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Characterization of a trypsin-like serine protease of activated B cells mediating the cleavage of surface proteins

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Abstract

Activated B cells may cleave their surface receptors due to the proteolytic activity on the cell membrane or in its vicinity. We attempted to isolate and characterize the protease(s) responsible for this cleavage. Zymograms prepared from the supernatant and the plasma membrane fraction of activated human B cells and BL41/95 cell line exhibited a 85–90 kDa doublet band with protease activity, while that of resting B cells did not. Soybean trypsin inhibitor (STI), *N*α-*p*-tosyl-L-lysine chloromethyl ketone (TLCK) and EDTA treatment abolished the activity of this protease. The excess of Zn^{2+} ions in EDTA did not restore the enzymatic activity, while it was completely recovered in the presence of Ca^{2+}. We affinity-purified a 85–90 kDa protease from the supernatant of BL41/95 cells using STI coupled to Sepharose 4B beads, and measured its kinetic parameters. For the arginyl substrate K_M was 358 ± 59 μM and for the lysyl substrate 582 ± 103 μM. TLCK and benzamidine inhibited the protease at micromolar, while STI at nanomolar concentrations. Both the inhibition profile and the substrate specificity suggest that it is a trypsin-like serine protease. We assume that the 85–90 kDa serine protease expressed on and secreted by activated B cells and BL41/95 cell line is responsible for the cleavage of various membrane proteins, including Fcγ receptors; thus it may play a crucial role in regulating B cell's function.

Keywords: Serine protease; Human; B cell; Shedding

1. Introduction

Serine proteases are present on a variety of cells and their involvement in different phases of the immune response has been demonstrated, including cell killing by cytotoxic T lymphocytes [1–3] and cleavage of receptors from the surface of leukocytes [4–7]. Many membrane proteins, such as CD14 [8], tumor necrosis factor receptors (TNFR) [9], L-selectin [10], CD23 [11], Fcγ receptors [4,5], IL-6 receptors [12], CD43 [13], and CD44 [14], have soluble forms, which are released from the cell membrane as a result of an enzymic cleavage. The release of these receptors is usually initiated by cell stimulation. This process may regulate the surface expression of membrane molecules on activated cells and may be responsible for the appearance of the soluble form of various receptors in the body fluids [15]. Soluble receptors, interacting with their ligands, fulfill important immunoregulatory function. Thus, the abnormal level of soluble receptors in the serum often associates with pathological conditions [16–19]. Little is known about the proteases involved in this process. It was shown earlier that cell surface peptidases, like dipeptidyl peptidase IV (CD26), may modulate signal transduction in T cells [20], while trypsin may act as a polyclonal

Abbreviations: AMC, aminomethyl cumarin; BCR, B cell receptors; FcγRII, receptors binding the Fc part of IgG; HRPO, horse radish peroxidase; PBL, peripheral blood lymphocytes; PM, plasma membrane; RER, rough endoplasmic reticulum; STI, soybean trypsin inhibitor; TLCK, *N*α-*p*-tosyl-L-lysine chloromethyl ketone; TNFR, tumor necrosis factor receptor

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activator of B cells [21,22]. Protease inhibitors were reported to interfere with B cell activation [23].

Our previous studies have shown that activated human tonsil B lymphocytes and several B cell lines express a trypsin-like serine protease activity [6]. The expression of the protease activity correlated with the activation stage of the cells. The in vivo activated B cells exerted an elevated protease activity as compared to the resting subset. Furthermore, B cell lines with an activated phenotype, such as BL41/95, had high, while those with a resting phenotype (BL41) had low protease activity [6]. We have also reported that activated lymphocytes cleave and bind C3, while the resting subset has little capacity to do so [6,24]. Furthermore, we observed the cleavage and release of Fcγ receptor type IIb [FcγRIIb], when activated human B cells were incubated at 37 °C under serum-free conditions [25]. Specific inhibitor of trypsin-like serine proteases, *N*α-*p*-tosyl-L-lysine chloromethyl ketone (TLCK), blocked both the shedding of FcγRII and the cleavage the serine protease substrate by activated B cell extract [5]. In an attempt to characterize this protease, we partially purified it from the supernatant of BL41/95 cells. The results revealed that a 85–90 kDa trypsin-like serine protease represents the major protease activity on the surface and in the supernatants of activated B cells and BL41/95 cell line. This protease may cleave cell surface proteins such as FcγRII and B cell receptors (BCR) upon cell activation.

2. Materials and methods

2.1. Reagents

Substrates, inhibitors and most of the other reagents were obtained from Sigma. Protein Assay Kit and Gelcode Blue Stain reagent were purchased from Bio-Rad, enhanced chemiluminescent substrate from Pierce.

2.2. Cell culture conditions

BL41/95 and BL41 cells were cultured in RPMI 1640 media supplemented with 10% fetal calf serum, 2 mM glutamine, 10^5 IU/l penicillin, 100 mg/l streptomycin in 5% CO_2 containing atmosphere.

2.3. Separation of activated B cells

Lymphocytes were separated from fresh, dispersed human tonsils as previously described. The mononuclear cells were purified by density centrifugation on Ficoll-Hypaque (1.077) [26]. T cells were removed by AET (2-aminoethylisothio-uronium bromide)-treated sheep erythrocyte rosetting followed by separation of the rosetted cells on Ficoll. The low-density [in vivo activated] and high-density [resting] cells were separated on a two-step Percoll (50% and 65%) gradient [27].

