A Cellulose-Binding Domain-Fused Recombinant Human T Cell Connective Tissue-Activating Peptide-III Manifests Heparanase Activity

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The chemokine connective tissue-activating peptide (CTAP)-III, which belongs to the leukocyte-derived growth factor family of mediators, was previously shown to be mitogenic for fibroblasts. However, it has recently been shown that CTAP-III, released from platelets, can act like a heparanase enzyme and degrade heparan sulfate. This suggests that CTAP-III may also function as a proinflammatory mediator. We have successfully cloned CTAP-III from a λgt11 cDNA library of PHA-activated human CD4+ T cells and produced recombinant CTAP-III as a fusion protein with a cellulose-binding domain moiety. This recombinant CTAP-III exhibited heparanase activity and released degradation products from metabolically labeled, naturally produced extracellular matrix. We have also developed polyclonal and monoclonal antibodies, and these antibodies against the recombinant CTAP-III detected the CTAP-III molecule in human T cells, polymorphonuclear leukocytes, and placental extracts. Thus, our study provides tools to examine further immune cell behavior in inflamed sites rich with extracellular moieties and proinflammatory mediators.

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Chemokines are a recently characterized family of pro-inflammatory mediators that function biologically in both blood vessels and in extracellular matrix sites to promote immune cell adhesion and migration (1-4).

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Abbreviations used: CTAP-III, connective tissue activating peptide-III; LDGF, leukocyte derived growth factor; NAP-2, neutrophil activating peptide-2; HSPG, heparan sulfate proteoglycans; ECM, extracellular matrix; CBD, cellulose binding domain.
the ECM by heparanase facilitates cell movement and migration through extravascular tissues (16). Heparanase activity has been found in various activated blood-borne cells, such as macrophages, neutrophils, activated CD4+ T cells, mast cells and platelets (17-20). Partially purified heparanase obtained from human platelets, melanoma cells, and placenta has been used in many studies (18,21,22), but the effects of contaminants in these preparations cannot be ruled out. Despite much effort, an active recombinant immune cell-derived heparanase has not yet been reported, and only bacterial heparinases have been produced as recombinant enzymes (23). Recently, another human platelet heparanase has been described, although its amino acid sequence has not yet been identified (24).

Although many recombinant proteins have been successfully expressed in Escherichia coli (E. coli), small recombinant polypeptides (<10 kDa) may be unstable and degrade rapidly in the bacteria. Therefore, fusion proteins containing the desired small polypeptides have an advantage when producing such polypeptides (25). A cellulose-binding protein (CBPA) was isolated from the cellulolytic bacterium Clostridium cellulovorans by Shoseyov and Doi (26). This protein, which is a major subunit of the bacterial cellulase complex, binds to cellulose, but does not express any hydrolytic activity. The gene (cbpA) for the CBPA protein was cloned and sequenced (27). Subsequently, the cellulose-binding domain (cbd) of cbpA was purified using PCR primers flanking the cbd. Cloning of this construct into an over-expression vector enabled overproduction of the 17-kDa cellulose binding domain (CBD), which exhibits a strong affinity for cellulose (28). CBD was found useful as affinity tag for the production of recombinant proteins (29,30). Here, we describe the production of a recombinant human T cell-derived CTAP-III fused to CBD and its heparan sulfate degrading activity.

MATERIALS AND METHODS

Animals. BALB/c mice (8-week-old females) and New Zealand rabbits (8-week-old females) were obtained from the Experimental Animal Center of the Weizmann Institute of Science.

