Broad Spectrum Activity of a Lectin-Like Bacterial Serine Protease Family on Human Leukocytes

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Abstract

The serine protease autotransporter from Enterobacteriaceae (SPATE) family, which number more than 25 proteases with apparent diverse functions, have been phylogenetically divided into two distinct classes, designated 1 and 2. We recently demonstrated that Pic and Tsh, two members of the class-2 SPATE family produced by intestinal and extraintestinal pathogenic E. coli, were able to cleave a number of O-glycosylated proteins on neutrophils and lymphocytes resulting in impaired leukocyte functions. Here we show that most members of the class-2 SPATE family have lectin-like properties and exhibit differential protease activity reliant on glycoprotein type and cell lineage. Protease activity was seen in virtually all tested O-glycosylated proteins including CD34, CD55, CD164, TIM1, TIM3, TIM4 and C1-INH. We also show that although SPATE proteins bound and cleaved glycoproteins more efficiently on granulocytes and monocytes, they also targeted glycoproteins on B, T and natural killer lymphocytes. Finally, we found that the characteristic domain-2 of class-2 SPATEs is not required for glycoprotease activity, but single amino acid mutations in Pic domain-1 to those residues naturally occurring in domain-1 of SepA, were sufficient to hamper Pic glycoprotease activity. This study shows that most class-2 SPATEs have redundant activities and suggest that they may function as immunomodulators at several levels of the immune system.

Introduction

The most abundant and functionally diverse proteolytic enzymes in living organisms are the serine proteases [1]. The serine protease autotransporters from Enterobacteriaceae (SPATE) constitute a superfamily of virulence factors whose members resemble those belonging to the trypsin-like superfamily of serine proteases [2,3]. SPATE proteins are produced by enteric pathogens including E. coli, Shigella and recently, also found in Salmonella, Edwardsiella, and Citrobacter species [2,3]. Interestingly, SPATEs are produced by all recognized pathogenic E. coli strains including enteropathogenic E. coli (EPEC), shiga toxin-producing E. coli (STE), enterotoxigenic E. coli (ETEC), enterohemorrhagic E. coli (EHEC), enteroinvasive E. coli (EIEC), enteroaegregative E. coli (EAEC), adherent-entererotoxigenic E. coli (AIEC), uropathogenic E. coli (UPEC) and by the animal pathogens: avian pathogenic E. coli (APEC) and rabbit pathogenic E. coli (REPEC), all agents of enteric/diarrheal disease [2,3].

SPATEs are secreted members of the autotransporter family, whose secretion involves the excision of the N-terminal region, known as the “passenger domain”, from the C-terminal region or “β-domain region”, and subsequent release of the passenger domain (which harbors the proteolytic activity) into the cell surroundings [2,4].

The SPATE family, which now includes more than 25 proteases, has been phylogenetically divided into class-1 and class-2 based on the amino acid sequence of the passenger domain [2,3,5]. The bifurcation of SPATEs into two classes is consistent with structural differences and biological effects. Class-1 SPATEs such as Pet, SigA, Sat, and EspC for example, display similar substrate specificity, consistent cytotoxic effects on cultured cells, and enterotoxin activity on intestinal tissues (reviewed in reference [2]). On the other hand, most knowledge on class 2 SPATEs comes from two members of this family: Tsh/Hbp and the Pic protease. Tsh (Temperature-sensitive hemagglutinin), the first class-2 SPATE isolated from a septicemic pathogen in poultry [6], was found to confer hemagglutination and binding to extracellular matrix proteins, such as fibronectin and collagen IV. Subsequently, Hbp (Haemoglobin binding protein) identified in the E. coli strain (EB1) [7], isolated from a human wound infection, was shown to differ from Tsh in only two residues. Hbp was able to interact with hemoglobin, to degrade it, and subsequently to bind the released heme suggesting a role for Hbp in heme acquisition [7]. Pic (Protease involved in intestinal colonization) originally identified in Shigella flexneri 2a and EAEC [8], was found to cleave mucin from numerous sources, to induce mucus release [9],

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and to confer a subtle competitive advantage in mucosal colonization [10]. We recently showed that Pic and Tsh/Hb cleave a variety of leukocyte surface glycoproteins with diverse roles in numerous cellular and immune functions, and which were substituted with carbohydrates structurally similar to those found on human mucin glycoproteins [11]. More importantly, cleavage