2.4. Flow cytometric analysis of cell surface serine proteases

BL41/95 cells (10^6) were washed with PBS and incubated with biotinylated soybean trypsin inhibitor (STI) for 1 h at 37 °C. Biotinylation of STI was carried out according to Kido et al. [28]. The cells were incubated with streptavidin–FITC and were analyzed by a FACSCalibur flow cytometer. Binding of STI was compared in samples incubated in the presence of 5 mM, 0.5 mM or 50 μM TLCK, or various amounts of unlabeled STI.

2.5. Preparation of cell fractions

The BL41/95, BL41 and B cells were fractionated with the method described by Saraste et al. [29]. All steps were carried out at 0–4 °C. Cells ($2–5 \times 10^8$) were homogenized in 1.1 M sucrose solution with 15–20 strokes in a Potter type homogenizer at 1300 rpm. The homogenates were centrifuged at $600 \times g$ for 10 min to remove the nuclei and cell debris. The supernatant was centrifuged at $5500 \times g$ to remove the mitochondria. The resulting supernatant was then centrifuged at $90\,000 \times g$ for 30 min to pellet the membranes. The pellet was resuspended in 2-ml 10% sucrose and layered on a gradient consisting of 25%, 30%, 35%, 40%, and 45% sucrose. The gradient was centrifuged for 17 h at $90\,000 \times g$, the fractions collected, and pelleted at $90\,000 \times g$. Samples were taken of all the fractions, and analyzed by electron microscopy. We found the plasma membrane in the 10–25% sucrose layers, the Golgi and smooth endoplasmic reticulum in the 30–40% sucrose, and the rough endoplasmic reticulum (RER) in the 45% sucrose. The pellet consisted of electron dense bodies.

2.6. Protease activity measurements in the cell fractions

Ten to one hundred microliters of each fraction was incubated with 40 μM substrate Suc-Ala-Ala-Pro-Arg-AMC in 30 mM Tris buffer pH 8, containing 10 mM $CaCl_2$ and 0.1% Triton X-100. The generated AMC was monitored fluorimetrically (excitation: 380 nm, emission: 460 nm), and the results were compared to a calibration curve. Protein content of fractions was measured by Bio-Rad Protein Assay Kit and protease activity was given as pmol AMC/min/mg protein. Samples of fractions were also analyzed by non-reducing SDS-polyacrylamide gel electrophoresis (SDS-PAGE) followed by gelatin zymography.

2.7. Preparation of cell supernatants

Cells (10^7/ml) were washed three times in buffer at pH 7.6 (100 mM HEPES, 120 mM NaCl, 5 mM KCl, 1,2 mM $MgSO_4$ and 8 mM glucose), and kept at room temperature for 2 h. The cells were then centrifuged, the supernatant was filtered (Millipore, pore size 0.45 μm) and 20 mM ε-aminocaproic acid was added to prevent autolysis.

2.8. Zymogram analysis

Protease activity in the supernatants of cells, in the plasma membrane and in the intracellular fractions was assayed by electrophoresis in SDS-polyacrylamide gels containing 0.05% gelatin as substrate. Samples (corresponding to 1.25-μg protein as measured by the Bio-Rad Protein Assay Kit) were treated with sample buffer containing no mercaptoethanol, and kept at 65 °C for 5 min. As positive control, 0.3 μg trypsin was applied. After electrophoresis, gels were soaked in 2.5% Triton X-100 to remove SDS and incubated for 24 h in activity buffer containing 50 mM Tris–HCl pH 8.0, 10 mM $CaCl_2$ and 0.1 mM NaCl. The gels were rinsed and stained by Coomassie brilliant blue. For inhibition analysis the activity buffer contained 20 μM STI or 10 mM EDTA.

2.9. Affinity purification of the protease

STI was covalently coupled to CNBr activated Sepharose 4B beads according to the manufacturer's instruction. Forty-five-milliliter supernatant of BL41/95 cells [10^7 cells/ml] was incubated overnight at 4 °C by continuous mixing with 1-ml STI-coated Sepharose 4B beads in the presence of 20 mM ε-amino-caproic acid. The beads were pelleted and washed with PBS until protein was not detected in the effluent. The bound proteins were eluted by 7.5 mM HCl. Fractions were neutralized, the protein content was determined by Bio-Rad Protein Assay Kit and analyzed by gelatin zymography or used in subsequent experiments.

2.10. Western blot analysis

The fractions eluted from immobilized STI showing enzymatic activity were pooled and further analyzed by Western blots [28]. Samples containing 5–10 μg protein were electrophoresed, transferred onto nitrocellulose membranes and probed with biotinylated STI, followed by streptavidin–horse radish peroxidase (HRPO). The blots were developed by enhanced chemiluminescence (ECL) (Pierce).

2.11. Detection of proteolytic activity on the cell surface

Surface proteins on BL41/95 and BL41 cells were labeled with biotin; the cells were washed five times and incubated in serum-free buffer for different time intervals. After centrifugation the supernatants were collected, electrophoresed, blotted on nitrocellulose membrane and the biotinylated proteins released by the cells were detected by avidin–peroxidase or with monoclonal antibodies recognizing FcγRIIb followed by HRPO conjugated anti-mouse IgG.