Reagents. Reagents were obtained as follows: β-Thromboglobulin (Calbiochem, San Diego, CA); Phytohemagglutinin (PHA) -activated liquid scintillation cocktail (Packard Instrument Co., Meriden, CT); Madison, WI); complete Freund's adjuvant (Difco Lab., Detroit, MI); pET-34b and pET-3c plasmids (Novagen, Chemical Co., St. Louis, MO); Jetsorb kit (Genomed, Bad-
Preparation of human T cells homogenates. Human T cells were purified from the peripheral blood of healthy donors according to a previously described procedure (18). The cultured cells (about 2.5 × 10⁶ cells) were washed with 50 mM Tris, pH 6.8, containing 10 mM EDTA, centrifuged (120 × g, 10 min, 25°C), and the pellet stored at −20°C. Homogenates of human T cells were obtained by 5 cycles of freezing and thawing T cell pellets in 2 ml of 50 mM Tris, pH 6.8, containing 1 mM EDTA, pepstatin A (1 μg/ml) and leupeptin (2 μg/ml; buffer A). The resulting homogenate was centrifuged (750 × g, 10 min, 25°C) and the supernatant filtered through a 0.2μm cellulose acetate filter.

Preparation of supernatants from PMN lymphocytes. Human PMNs were purified from the peripheral blood of healthy donors. Briefly, fresh blood (−30 ml) was incubated (1 h, room temperature) with 0.9% NaCl (75 ml) and 6% dextran T-70 (50 ml). Equivalent amounts of the mixture were then placed in four 50 ml plastic tubes and centrifuged (190 × g, 10 min). Afterwards the supernatants were discarded, and the pellets resuspended in 6% KCl (3 ml) and centrifuged (190 × g, 10 min, 25°C). The supernatants were again discarded, and the neutrophils thus purified were resuspended in PBS containing 0.01% CaCl₂ and 0.01% MgCl₂ to a final dilution of 5 × 10⁸ cells/ml and incubated overnight on ice. Subsequently the cells were centrifuged (750 × g, 20 min, 25°C) and the supernatants (PMN supernatants) used for the immunoprecipitation.

Immunoprecipitation of CTAP-III from T cell homogenates and PMN supernatants. T cells supernatant, corresponding to 5 × 10⁶ cells, was added to each tube and centrifuged (13,000 × g, 5 min, 25°C).

For Western blotting, 5 μl samples of the immunoprecipitated proteins were subjected to 15% SDS-PAGE and transferred to a cellulose nitrate membrane. The primary antibody was anti-CTAP-III mAb (hybridoma culture supernatant designated clone 792; diluted 1:20 in 20 mM Tris, pH 7.6, containing 80 mM NaCl, 5 mM HCl, 0.1% Tween-20 containing 7.5% milk powder) and goat anti-mouse pAb, conjugated to alkaline phosphatase (diluted 1:10,000) was used as a secondary antibody.

Coating of tissue culture dishes with ECM. ³²S-labeled bovine corneal endothelial cell ECM-coated plates were prepared as previously described (33). The ECM thus exposed remained intact, free of cellular debris, and firmly attached to the entire area of the tissue culture plates (33).

Preparation of soluble high molecular weight sulfate-labeled proteoglycans. ³²S-labeled soluble high molecular weight proteoglycans (peak I) were prepared as previously described (34).

Heparanase activity assay. Heparanase activity was determined by measuring the degradation of either immobilized ³²S-labeled ECM or soluble ³²S-labeled heparan sulfate proteoglycans. For immobilized preparations, aliquots (1 ml of ~150 μg CBD-CTAP-III or CBD immobilized on 80 mg of cellulose) in 80 mM phosphate-citrate buffer, pH 5.5, containing 50 mM NaCl and 1 mM CaCl₂, were added to ³²S-labeled ECM-coated plates with or without 50 μg/ml heparin. The plates were then incubated (37°C, 18 h), the ³²S-labeled material that was released into the incubation medium was collected and eluted by centrifugation (13,000 × g, 5 min, 25°C).

In addition, heparanase activity was evaluated by adding 40 μl of the Immunoprecipitates derived from human T cells and PMN to ³²S-labeled peak I material (10−20 μl; ~20,000 cpn) in 20 mM phosphate citrate buffer, pH 6.2 (1 ml), and the reaction mixtures were incubated (37°C, 18 h). Both degradation results were analyzed by gel filtration on a Sepharose CL-6B column (0.9x25 cm) equilibrated with PBS. Fractions (0.3 ml) were eluted with PBS (flow rate of 15 ml/h) and their radioactivity was determined. The excluded (Vo) and total included (Vt) volumes were marked by blue dextran and phenol red, respectively.