Table 1. Primer sequences used in this study.

| Primer Name | Sequence | Use |
|-------------|----------|-----|
| S124T sense | 5′-gtatcgcctctcattcatctgcgaagctgaatcctgac-3′ | Site mutagenesis |
| S124T antisense | 5′-cgtgtatgtttaatgacgatctctgctgtctgcaagtc-3′ | Site mutagenesis |
| N128V sense | 5′-cagttatatgtcagctgaacgctgagctgagctgac-3′ | Site mutagenesis |
| N128V antisense | 5′-tcctgatactccactctgctgatctgctgctgctgcaagtc-3′ | Site mutagenesis |
| Y131S sense | 5′-cagctcaaccctcactctgctgatctgctgctgcaagtc-3′ | Site mutagenesis |
| Y131S antisense | 5′-aaagctcaaccctcactctgctgatctgctgcaagtc-3′ | Site mutagenesis |
| N182Y sense | 5′-ggaatatgtattttttccatctaaccctcactctgctgcaagtc-3′ | Site mutagenesis |
| N182Y antisense | 5′-tcgaggtggtgtattttttccatctaaccctcactctgctgcaagtc-3′ | Site mutagenesis |
| L145A sense | 5′-aagggtggttattacgccagggaatgattatcttcttctccattac-3′ | Site mutagenesis |
| L145A antisense | 5′-gtaatggttaacccataatctccgcgttgacgcgttaaaccctcattc-3′ | Site mutagenesis |
| N153G sense | 5′-ctgagctggaagctgaaccctcactctgctgcaagtc-3′ | Site mutagenesis |
| N153G antisense | 5′-gaagctggaagctgaaccctcactctgctgcaagtc-3′ | Site mutagenesis |
| G253L sense | 5′-aagggccttaactgcagtttctccgttgacgcgttaaaccctcattc-3′ | Site mutagenesis |
| G253L antisense | 5′-cccaggggttaaagctgctagaacgccggttaaaccctcattc-3′ | Site mutagenesis |
| pBADadcA sense | 5′-cgagccggtgaaggcttgctgctgctgctgctgcaagtc-3′ | AdcA cloning |
| pBADadcA antisense | 5′-taagctggaagcttgctgctgctgctgctgcaagtc-3′ | AdcA cloning |
| pBADcrc2sp sense | 5′-cgagccggtgaaggcttgctgctgctgctgcaagtc-3′ | Crc2sp cloning |
| pBADcrc2 antisense | 5′-taagctggaagcttgctgctgctgctgcaagtc-3′ | Crc2sp cloning |

Figure 1. Sequence relationships of class-2 SPATE proteins. The phylogenetic analysis of the aminoacidic sequence of class-2 SPATEs shown here was modified from reference [2], with permission of the Editorial board. The phylogenetic tree shows allelic variants of SPATEs in clusters (colored branches) Bacterial species and pathotypes from which SPATE sequences were derived are shown on the right side. Class-2 SPATEs also include a cluster of proteases found mostly in animal pathogens, which lack of the classic domain-2 (green branches, see text). SPATE members of each major cluster purified in this study are indicated with asterisks.

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of those glycoproteins triggered adverse effects on leukocyte functions such as chemotaxis, transmigration, activation and apoptosis [11]. Therefore, we have proposed a main role for Pic and Tsh/Hbp in immune evasion [11]. Here, we show that the class-2 SPATE family shares a lectin-like property and have proteolytic activity against a large variety of O-linked glycoproteins in the hematopoietic cell lineage, and in both, innate and adaptive immunity.

Figure 2. Class-2 SPATEs trigger leukoagglutination. $1 \times 10^5$ Jurkat T cell were incubated with 2 µM of each class-2 SPATE at 37°C in a CO$_2$ incubator. One hour following initial treatment, cells were analyzed in an inverted light microscopy. Micrographs were taken in the light field at 20× magnification.