2.12. Kinetic and inhibition studies

In the kinetic studies, 40 μl affinity-purified enzyme was incubated with increasing amounts of Suc-Ala-Ala-Pro-Arg-AMC [50–1000 μM], Suc-Ala-Ala-Pro-Lys-AMC [50–1000 μM], and Suc-Ala-Ala-Pro-Phe-AMC at 200 μM in 30 mM Tris buffer pH 8, containing 10 mM $CaCl_2$ and 0.1% Triton X-100 in a final volume of 2 ml. The generated AMC was monitored fluorimetrically. The results were evaluated by Enzfitter program.

In the inhibition studies the system described above was used, with increasing amounts of inhibitor added to the affinity-purified enzyme. Inhibitors were used in the following concentrations: TLCK 10 μM–1.8 mM, STI 50 nM–2 μM, benzamidine 10 μM–1.8 mM. TLCK was preincubated with the enzyme for 1 h as it binds covalently; STI and benzamidine were added simultaneously with 100–300 μM Suc-Ala-Ala-Pro-Lys-AMC substrate.

2.13. pH dependence of the affinity-purified enzyme

Thirty-five-microliter affinity-purified enzyme (0.4 mg/ml) was incubated in a final volume of 2 ml with buffers of different pH together with 50 μM Suc-Ala-Ala-Pro-Lys-AMC substrate. The buffers used were the following: 50 mM MES buffer (pH 6 and 6.5), 50 mM MOPS buffer (pH 7 and 7.5), 50 mM tricine buffer (pH 8 and 8.5), 50 mM CHES buffer (pH 9, 9.5 and 10). All buffers contained 10 mM $CaCl_2$, 100 mM NaCl, and 0.005% Triton X-100.

3. Results

3.1. Flow cytometric analysis of cell surface serine proteases on BL41/95 cells

BL41/95 cells were labeled with biotinylated STI followed by incubation with streptavidin–FITC and analyzed by a FACSCalibur flow cytometer. The specificity of binding of STI to cell surface proteases was assayed by inhibiting it with TLCK (Fig. 1). Soybean trypsin inhibitor bound to the surface of BL41/95 cells, and this was dose-dependently inhibited by TLCK. When TLCK concentration was lowered further (50 μM TLCK), the inhibition ceased. Similarly to TLCK, unlabeled STI also dose-dependently inhibited the binding of biotinylated STI to BL41/95 cells (data not shown), indicating that trypsin-like serine protease(s) are expressed on the membrane of these cells. BL41 cells having resting phenotype [6] exhibited little binding of STI (data not shown).

3.2. Protease activity of cell fractions

In order to localize the protease activity inside the cells, we fractionated BL41/95 and BL41 cells on sucrose gradient. The specific activities of the intracellular fractions of BL41/95 and BL41 cells as well as that of the supernatant of cells were compared. The protease activity was assayed on Suc-Ala-Ala-Pro-Arg-AMC substrate. The specific activities were the following: supernatant, 67 pmol/min/mg;