RESULTS

Cloning of human T cell CTAP-III in E. coli expression vectors. In order to clone the T cell CTAP-III, a λgt11 cDNA library of PHA-activated human CD4⁺ T cells was used as a template for the PCR reaction, using CTAP-III-specific primers. The 260 bp PCR product was cloned into the pET-3c plasmid. Sequence analysis revealed that the T cell CTAP-III cDNA sequence was identical to that of the human platelet CTAP-III cDNA (13). The pET-CBD, pET-CTAP-III, and pET-CTAP-III plasmids, respectively. Fifteen μl of the cell lysates were subjected to 15% SDS-PAGE.
protein could be purified on a cellulose-containing affinity column (29,30).

CTAP-III contains four cysteines, which form two disulfide bonds. A refolding-oxidation protocol that enabled the production of soluble CBD-CTAP-III was developed, since substantial oxidation of the recombinant protein failed to occur in the bacterial cytoplasm and most of the protein accumulated in non-soluble inclusion bodies. In this protocol, the bacterial cells were disrupted by sonication, and the washed inclusion bodies were solubilized using guanidine-HCl, and allowed to oxidize slowly during gradual dialysis. Finally, the recombinant CBD-CTAP-III was affinity purified on cellulose (Figure 2).

Immunoprecipitation of heparanase activity from human T cells and PMN. Immnoprecipitation of heparan sulfate-degrading activity from T cells and PMN, using anti-CBD-CTAP-III antibodies, would be consistent with CTAP-III being a T cell heparanase. For this purpose, the fusion protein CBD-CTAP-III was used to immunize rabbits and mice. The fusion protein was immobilized on cellulose, which facilitated its purification. For production of polyclonal Abs, rabbits were injected with either the immobilized CBD-CTAP-III or with the soluble form after its release from cellulose. For production of mAb, BALB/c mice were injected with the soluble CBD-CTAP-III.

Screening by Western blot analysis (Figure 3) of the mAb indicated that clone number 340 was specific for CBD, whereas mAb clone number 792 identified CBD-CTAP-III but not CBD. Thus, antibodies specific for CTAP-III could be generated and used to clarify the biological role of this molecule.

Immunoprecipitates derived from the human T cells and PMN using rabbit sera (anti-CBD-CTAP-III, preimmune) and protein-A-conjugated agarose beads were subjected to Western blot analysis. Immunoprecipitation of CTAP-III occurred only when the polyclonal anti-CBD-CTAP-III Abs were used (Figure 4). The two bands of about 25 and 55 kDa, which appeared with both the anti-CBD-CTAP-III and preimmune (control) sera, are caused by antibody chains that cross-react with the goat anti-mouse IgG used as the secondary antibody. CTAP-III was also identified in the heparanase preparation of human placenta when anti-CBD-CTAP-III pAb was used for the immunoprecipitation assay (data not shown). Finally, when mouse mAbs were used to immunoprecipitate T cell lysates, the anti-CTAP-III mAb (clone 792), but not the anti-CBD mAb (clone 340), precipitated CTAP-III (data not shown). The presence of platelets CTAP-III in the T cell lysates used throughout our studies was minimized by washing the purified T cells several times at low speed, also in the presence of EDTA, thus eliminating the binding of platelets to T cells by cation bridges. Thus, both pAb and mAb against CTAP-III appear to recognize, bind, and precipitate human CTAP-III derived from human peripheral blood immune cells, as well as from human placenta.