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**Materials and Methods**

Recombinant proteins and antibodies. Human glycosylated recombinant proteins: C1-INH, CX3CL1, CD44, CD45, CD55, CD97, CD99, CD162, TIM1, TIM3, TIM4, fibronectin, E-selectin and human Integrin alpha M beta 2 were obtained from R&D systems Inc. (Minneapolis, MN 55413), while the human glycosylated recombinant proteins: CD34, CD43, CD93, and CD164 were obtained from Sino Biologicals (Beijing 100176 P. R. China). Antibodies used in this study were dye conjugated mouse anti human CD3, CD4, CD8, CD14, CD16, CD56, CD43, CD44, CD45 and CD162 from BD and Invitrogen). Rabbit anti-sialic acid antibody was purchased from Cloud Clone Corp, Houston, TX.

**Cell lines and human primary cells**

Binding experiments and immunofluorescence assays were performed in the Jurkat T cell line (Clone E6-1, American Type Culture Collection, Manassas, VA,20110. USA). Jurkat cells were routinely cultured in RPMI 1640 medium, supplemented with 10% heat inactivated fetal bovine serum (FBS) and antibiotics: 100 U/ml penicillin, and 100 μg/ml streptomycin at 37°C in a humidified 95% air and 5% CO2. Fresh human blood to isolate primary cells were purchased from Allcells LLC. (Alameda, CA 94502).

**Leukagglutination assays**

Jurkat cells were incubated in 48-well plates at a concentration of 5x10^5 cells/200 μl in RPMI medium containing 10% FBS and incubated at 37°C and CO2 with different SPATEs and observed in an inverted microscope at 1 and 3 hrs.

**Deglycosylation of glycoproteins**

Glycoproteins were deglycosylated with deglycosylation enzyme cocktail (New England BioLab) under denaturing and non-denaturing conditions, and following the manufacturer’s recommendations. Denaturing conditions: briefly, 20 μg of glycoproteins in denaturing buffer were denatured by heating at 100°C for 10 minutes. Denatured proteins were treated with G7 reaction buffer, NP40, and 5 μl deglycosylation enzyme cocktail [Neuraminidase (12,500), O-Glycosidase (10000000 units), β1-4-Galactosidase (2000 units), β-N-acetylglucosaminidase (1000 units), PNGase-F (125000 units)], and incubated overnight at 37°C. Non denaturing conditions: 20 μg of glycoproteins in G7 reaction buffer were treated with 5 μl of deglycosylation enzyme cocktail, mixed gently and incubated overnight at 37°C. To determine the extent of deglycosylation by both protocols, samples were analyzed by mobility shifts on SDS-PAGE gels.

**Immunofluorescence assays**

Following incubation of Jurkat cells with SPATEs, cells were washed and suspended in flow cytometry buffer (HBSS without Ca2+/Mg2+ and 0.2% BSA) and blocked with human IgG. Then, cells were incubated for 1 hour with FITC anti-CD43 and PE anti-CD162, washed twice with FACS buffer and fixed with 2.5% formalin. 10 μl were extended on slides and allowed to dry out, and counterstained with ProLong Gold antifade reagent with DAPI (Invitrogen). Samples were analyzed in a Leica fluorescence microscope.

**Binding of SPATEs to leukocytes**

To determine binding of SPATEs to different leukocyte subpopulations, SPATEs were labeled with FITC (Pierce Biotechnology). Therefore, 1 mg/mL of each SPATE were incubated with 100 μg/mL FITC in 0.1 M sodium carbonate buffer (pH 9.6) for 1 hour at room temperature. Using desalting columns from the same manufacturer, FITC-labeled SPATE (SPATE-FITC) were separated from unbound FITC. For binding of SPATEs-FITC to leukocytes, 1 million total leukocytes were incubated with increasing concentrations of SPATE-FITC in HBSS/1% BSA buffer for 30 minutes on ice. Alternately, cells were incubated with heat-denatured SPATE labeled with FITC or a combination of equimolar concentrations of unlabeled SPATE and SPATE-FITC. SPATE binding on specific cell types was identified through “forward and side scatter” gates plus cell type-specific lineage markers including anti-CD3, -CD14, -CD16, -CD19, and -CD56 ([12]) (Figure S1). After washing the cells twice with HBSS/1% BSA, fluorescence was measured on a flow cytometer (Beckmann Coulter Cyan ADP LX, Brea, California) and data were analyzed with Flow version 4.5 (TreeStar, Ashland, OR).