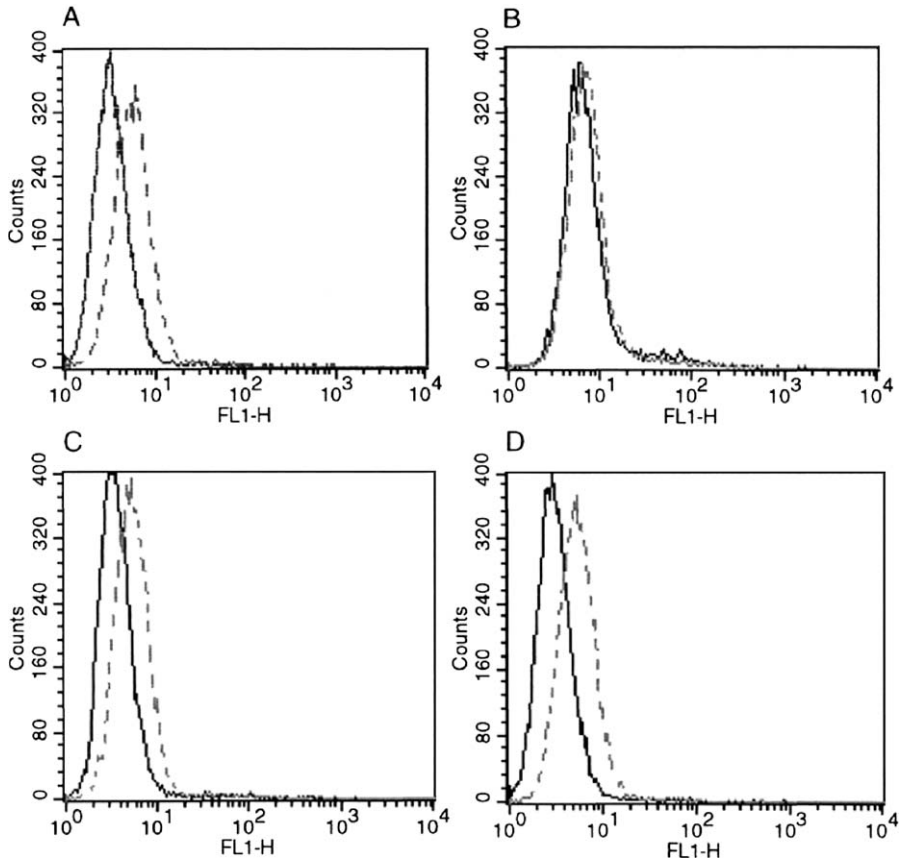

Fig. 1. Flow cytometric analysis of cell surface serine protease expression on BL41/95 cells. The cells were labeled with biotinylated STI followed by the addition of streptavidin–FITC and the samples were analyzed by FACSCalibur flow cytometer. The specificity of binding of STI to cell surface proteases was assayed by inhibiting the binding with TLCK. Every sample was compared to its own control containing the appropriate concentration of TLCK (solid line). Panel A shows the labeling with STI (dashed line) in comparison to the negative control (solid line). The cells were treated with: panel B, 5 mM; panel C, 0.5 mM; and panel D, 50 μM TLCK.

homogenate, 30 pmol/min/mg; plasma membrane, 26 pmol/min/mg; and RER, 231 pmol/min/mg. The Golgi fraction exhibited a great increase in activity, giving a value of 343 pmol/min/mg protein. The fractions derived from BL41 cells showed a similar distribution, but all values were lower.

3.3. Zymogram analysis

The protease activities of the subcellular fractions were compared by zymogram analysis. Zymograms containing 0.05% gelatin were prepared from the supernatants and the subcellular fractions of BL41/95 cell line or activated, low-density tonsil B cells (Fig. 2a). Both the supernatant and all fractions of BL41/95 cells as well as tonsil B cell fractions exhibited a doublet protease band at 85–90 kDa. Only a weak band appeared in the B cell supernatant; on the other hand, it contained a 68-kDa protease. The B cell plasma membrane and RER as well as the BL41/95 plasma membrane fractions contained further proteases of 25 and 48 kDa.

In order to check the specificity of the proteases, the zymograms were treated with STI. The activity of the 85–90 kDa protease was inhibited by the presence of 20 μM STI in the buffer (Fig. 2b) and so was the protease of 25 kDa molecular mass. The 48 kDa and 68 kDa enzymes on the other hand were not affected by the presence of STI. Trypsin was used as positive control in these experiments.

The requirement of proteases for bivalent cations was tested on zymograms of low-density tonsil B cells. The gels were soaked in activity buffer containing 10 mM EDTA. Both the 85–90 kDa and the 68 kDa proteases were inhibited by the presence of EDTA, while the 45 kDa and 25 kDa proteases were not affected (Fig. 2c).

The supernatants obtained from 10^8 BL41/95 and BL41 cells having a resting phenotype, respectively, were 50 times concentrated and the protease activities released by these cells were further studied by zymogram analysis (Fig. 3). Only the supernatant of BL41/95 cells exerted a considerable proteolytic activity, which could not be detected in the absence of Ca^{2+} (Fig. 3a). STI and TLCK completely blocked the enzymatic activity, and the majority of protease activity was also inhibited by benzamidine (Fig. 3b). Addition of excess of Ca^{2+} to the EDTA containing buffer completely reestablished enzymatic activity, while the inhibitory effect of EDTA could not be restored when Zn^{2+} ions were added (Fig. 3c).

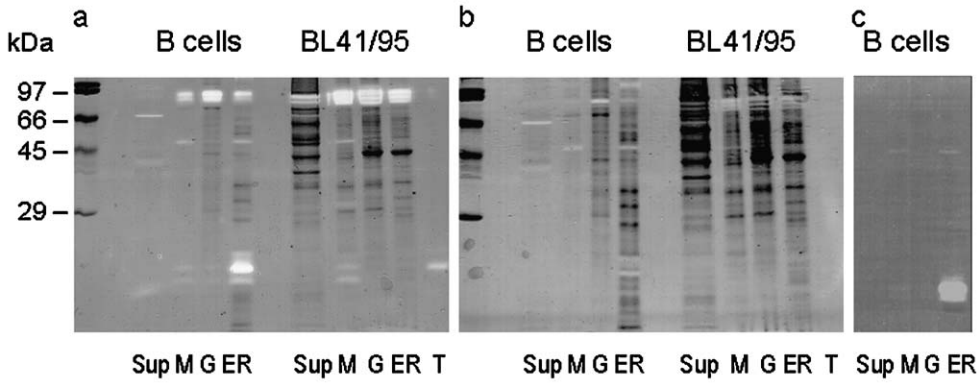

Fig. 2. Zymogram analysis of the supernatant and subcellular fractions of activated (low density) tonsil B cells and BL41/95 cells. Cell fractions were electrophoresed in polyacrylamide gels containing gelatin, kept in activity buffer overnight and stained with Coomassie Brilliant Blue. All fractions contained equal amounts of protein, determined by Bio-Rad Protein Assay kit, corresponding to 1.5 μg/ml bovine serum albumin. Sup, supernatant; M, plasma membrane fraction; G, Golgi apparatus; ER, rough endoplasmic reticulum; T, trypsin control. Panel a: untreated zymogram; panel b: zymogram treated with 20 μM STI in the activity buffer; panel c: zymogram of samples from B cells treated with 10 mM EDTA in the activity buffer.

