Human Eosinophil Cytotoxicity-enhancing Factor

EOSINOPHIL-STIMULATING AND DITHIOL REDUCTASE ACTIVITIES OF BIOSYNTHETIC (RECOMBINANT) SPECIES WITH COOH-TERMINAL DELETIONS*

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U937 cells produce eosinophil cytotoxicity-enhancing factor (ECEF) polypeptides of 14 and 10 kDa that have identical NH₂-terminal amino acid sequences. The 10-kDa form has greater eosinophil-stimulating activity (half-maximal at >20-fold lower concentrations). We considered the hypothesis that there is a precursor-product relationship between the 14- and 10-kDa species. Recombinant 14-kDa 104-amino acid ECEF (rECEF-104) had a slight stimulatory effect on eosinophil cytotoxic function at concentrations of 160 nm and above. In contrast, two species, rECEF-80 and rECEF-84, representing cleavage products of approximately 10 kDa had substantial statistically significant cytotoxicity-enhancing activity at concentrations as low as 10 pm. This evidence demonstrates the potential to generate the high-activity ECEF species by proteolytic cleavage of the 104-amino acid species. Another feature of this cytokine is the sequence from amino acids 31 to 34, which constitutes the conserved and active site of the enzyme thioredoxin. When tested for dithiol reductase enzymatic activity, rECEF-104 was active in a dose- and time-dependent manner, whereas the truncated forms of the molecule had no dithiol reductase activity. Thus the eosinophil-stimulating functions of the molecule do not correlate with its enzymatic activity. The evidence shows that the enzymatic activity is not essential for the initial interaction of ECEF with the eosinophil, and it suggests that the ECEF molecule functions by means of two discrete mechanisms.

The proinflammatory functions of human eosinophils are subject to regulation by cytokines. In terms of units of activity produced in vitro by monocytes or U937 cells, the predominant cytokine affecting eosinophil functions is an acidic heat-stable polypeptide, designated ECEF. This material augments both eosinophil cytotoxic function (1-3) and leukotriene synthesis (4).

ECEF derived from U937 cells consists of at least two polypeptide species, including a more abundant 14-kDa form and a less abundant 10-kDa form. The 14-kDa form is preferentially released into surrounding culture medium, whereas the 10-kDa form is preferentially retained in the cell membrane, externally oriented. With respect to eosinophil cytotoxic function, the 10-kDa form has >20 times higher activity. Both 14- and 10-kDa species have the same NH₂-terminal amino acid sequence, and neither is glycosylated (5).

14-kDa ECEF appears to be identical to the cytokine/enzyme known as “thioredoxin” or “adult T cell leukemia-derived factor” that has been studied (and its cDNA cloned and expressed) for its ability to regulate the growth or development of certain lymphocyte cell lines (6-9) and to protect U937 cells from the toxic effects of tumor necrosis factor (10). An interesting structural feature of this cytokine/enzyme is the presence of the conserved and active dithiol reductase moiety from the enzyme thioredoxin, which may be involved in the mechanism of signal transduction. 14-kDa ECEF consists of a single polypeptide chain of 104 amino acids (8, 9). Except for the 20 NH₂-terminal amino acids, the structure of 10-kDa ECEF is unknown.

One model to account for these observations would be that there is a precursor-product relationship between the ECEF species, such that the 10-kDa polypeptide is derived by proteolytic cleavage of the 14-kDa precursor. Such a processing event has been demonstrated for many cytokines and prohormones (see Refs. 11 and 12) and, in U937 cells, for the specific examples of the cytokine ontocstatin M (13). If this is the case for the ECEF species, then it should be possible to prepare a 10-kDa ECEF polypeptide equivalent to a truncated 14-kDa species and to demonstrate its enhanced activity. Our approach was to prepare recombinant ECEF polypeptides representing the full-length 14 kDa molecule and two (approximately 10 kDa) species truncated at the COOH terminus. We tested the activity of these substances in the assay of eosinophil cytotoxic function. Since 14-kDa ECEF (thioredoxin) is known to possess dithiol reductase activity (8), we evaluated the catalytic activity of the truncated rECEF polypeptides.

MATERIALS AND METHODS

cDNA Encoding ECEF-104—cDNA clones encoding ECEF-104 (full-length) were isolated from a PMA-stimulated U937 Agt10 cDNA library (Clontech, Palo Alto, CA). The probe for screening of the library was generated by polymerase chain reaction, using sequence information from Ref. 8. The primers 5’-GTGAAGCAGATCGA

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GAGCAAG-3' (+strand NH₂ terminus) and 5'-GACTAATTCATGACATGCG-3' (-strand COOH terminus) were used to amplify a sequence from total amplified library phage DNA (0.5 μg), resulting in a single amplified species of the appropriate size (312 base pairs). This fragment was labeled with ³²P by random priming (kit from Boehringer Mannheim) and used to screen the same U937 cell cDNA library.