**Preparation of total leukocyte cells**

Fresh heparinized blood from healthy human donors purchased from AllCells LLC. (Alameda, CA 94502) was mixed 1:1 with dextran/0.9% NaCl (final concentration, 1.5% dextran) and allowed to settle undisturbed for 30 min. To remove residual erythrocytes, the cell fraction from the leukocyte-rich plasma was spun down at 1500 rpm/15 min and resuspended in 12 ml of cold sterile water for 25 seconds followed by addition of 4 ml of 3% NaCl. Red cell debris was removed by centrifugation at 1500 rpm/10 min, followed by a wash with HBSS buffer without Ca++/Mg++ (Invitro gene). Total leukocytes (this fraction included lymphocytes, NK, monocytes and granulocytes) were enumerated by using a hemocytometer.

**Purification of SPATE proteins**

Expression and purification of SPATE proteins from minimal clones in E. coli HB101 have been described previously [13]. Anion-exchange purified proteins were treated with Endo-Trap Blue (Hyglos GmbH. 82347 Bernried am Starnberger See Germany) to remove LPS according to the manufacturer’s specifications, dialyzed in 1× PBS pH 7.0, and stored in small aliquots at −80°C.

**Construction of pBAD30-Crc2sp and pBAD30-AdcA**

Most of the SPATE minimal clones used in this study were published previously [13], except the Crc2sp and AdcA clones.
which were constructed in this study. The crc2sp (NCBI accession No. YP_003368482.1) [2] and adcA (NCBI accession No. YP_003367548.1 [2,14], genes were amplified by PCR from the C. rodentium genome (American Type Culture Collection, Manassas, VA,20110. USA) using Pfx platinum DNA polymerase (Invitrogen) and primers in Table-1. The amplification products were digested with SacI and XhoI restriction enzymes and cloned under the pBAD promoter in the pBAD30 plasmid previously digested with SacI and SalI enzymes. The PCR cycling conditions were as follows: denaturing at 95°C for 5 min; followed by 30 cycles of denaturing at 94°C for 2 min, annealing at 60°C for 30 s, and extension at 68°C for 2 min; and a final cycle of 68°C for 10 min.

Site-directed mutagenesis

Site-directed mutagenesis was performed following the Quik-Change protocol (Stratagene, Cedar Creek, TX) and with the PfuTurbo (Stratagene) high-fidelity polymerase. The pACYC184-Pic template was used at 25 to 50 ng per reaction with 10 pmol of each of the complementary primers. Reactions were carried out according to the manufacturer’s protocol. Primers used to generate the single point mutations are shown in table-1. All constructs were verified by sequencing at the University of Virginia DNA Core Facility.

Results

Phylogenetic analysis of the Class-2 SPATE family

The SPATE family has been divided into two classes. Figure-1 shows an updated phylogenetic tree of the class-2 SPATE subfamily (reviewed in [2]). Members of this class show high degree of allelic variation and are grouped into clusters (Figure 1, colored branches). Among class-2 SPATEs, there is a subset of hypothetical allelic SPATE variants that do not exhibit the characteristic N-terminal protein protuberance termed domain-2 [15], exhibited by Tsh/Hbp and many other class-2 SPATEs, and which are mainly distributed among animal pathogens (Figure 1,
Figure 5. Class-2 SPATEs do not degrade non mucin-type, O-linked glycoproteins. A, In order to assess the proteolytic activity of class-2 SPATEs on other heavily glycosylated proteins, we treated CD97, CD99, E-selectin, αv-integrin and fibronectin with 2 μM class-2 SPATEs by overnight incubation at 37 C. Samples were analyzed by SDS-PAGE and Blue Commassie staining. B, Proteolytic activity of SPATEs was also evaluated on...
green branch). In order to determine whether the class-2 family share similar properties with Pic and Tsh/Hbp, we purified one or more members of each major phylogenetic cluster (Figure 1, indicated with an asterisk) including Pic, PicU, Crc2sp, Tsh/Hbp, Vat-Ex, AdcA, EpeA and SepA from minimal clones in E. coli HB101. PicU and Crc2sp share 95.7/97.6% and 74.6/85.0% identity/similarity to Pic, respectively; AdcA and Vat-Ex share 45.6/62.4% and 69.6/79.2% identity/similarity to Tsh/Hbp, respectively. SepA and EpeA still belonging to the class-2 SPATE family occupy more distant branches, sharing 49.5/67.4% and 43.7/62.4% identity/similarity to Pic, respectively.