To exclude the presence of metalloproteinases (MMP), 50 times concentrated supernatants of 10^7 BL41/95 cells or 2×10^6 of rat basophylic leukocytes (RBL), which are known to express several proteases [30], were compared. Proteolytic activities were detected on the zymograms of both samples, but at different electrophoretic mobilities. Western blot experiments revealed that only the supernatant of RBL contained MMP-9 as detected by specific antibodies (Fig. 4).

Together these data indicate that the 85–90 kDa protease detected in the cell fractions and in the supernatants of BL41/95 cells and activated B cells is a trypsin-like serine protease, which requires the presence of Ca^{2+} for its activity.

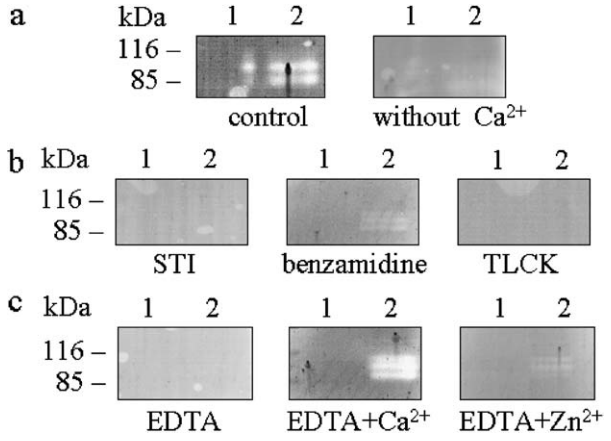

Fig. 3. Zymogram analysis of gelatinolytic activities of the supernatants in the presence of various inhibitors. Supernatants of 10^7 BL41(left lanes) and BL41/95 cells (right lanes), respectively, were electrophoresed on gelatin containing polyacrylamide gels. The gels were treated overnight with: panel a, activity buffer with or without Ca^{2+}; panel b, 20 μM STI or 10 mM benzamidine; panel c, 10 mM EDTA without or in the presence of 100 mM $CaCl_2$ and 100 mM $ZnCL_2$, respectively. 10 mM TLCK was added directly to the supernatant before the electrophoresis. The zymograms were stained with Coomassie Brilliant Blue.

3.4. Affinity purification of the protease activity from the supernatant of BL41/95 cells

STI covalently bound to Sepharose beads was used for affinity isolation of the protease from the supernatant of BL41 and BL41/95 cells. The eluted proteins were assayed by zymograms using polyacrylamide gel containing 0.05% gelatin (Fig. 5a, panel 1). The samples from BL41/95 cells were also electrophoresed on SDS-polyacrylamide gels without gelatin, blotted onto nitrocellulose membranes and probed with biotinylated STI followed by streptavidin–HRPO (Fig. 5a, panel 2). The doublet bands at 85–90 kDa were detected by both methods, corresponding to the previously observed protease activity in the supernatant of BL41/95 cells. Similarly to tonsil B cells, the supernatant of BL41 cells did not exhibit a considerable protease activity. When the polyacrylamide gel containing the protease samples isolated from BL41/95 cell's supernatants was stained with Gelcode-Blue, a doublet protein band with the apparent

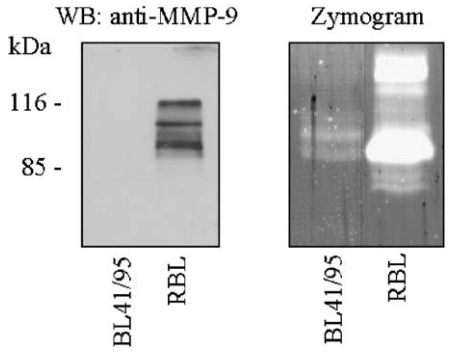

Fig. 4. Comparison proteases in the supernatants of BL41/95 and RBL cells. Supernatants of 10^7 cells were electrophoresed on SDS-PAGE, blotted onto nitrocellulose membrane and probed with MMP-9 specific antibodies. Zymogram analysis was carried out as described at Fig. 2.

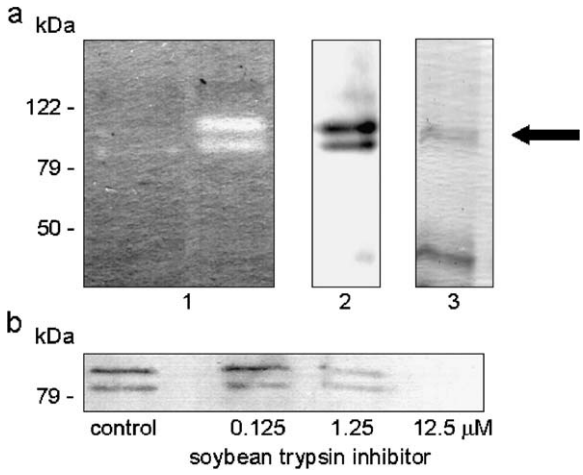

Fig. 5. Analysis of the serine protease affinity-isolated by STI covalently bound to Sepharose 4B. (Panel a) 1: zymogram of the protease samples affinity-purified from the supernatant of BL41 cells (first lane) and BL41/95 cells (second lane); 2: the isolated protease from the BL41/95 cells was exposed to SDS-PAGE, transferred onto nitrocellulose membrane, and probed with biotinylated STI, followed by the addition of streptavidin–HRPO. The blot was developed by enhanced chemiluminescence; 3: the affinity-purified protease from BL41/95 cells was electrophoresed and the proteins in the gel were stained by Gelcode Blue Stain. (Panel b) Western blot analysis of the isolated protease as described at panel a-2; inhibition of the biotinylated STI binding by different doses of unlabeled STI.

molecular mass of 85–90 kDa was detected in the first fraction eluted from the STI-Sepharose column. This doublet corresponded to the bands binding STI on Western blots and possessing proteolytic activity on zymograms (Fig. 5a, panel 3). These data indicate that the 85–90 kDa protease is the only serine protease in the supernatant of BL41/95 cells.