Determination of heparanase activity in the immunoprecipitates and recombinant CBD-CTAP-III. Heparanase activity was determined by measuring the degradation of either soluble 35S-labeled proteoglycans, designated peak I, or immobilized 35S-labeled ECM. Immunoprecipitates released from the agarose beads degraded soluble 35S-labeled heparan sulfate proteoglycans only when the anti-CBD-CTAP-III pAb was used for the immunoprecipitation (Figures 5A and 5B).
Because the heparanase activity immunoprecipitated with CTAP-III, we examined the heparanase activity of the recombinant CBD-CTAP-III. CBD-CTAP-III or CBD (added as control) immobilized on cellulose were added to 35S-labeled ECM-coated plates, with or without heparin, a non-radioabeled substrate of heparanase. Gel filtration chromatography of the reaction mixtures revealed a second peak of low molecular weight 35S-labeled degradation products only when CBD-CTAP-III was added (Figure 6A). This second peak was not produced when heparin was included together with the CBD-CTAP-III preparation (Figure 6B). Soluble CBD and CBD-CTAP-III before immobilization did not show heparan sulfate-degrading activity (data not shown). Thus, CBD-CTAP-III exhibited heparan sulfate-degrading activity reminiscent of heparanase preparations from different sources (17-19).

DISCUSSION

In this study we have cloned CTAP-III from a λgt11 cDNA library of PHA-activated human CD4+ T cells. The cDNA sequence of this chemokine was found to be identical to that previously published for human platelet-derived CTAP-III (13). Moreover, in agreement with our finding, it has been recently reported that CTAP-III is expressed by human T cells (12).

When CTAP-III and the fusion protein CBD-CTAP-III were over-expressed in E. coli, the fusion protein, but not CTAP-III, was produced in large quantities and appeared resistant to proteolysis in the bacteria. The apparent resistance from degradation of CBD-CTAP-III was probably due to its accumulation within inclusion bodies.

Immunoprecipitation of heparanase activity from human T cells and PMN with anti-CTAP-III antibodies, (Figure 5) substantiates the report of Hoogewerf et al (14) that native CTAP-III, purified from human platelets, exhibits heparanase activity. Moreover, the possibility that the platelet-derived heparanase was co-purified with native CTAP-III has been ruled out by our present production of recombinant heparanase.

It is highly unlikely that the heparanase activity, manifested by the doned CTAP-III, was due to the actual existence in E.coli of molecules capable of degrading heparan sulfate since: (1) all cytosolic-soluble material and membrane fragments were separated from the insoluble inclusion bodies by washing with a detergent. (2) the recombinant CBD-CTAP-III and CBD were affinity-bound to cellulose and the columns were washed with high stringency (the buffer contained 1M NaCl). And, finally, (3) CBD was expressed as a control for the recombinant CBD-CTAP-III; CBD was treated the same as CBD-CTAP-III, but showed no heparanase activity. Therefore, our results clearly demonstrate that the heparan sulfate-degrading protein expressed by T cells is actually the chemokine CTAP-III.

The identification of CTAP-III in heparanase preparations from human placenta may indicate that CTAP-III is also at least one of the heparanases found in the human placenta.

Heparanase plays an important role in the extravasation of blood-borne tumor cells and of activated cells of the immune system and hence in tumor metastasis, wound healing, and inflammation (35). This study proves that at least one of the heparanases found in T cells and PMN is CTAP-III and suggests that CTAP-III is also at least one of the heparanases found in the placenta. However, whether CTAP-III can express its
heparanase activity only when found in the tetrameric form (which is required for its chemokine activity) is not yet known.

Production of a recombinant CBD-CTAP-III exhibiting heparanase activity not only proves that CTAP-III has heparanase activity, but should further elucidate the multiple activities of this sub-family of chemokines. The antibodies produced and characterized herein may be studied for their ability to inhibit the enzymatic or chemokinic activity of CTAP-III in-vivo in inflammatory reactions in laboratory animals, as well as their application in staining tissues obtained from mammals undergoing wound healing, inflammation, or tumor growth. Our findings may thus lead to additional studies, not only supporting the versatility of chemokines and ECM-degrading enzymes, but also regarding the clinical significance of these mediators.

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REFERENCES