Class-2 SPATEs agglutinate white cells

Pic and Tsh have been previously shown to display lectin-like activity, agglutinating red cells and cleaving leukocyte glycoproteins. To assess agglutination ability to leukocytes, we incubated Jurkat T cells with 2 μM of purified SPATE proteins for 1 h and recorded the agglutination phenotype microscopically (Figure 2). We noticed that although most class-2 SPATEs were able to agglutinate white cells, a heat-denatured Pic, the Pic258A mutant (a protease deficient Pic), SepA and EpeA did not display this activity. Likewise, the class-1 SPATE, Sat did not show this phenotype (Figure 2). Since the agglutinating activity seemed to be a consequence of protease activity, we next looked for direct binding of SPATEs to leukocytes.

Binding of SPATEs to human Leukocyte subpopulations

To further document direct binding of SPATEs to leukocytes, we incubated total human leukocytes with FITC-labeled Pic, the protease deficient Pic258A and Tsh for 30 minutes in ice. Specific cell types were identified through scatter gates and cell type-specific lineage markers by flow cytometry (Figure 3) [12]. SPATEs bound efficiently to monocytes (>60% CD14+) and granulocytes (>97%), whereas binding of SPATEs to T (>10%, CD3+) and B (>11%, CD19+) lymphocytes was to a lesser degree (Figure 3A). In order to determine the specificity of this binding we incubated leukocyte populations with FITC-labeled heat-denatured Pic258A, Tsh or SepA. In a separate experiment we incubated leukocytes with an equimolar combination of FITC-labeled and unlabeled SPATEs, and determined the percentage of leukocyte demonstrating FITC-SPATE binding. We observed substantial reduction of Pic258A and Tsh/Hbp binding to granulocytes and lymphocytes when incubating with denatured SPATEs or when competing with unlabeled SPATEs, while SepA showed only background binding levels to leukocytes, which was not affected when using denatured SepA or when competing with unlabeled SepA (Figure 3B).

Broad spectrum activity of class-2 SPATEs on mucin-type O-glycoproteins of the immune system

We previously showed that Pic and Tsh/Hbp were able to cleave O-linked glycoproteins such as CD43, CD44, CD45, CD93, CD162 and CX3CL on human neutrophils and lymphocytes. Here we tested all other class-2 SPATEs on the same and new glycoproteins involved in diverse functions of the immune system (Figure 4). Three μg of glycoproteins were incubated with 2 μM of each SPATE for 1 h at 37°C, and analyzed by SDS-PAGE and Commassie staining. We found that most class-2 SPATEs tested except SepA, EpeA or the Pic258A mutant efficiently cleaved the substrates mentioned above, and many others including CD34, CD55, CD164, TIM1, TIM2, TIM3 and C1-INH, all of them known to possess O-linked carbohydrates (Figure 4) [16,17,18,19,20]. Pic, PicU and Crc2sp showed the most astringent protease activity as we noticed extensive cleavage in almost all glycoproteins tested. Tsh/Hbp and Vat-Ex showed comparable activities. The differential cleavage pattern seen on glycoproteins could be explained by the glycosylation level of the molecules, as some glycoproteins such as CD43, CD93, CD162 and CX3CL are known to be extensively O-glycosylated [21,22,23,24], and which were almost completely digested by SPATEs, suggesting the presence of multiple cleavage sites. CD44 and CD45 are known to be just partially glycosylated reliant on isoforms [21,25], whereas CD55 and C1-INH have limited sites of O-glycosylation [19,20,25]. Extensive degradation of slightly glycosylated substrates by SPATEs was not seen despite prolonged incubation times (overnight) or with higher SPATE concentrations (>2 uM). SepA, EpeA or the PicS258A did not show any protease activity under these conditions (Data not shown).

In our previous study we found that the Pic protease activity was dependent on mucin-type O-glycosylation [11]. In order to determine whether other glycoprotein types are also susceptible to SPATE proteolytic activity, we incubated CD97 and CD99 [26,27]; two non-mucin type glycoproteins, and three heavily N-linked glycosylated proteins such as the fibronectin, E-selectin and the alpha (M)beta (2) integrin with SPATEs overnight at 37°C and analyzed them by SDS-PAGE for proteolytic cleavage. Under these conditions, none of the these molecules were susceptible to SPATEs (Figure 5A). Furthermore, SPATE proteins had no effect on leukocyte molecules such as CD16, CD14, CD19, CD3 and CD8 as assessed by flow cytometry (Figure 5B).