To reveal the specificity of the interaction of STI with the isolated protease on the nitrocellulose membrane, the effect of unlabeled inhibitor was tested. The binding of biotinylated STI to the isolated protease was dose-dependently inhibited by unlabeled STI. Ten times excess of the cold reagent (12.5 μM) completely abolished the detection (Fig. 5b).

3.5. Protease inhibition studies

We compared the effect of TLCK, benzamidine and STI on the protease affinity-isolated from the supernatant of BL41/95 cells using 100–300 μM Suc-Ala-Ala-Pro-Lys-AMC as substrate. TLCK and benzamidine inhibited the protease at micromolar, while STI at nanomolar concentrations (Fig. 6).

3.6. Kinetic studies and pH dependence of the affinity-purified protease

Kinetic studies were done on the affinity-purified enzyme from the supernatant of the BL41/95 cells. Increased concentrations of Suc-Ala-Ala-Pro-Arg-AMC and Suc-Ala-Ala-Pro-Lys-AMC substrates were added to the enzyme and the released AMC was measured fluorimetrically. The Michaelis constants of the enzyme were calculated by Enzfitter program. For the arginyl substrate K_M was 358 ± 59 μM and $K_{cat} = 8.0 \times 10^{-3}$ $s^{-1} \pm 0.7 \times 10^{-3}$ and for the lysyl substrate $K_M = 582 \pm 103$ μM and $K_{cat} = 1.16 \times 10^{-2}$ $s^{-1} \pm 0.2 \times 10^{-2}$. Suc-Ala-Ala-Pro-Phe-AMC substrate was not cleaved.

The affinity-purified 85–90 kDa protease was assayed for pH dependency using Suc-Ala-Ala-Pro-Lys-AMC substrate. The pH optimum of the protease was found to be between 8–9 pH (data not shown).

3.7. Shedding of surface proteins from BL41/95 cells

To examine further the surface proteinase activity of BL41/95 cells, the membrane proteins were biotinylated and the cells were incubated at 37 °C for different time intervals. The supernatants were assayed by SDS-PAGE followed by Western blotting and the shed biotinylated proteins were detected by streptavidin–HRPO. Several bands appeared in the supernatants of BL41/95 cells, indicating that proteins were cleaved and released from the cell membrane. The release of surface proteins showed a maximum at 2–4 h of incubation, then the intensity gradually decreased. BL41 cells with a resting phenotype did not show a significant shedding of surface molecules. However, when the supernatants of BL41/95 cells were added to the biotinylated BL41 cells, a release of surface proteins with a similar

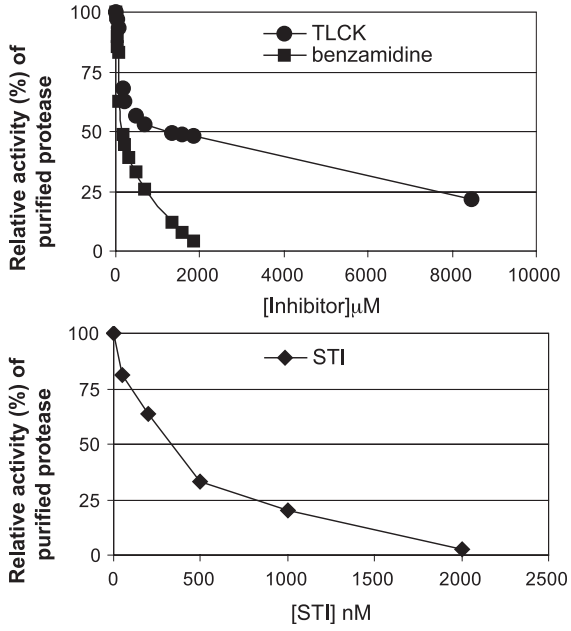

Fig. 6. Inhibition of the affinity-purified serine protease from the supernatant of BL41/95 cells by TLCK (●), benzamidine (■) and STI (◆). Protease activity was measured on Suc-Ala-Ala-Pro-Lys-AMC substrate. The generated AMC was detected by fluorimetry.

