution of dimers and polymers of MUC2, respectively (49). Additional study will be needed to determine whether EatA cleaves MUC2 at either the unprotected amino or carboxy termini, similar to a cysteine protease of Entamoeba histolytica (49).

Our studies demonstrate that vaccination with the passenger domain of EatA, which possesses MUC2-degrading activity, afforded significant protection against infection of the small intestinal mucosa in the murine model. However, we should point out that it is not clear from these studies that this relates directly to neutralization of EatA enzymatic activity. Earlier studies of the ability of EatA to modulate adhesion, toxin delivery, and intestinal colonization by degrading the EtpA adhesin (25) would suggest formation of dimers and polymers of MUC2, respectively (49). Additional study will be needed to determine whether EatA cleaves MUC2 at either the unprotected amino or carboxy termini, similar to a cysteine protease of Entamoeba histolytica (49).
of E. coli and other antigens to virulence and protective immunity should inform rational approaches to ETEC vaccinology.

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