Class-2 SPATEs target glycoproteins on all hematopoietic lineage

To evaluate the proteolytic activity of SPATEs on native leukocyte glycoproteins, we isolated white cells from healthy donors and incubated them with equimolar concentration of SPATEs for 1 h at 37°C, followed by assessment of cleavage by flow cytometry. Specific cell types and glycoproteins were identified through scatter gates, cell type-specific lineage markers, and dye-conjugated antibodies specific to SPATE-susceptible glycoproteins such as CD43, CD44, CD45 and CD162. As expected, most SPATEs except EpeA, SepA or the non-class-2 SPATE EspP were able to cleave glycoproteins on the surface of granulocytes, monocytes (CD14+) and T(CD3+), BCD19+) and natural killer(CD3−, CD16+, CD56+) lymphocytes (Figure 6). Overall, we observed efficient cleavage of O-linked glycoproteins in all cell populations, however the cleavage efficiency was more pronounced on granulocytes and monocytes than the lymphoid populations. Also, the cleavage pattern of CD162 and CD43 were in agreement with the extensive cleavage of these proteins seen by SDS-PAGE with recombinant proteins. Interestingly, the AdcA SPATE protein which lacks of “Domain-2”; characteristic in other class-2 SPATE members, cleaved glycoproteins in all cell populations suggesting that domain-2 is not involved in the glycoprotease activity (Figure 6). Pic displayed the most efficient protease activity and AdcA the lesser; the overall performance was: Pic>PicU>Tsh/Hbp>CrC2sp>Vat-Ex>AdcA (Figure 4 and...
Figure 6. Class-2 SPATEs degrades the extracellular domain of mucin-type O-glycoproteins on human leukocytes subpopulation.

$1 \times 10^6$ total leukocytes isolated from human blood were incubated with $2 \mu M$ of purified SPATEs at $37^\circ C$ for 1 h. Multi-color flow cytometry was used to analyze cleavage of glycoproteins in different leukocyte subpopulations. Cell types were identified through “forward and side scatter” gates plus cell type-specific lineage markers. Monocytes, T, and B lymphocytes were concurrently stained with conjugated antibodies directed against CD14, CD3, and CD19, respectively. Granulocytes were selected by gating. NK cells were first negatively selected for binding of anti-CD3 and then positively selected for binding of anti-CD16 and anti-CD56. The data show cleavage profiles of CD43, CD44, CD45, and CD162, and are representative of 3 independent experiments.

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Figure 7. Cleavage of CD43 and CD162 glycoproteins in Jurkat T cells by class-2 SPATEs. To visualize degradation of mucin-type glycoproteins by SPATEs, Jurkat T cells were treated with 2 μM of each SPATE for 1 h at 37 °C. T cells were stained using fluorescent monoclonal antibodies against human CD43 (green) and CD162 (red) simultaneously, and analyzed by fluorescence microscopy. CD43 shows up in green, CD162 in red and co-localization of both, CD43 and CD162 is in yellow. DAPI was used to visualize cell nucleus (blue). Micrographs were taken at 40× magnification.
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6). Loss of CD43 and CD162 extracellular domains following T cell treatment with SPATEs was also confirmed by fluorescence microscopy (Figure 7).

Reduced glycoprotease activity on deglycosylated substrates

We and other groups have previously shown that Pic activity on glycoproteins relies on the saccharide nature of substrates. Pic displays reduced proteolytic activity on deglycosylated substrates, and reduced binding ability to mucin in presence of free monosaccharides [11,28]. In order to determine if other class-2 members share this property, we treated CD44, CD162 and C1-inh with a deglycosylation enzyme cocktail followed by incubation with class-2 SPATEs for two hours at 37°C (Figure 8A). We observed reduced cleavage of deglycosylated proteins by all class-2 SPATEs when compared with intact glycoproteins treated for only one hour (Figure 4 and 8A). Although Tsh/Hbp and Vat-Ex showed reduced protease activity on deglycosylated CD44 and C1-inh, they efficiently cleaved deglycosylated CD162 (Figure 8A), which seemed to be partially deglycosylated, as judged by the presence of bands with higher molecular weight similar to the glycosylated protein (Figure 8A). Crc2sp showed lesser proteolytic activity on deglycosylated proteins than other class-2 SPATEs. Pic and PicU showed similar activity on deglycosylated proteins, with some degree of degradation of higher molecular weight species of deglycosylated CD162. However, all deglycosylated proteins were degraded after overnight incubation with class-2 SPATEs except SepA and EpeA (data not shown).