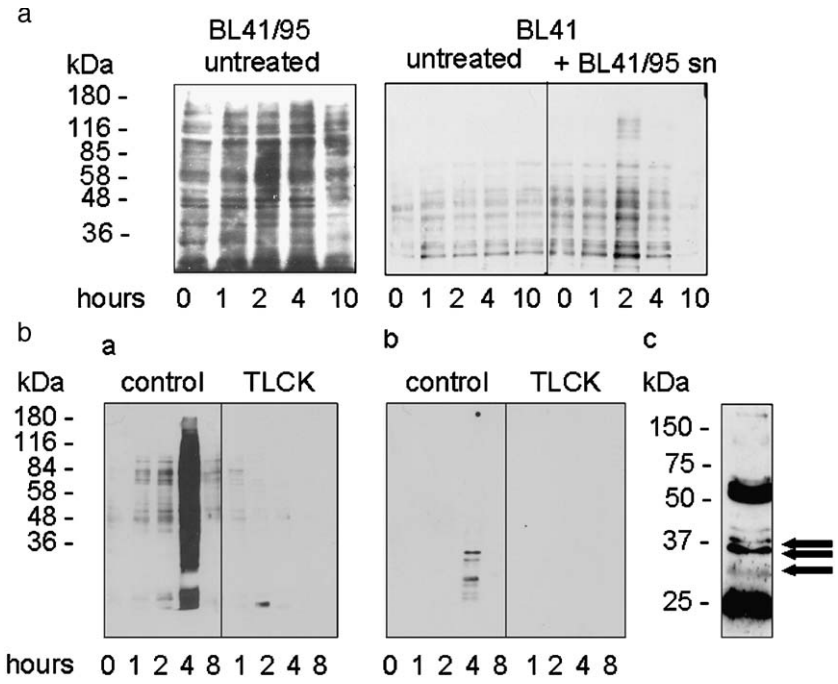

Fig. 7. Shedding of proteins from the cell membrane as a result of proteolytic activity of BL41/95 cells. (a) BL41/95 and BL41 cells were surface labeled with biotin, then 10^7 cells were incubated at 37 °C for 1, 2, 4 and 10 h, respectively, in 30-μl serum-free buffer. Second panel: surface biotinylated BL41 cells were treated with the 3 h supernatant of BL41/95 cells. The supernatants of cells containing the cleaved surface proteins were collected, electrophoresed, blotted onto nitrocellulose membrane, and developed by streptavidin–HRPO and ECL. (b) Panel a: TLCK inhibits the proteolytic activity and the release of FcγRIIb fragments from BL41/95 cells. The cells were labeled and treated as described in 7a, and the cells were incubated with or without 10 mM TLCK. Panel a was developed with HRPO–streptavidin; panel b: the same membrane was developed with monoclonal antibody specific for FcγRIIb. Panel c: Western blot analysis of proteins affinity-purified by FcγRIIb-specific monoclonal antibody from the supernatant of BL41/95 cells. The proteins were immunoprecipitated by FcγRIIb-specific monoclonal antibody coupled to Protein G beads; the eluted samples were electrophoresed, blotted onto nitrocellulose membrane and probed with the same anti-FcγRIIb-specific antibody.

kinetics was observed (Fig. 7a). The shedding of proteins from BL41/95 cells was completely inhibited by TLCK, suggesting that it was a result of serine protease cleavage (Fig. 7b, panel a). We attempted to identify one of the proteins cleaved from the cell surface. Using a monoclonal antibody specific for the type II FcγR, we detected several bands in the supernatants of cells, which most probably correspond to the cleaved fragments of FcγRIIb (Fig. 7b, panel b). This was confirmed after affinity isolation of proteins from the cell supernatants by anti-FcγRIIb and probing the membrane with the same antibody. Proteins with apparent Mr 30 kDa, 35 kDa and 37 kDa proteins were specifically recognized as FcγRII fragments. The 50 kDa and 25 kDa bands represent the heavy and light chains of the immunoprecipitating antibody (Fig. 7b, panel c).

4. Discussion

We describe here a previously unknown 85–90 kDa trypsin-like serine protease, expressed on activated human B cells and BL41/95 Burkitt's lymphoma cell line. This protease can be detected both on the cell surface and as a secreted protein in the supernatant of BL41/95 cells and activated B cells.

A number of cell surface receptors have soluble forms, which are produced by alternative splicing or limited proteolysis [8,14,15,18]. Surface proteases as well as extracellular proteases might be responsible for the latter effect. TNFα receptors [9], IL-6 receptors [12], intracellular adhesion molecule I [10], FcγRII [4,5,31] and FcγRIII [11] are representative examples of receptors on immune competent cells having a soluble form. These soluble receptors have an important regulatory function on ligand-mediated cell activation processes. Cell surface expressed and extracellular proteases thus play a crucial role in modulating the function of both membrane-bound and soluble receptors [15].

We reported previously that human peripheral blood lymphocytes (PBL) shed some of the surface receptors including FcγRII, most probably as a result of the expression of trypsin-like serine protease activity on these cells [4,5,25]. It was also shown that activated human tonsil B lymphocytes and several B cell lines express trypsin-like serine protease activity, which enables the cells to cleave C3 [6,32]. Our aim was to characterize the protease(s) responsible for these events. Cell fractionation and monitoring the protease activities on zymograms revealed that both the plasma membrane and the intracellular fractions (RER, Golgi) of BL41/95 cells and low-density, activated tonsil B cells contained a doublet protease band at 85–90 kDa.

The same bands were observed in the supernatant of BL41/95 cells, while the supernatant of tonsil B cells contained only a minor 68 kDa protease. The plasma membrane and RER of activated tonsil B cells, as well as the BL41/95 plasma membrane fraction contained proteases of 25 and 48 kDa. Only the activity of the 85–90 kDa and 25 kDa proteases was blocked by STI, suggesting that these are trypsin-like serine proteases.

The 85–90 kDa protease but not the 25 kDa one was sensitive to EDTA. It was previously described that B cells may synthesize a 92 kDa metalloprotease, identical to MMP-9 [33,34]. Since Zn^{2+} ions did not restore the protease activity of the 85–90 kDa protein, while it was recovered in the presence of Ca^{2+}, we suppose that the protease released by BL41/95 cells is not a metalloprotease. Western blot experiments confirmed that this protease is not identical with MMP-9, since MMP-9 specific antibody did not recognize any protein in the supernatant of BL41/95 cells. It is possible that the B cell serine protease requires Ca^{2+} ions to regain its native conformation after SDS-PAGE. Based on their identical properties, we suggest that the doublet bands at 85–90 kDa may represent two isoforms of the same protease.

TLCK and STI, specific inhibitors of trypsin-like serine proteases, efficiently blocked gelatinolytic activity of the protease, suggesting that it is indeed a trypsin-like serine protease. Since this is the dominant trypsin-like serine protease on the membrane of both activated tonsil B cells and BL41/95 cells, while it was not detected in the supernatant or on the surface of BL41 cells representing the resting phenotype, we assume that the 85–90 kDa protease is responsible for the activities described earlier, such as the cleavage of C3 and shedding of FcγRIIb by activated B cells.

A p57 serine protease cleaving C3 was isolated earlier from human erythrocyte membranes [35]. Amino acid analysis of this protease showed that it was generated from ankyrin by limited proteolysis. Fujino et al. purified and characterized an 80-kDa serine protease which was present in erythrocyte cytosol. The protease became adherent to membranes upon cell oxidation and had high selectivity for proteins modified by oxidation and glycation [36]. In the case of lymphocytes, serine proteases of a similar molecular size have not been reported.

The 68 kDa protease released by activated B cells is not sensitive to STI, but EDTA inhibits it, suggesting that it might be a metalloprotease. Trocmé et al. reported that Epstein–Barr-immortalized human B lymphocytes are able to secrete a 92-kDa metalloprotease with gelatinolytic activity that was purified and identified as MMP-9. Metalloproteinases are known to be secreted as latent precursors of higher molecular mass than the mature enzyme, and MMP-9, during its activation, may produce active proteases of 86, 80 and 72 kDa molecular mass [33]. It has been shown that MMP-9 activity ceases when zymograms are incubated with metalloprotease inhibitor 1-10 phenanthroline (1 mM) or EDTA (10 mM), but serine or cystein protease inhibitors are not effective [37]. It is therefore possible that the 68 kDa protease found in the supernatant of the B lymphocytes and BL41/95 cells corresponds to an activated form of MMP-9. The 25 kDa protease present in the RER fraction of B cells may be identical with the protease isolated by Ku et al. [23] from activated B cells.

The 85–90 kDa protease is present in the plasma membrane of BL41/95 cells, in the Golgi apparatus, and RER membranes as well as in the supernatant of the cells. This suggests that the 85–90 kDa serine protease is synthesized in RER and transported to the cell surface via Golgi vesicles. Although BL41/95 cells are not actively secreting cell types, they release this protease into the supernatant. We could not detect differences in the size of surface expressed and secreted protease. The 85–90 kDa protease was present as an active enzyme in all fractions. We suppose that it might be activated due to the process of isolation from the subcellular fractions. Alternatively, its activity might be regulated inside the cell. The stable and specific binding of STI to the surface of BL41/95 cells as well as the detection of the 85–90 kDa protease in the isolated plasma membrane fraction of both BL41/95 and activated tonsil B cells suggest that the protease has an integral membrane protein form as well.

In order to check whether the protease on BL41/95 cells might be responsible for the production of soluble receptor forms, the shedding of membrane proteins was followed. Numerous released surface proteins were detected in the supernatant of BL41/95 cells already after 60-min incubation, and the appearance of these proteins was completely blocked by TLCK. This suggests that the shedding is a result of proteolytic cleavage by a serine protease. The earlier described shedding of FcγRIIb from activated B cells was reported to be a result of a trypsin-like serine protease activity expressed upon cell activation, since the release of FcγRIIb was inhibited by the specific inhibitor, TLCK [4]. Here we have shown that FcγRII-specific monoclonal antibody recognized several proteins in the supernatant of BL41/95 cells, which were not detected when the cells were treated with TLCK. Moreover, similar bands were observed when affinity-purified FcγRII from the supernatant was tested, indicating that cleaved fragments of the receptor are present. Since the 85–90 kDa serine protease was the only serine protease in the supernatant and in the cell membrane fraction detected by zymography, furthermore, this was the only protein which bound STI on Western blot, these data together strongly suggest that the 85–90 kDa affinity-isolated serine protease is identical with the enzyme cleaving FcγRIIb and most probably other receptors. A similar, activation-dependent proteolytic cleavage of FcαR (CD89) has been described recently [38], although the protease responsible for it was not characterized.

We suggest that the 85–90 kDa trypsin-like serine protease has a major role in regulating B cell function. Its

expression is regulated by cell activation; thus, via the cleavage of a variety of receptors, the 85–90 kDa protease may modulate the receptor profile on activated B cells, and may generate soluble, ligand binding forms of receptors.

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