Overall reduction of leukocyte surface carbohydrates by SPATEs

Removal of O-linked glycoproteins by SPATEs must also be reflected in loss of cell surface carbohydrates. To test this, we assessed the amount of sialic acid on the surface of leukocytes following SPATE treatment by flow cytometry. We observed more than 50% depletion of the sialic acid-bearing granulocytes and lymphocyte populations after one hour treatment with class-2 SPATEs (Figure 8B).

Single amino acid mutations in domain-1 of Pic protease abolishes glycoprotease activity

Alignment of the amino acid sequence of domain-1 (harboring the catalytic serine protease triad) of SepA, with all other class-2 SPATEs with glycoprotease activity using Clustal-Omega [29], revealed very conserved residues among class-2 members which were not shared with SepA (Figure 9A). To determine the relevance of those residues in glycoprotease activity, we mutated seven randomly selected residues in Pic, spanning the catalytic triad, to the residues naturally occurring in SepA by site directed mutagenesis (Figure 9A–C). Surprisingly, we found that even though secretion of the constructs harboring site mutations was not affected, mutation of conserved residues greatly reduced the glycoprotease activity of Pic on glycoproteins (Figure 9C).
Discussion and Conclusions

Pathogenic *E. coli* and *Shigella* are a significant cause of morbidity and mortality worldwide. They have been classified into pathotypes based in the clinical, pathological and epidemiological features of the disease they cause. Despite pathotypes harbor many diverse virulence factors, they all have in common the SPATEs, whose contributions have remained until recently obscure. We observed that most members of the class-2 SPATE family have the ability to agglutinate leukocytes, here evidenced in Jurkat T cells. The agglutination phenotype resembled that observed in a number of lectins produced by plants and microorganisms [30]. In addition, Tsh/Hbp and Pic have been previously shown to agglutinate red cells. Interestingly, we observed that the agglutinating activity appears rapidly upon contact with leukocytes, and increased over the time of exposure (Data not shown), but was dependent on the serine protease activity given that denatured Pic protease or the protease-deficient PicS258A failed to agglutinate leukocytes. Likewise, Sat, a class-1 SPATE, did not exhibit this phenotype.

We observed direct binding of most class-2 SPATEs to all leukocyte populations including granulocytes, monocytes, T, B and natural killer lymphocytes. Interestingly, binding efficiency was higher in granulocytes and monocytes than lymphocytes. We have previously shown that Pic protease activity against glycoproteins is reliant on the saccharide modification of the substrates since treatment of these substrates with neuraminidase greatly reduced or inhibited Pic protease activity [11]. The binding patterns could be explained by the fact that in leukocytes of the innate immune system such as the granulocytes, the glycosyltransferases involved in formation of the O-glycans are constitutively expressed, while in T cells, they are expressed only after appropriate activation. Moreover, it is known that B-cells express very low number of glycoproteins (such as CD43 and CD162) in the resting state [31,32], in agreement with our results (Figure 6). Surprisingly, we observed high binding capacity of the protease deficient PicS258A, but not by the heat-denatured Pic protease or SepA, suggesting that the agglutination phenotype is dependent on protease and not on binding activity.

We recently showed that Pic and Tsh/Hbp but not SepA were able to cleave O-linked glycoproteins such as CD43, CD44, CD45, CD93, CD162 and CX3CL1. In fact, in this study we show that most class-2 SPATEs except SepA and EpeA cleaved virtually all mucin-type O-linked glycoproteins, but not other non mucin-type, heavily glycosylated proteins such as E-selectin, αβ-integrin or fibronectin. In addition to previously reported substrates, we found that components of the complement system such as C1-INH and CD55, adhesion proteins CD34 and CD164, and members of the recently discovered Tim family [T cell, immunoglobulin and mucin domain] glycoproteins; Tim1, Tim3 and Tim4 were susceptible to SPATE digestion. Although most SPATEs cleaved mucin-type-glycoproteins they show different proteolytic strengths,
having Pic, its allelic variant PicU and its closest homologue Cx/C2sp produced by *C. rodentium*, as the most proteolytically efficient SPATEs, followed by Tsh/Hlp and its closer homologues Vat-Ex and AdcA. This latter, differs from other class-2 SPATEs in that AdcA does not possess domain-2 (Figure 9B); characteristic in all other members. We found the processed form of the AdcA passenger protein mostly associated to the bacterium, but concentrated supernatants from HB101 expressing AdcA was able to cleave all glycoproteins tested including native glycoproteins in human primary leukocytes (Figure 6).

We also observed that glycosylation of substrates greatly potentiate SPATE proteolytic activity, judged by the fact that reduced SPATE activity was observed on deglycosylated proteins. We noticed variable protease activity throughout all SPATEs on deglycosylated proteins, which may be the consequence of aminoacid variation in the binding-catalytic pocket site (domain-1, Figure 9B) of SPATEs and/or incomplete removal of complex O-linked glycans on deglycosylated substrates.

When the crystal structure of the first class-2 protease was solved it was hypothesized that domain-2 contributed to substrate recognition, but the lack of known biological substrates made difficult to address this proposition. The fact that AdcA, a domain-2-less SPATE cleaves glycoproteins, suggests that domain-2 does not play a role in substrate recognition.

Our purified SPATEs cleaved O-glycoproteins in both recombinant and native forms and in all leukocyte types. The extent of proteolytic activity was comparable to the binding activity observed in early experiments, with higher glycoprotein degradation in cells involved in the innate immune response, but also significantly in lymphocytes, including natural killer cells. It is predictable that cleavage of glycoproteins in lymphocytes may increase upon cell activation, where the expression level of glycoproteins and the enzymes involved in glycosylation processes are steadily enhanced. In fact, we previously observed that activated T cells treated with Pic undergo apoptosis, but not resting T cells [11].

SepA and EpeA, which are distantly located in the phylogenetic tree from other class-2 SPATEs (Figure 1), showed no glycoprotease activity on leukocytes, however, just recently a closer homologue of SepA; EatA, was shown to degrade other O-glycoproteins, such as those lining the intestinal mucosa, including Muc2 [33]. It is tempting to hypothesize that SepA deviated from the other class-2 SPATEs in such a way that the glycoprotease activity was narrowed to only those glycoproteins present in the intestinal mucosa. In fact, when we compared the amino acid sequence of domain-1 (where the H-D-S catalytic triad is located) among class-2 SPATEs, we found that many residues spanning the catalytic triad were conserved in all proteolytic active SPATEs, but not in SepA. We found that changing single residues in Pic for those naturally occurring in SepA, significantly reduced glycoprotease activity. Since the introduction of site mutations in Pic did not affect its secretion, and the fact that SepA is still proteolytically active in other substrates [13], this finding suggests that lectin activity is encoded in the region spanning the catalytic triad in domain-1.

In conclusion, we have here described a family of serine proteases with lectin-like properties, whose main role may lie in their ability to modulate the immune response at various levels. SPATEs cleave chemokines, complement proteins, adhesion proteins and co-stimulatory molecules, all involved in malignancy and inflammatory processes. We recognize that the broad specificity and functionality of class-2 SPATEs, in both innate and adaptive arms of the immune system suggests new therapeutic strategies against a large number of inflammatory disorders (patent # PCT/US2010/061758).

Supporting Information

Figure S1 Leukocyte subpopulations identified in whole blood by FACS analysis. The characteristic forward (FSC) and side (SSC) scatter profile was used to distinguish lymphocytes, monocytes and granulocytes. Within the lymphocyte population, B lymphocytes, T lymphocytes, and NK cells were identified using membrane markers CD19, CD3 and CD16/CD56, respectively. NK cells were first negatively selected for binding anti–CD3 and then positively selected for binding anti–CD16 and anti–CD56. Monocytes were CD14+ and neutrophils were selected only by gating on granulocytes (TIF)

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Author Contributions

Conceived and designed the experiments: JLAL AES FRP. Performed the experiments: JLAL VV MG RS. Analyzed the data: JLAL AES FRP. Wrote the paper: AES FRP.

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