From a total of approximately 400,000 phage clones, approximately 200 were positive on initial screening. Six of these were selected for further study and subcloned. The DNA sequence of the insert was determined, and for all six, the sequence of the coding region was identical to that described in Ref. 9. Phage DNA was purified by phenol/chloroform extraction of SDS/EDTA-treated polyethylene glycol-precipitated phage from plaque lysates (14).

Production of rECEF Species—DNA species were prepared for insertion in the p-MALc expression vector (New England Biolabs, Beverly, MA; except as specified, methods were according to the manufacturer's instructions) by polymerase chain reaction, using purified ECEF-104 cDNA clone as a template. The primers were designed to incorporate the coding region of the desired length, beginning with the NH₂-terminal valine, and followed by a stop codon at the COOH terminus might have on the Bradford assay. Contributions of factor Xa to the protein concentration was ignored. The major contributor to the absorbance was the maltose-binding protein, the factor Xa cleavage site, glycol-precipitated phage from plaque lysates (14).

The polymerase chain reaction fragments generated in the amplification, the double-stranded product was treated with exonuclease to digest the strand with the 5'-terminal phosphate. Hopp-Woods hydrophilicity analysis of the sequence in this region reveal that amino acids 72-79 are uncharged, whereas the sequence of amino acids from positions 80 through 84 (KKGQK) is highly charged, containing 3 lysines.

This lysine-rich region would appear to be a favorable site for a cleavage event, but it does not contain any of the recognized cleavage sequences employed in eukaryotic cells for the processing of prohormones or procytokines. Most notably, it contains no arginines (for review, see Ref. 11). Thus, there was no clear candidate for the position of the cleavage site, and it was decided to construct two truncated recombinant ECEF polypeptides: the first terminating at amino acid 80 (rECEF-80) and the second terminating at amino acid 84 (rECEF-84).

The full-length 104-amino acid polypeptide (rECEF-104) was also prepared for comparison of eosinophil-stimulating and dithiol reductase activities. Absorbance was monitored over time at room temperature at 630 nm, using an automated enzyme-linked immunosorbent assay plate reader (model MR 600, Dynatech Laboratories, Inc., Alexandria, VA).

RESULTS

The molecule known as 14-kDa ECEF, thioredoxin, or adult T cell leukemia-derived factor is a 104-amino acid polypeptide, with a calculated molecular weight of 12,744 (9), so that a cleavage event at the COOH terminus yielding a 10-kDa polypeptide would occur in the vicinity of amino acid 82. Visual inspection and Hopp-Woods hydrophilicity analysis of the sequence in this region reveal that amino acids 72-79 are uncharged, whereas the sequence of amino acids from positions 80 through 84 (KKGQK) is highly charged, containing 3 lysines.

The recombinant ECEF species were generated as fusion proteins with E. coli maltose-binding protein and an intervening factor Xα cleavage site. The construction was such that cleavage with factor Xα generated the ECEF NH₂ terminus, without any additional amino acids. After purification of the fusion protein by amylase resin affinity chromatography, the fusion proteins were cleaved with factor Xα. Analysis of the cleaved rECEF polypeptides showed that they were of the expected size (Fig. 1). Attempts to separate the free rECEF species from the maltose-binding protein by size exclusion or anion exchange high performance liquid chromatography or by an ad-
Fig. 1. SDS-PAGE analysis of rECEF preparations. The products were expressed as fusion proteins, purified by affinity chromatography on an amylose resin, and cleaved with factor Xa. Lane 1 contains approximately 50 times the amount of factor Xa as in the other samples in order to show its migration; lane 2, shows factor Xa-treated buffer diluted in a manner similar to the other samples; lane 3, maltose-binding protein (MBP) prepared from the pMAL-c plasmid with no insert; lane 4, the rECEF-104 preparation; lane 5, rECEF-84; lane 6, rECEF-80. The migration of molecular weight markers is indicated (in thousands). The molecular weight of the maltose-binding protein is 42,000. Factor Xa is barely discernible in these preparations.

Fig. 2. Eosinophil-stimulating activity of rECEF species. The cytotoxic function of eosinophils was evaluated in the standard 40-h antibody-dependent assay using Schistosoma mansoni larvae as targets. rECEF species were tested at the indicated concentrations (serial 5-fold from 800 nM at the left to 82 fm at the right). None of these substances had any direct toxicity to the targets. A representative experiment is shown. Data represent the means of triplicate determinations. Maltose-binding protein (MBP) treated with factor Xa and factor Xa alone (added to buffer, incubated, and diluted in a similar manner) were tested for activity at the three highest concentrations only. Increases in cytotoxic function were statistically significant (two-tailed t test for independent measurements) with respect to the "no ECEF" control for rECEF-104 at 800 and 160 nM only and for rECEF-84 and rECEF-80 at 10 PM and higher.

Additional passage over the amylose resin were unsuccessful, with rECEF and maltose-binding protein co-eluting in all fractions. When rECEF species were separated by preparative SDS-PAGE, the polypeptides tended to precipitate from solution in the absence of detergent. This did not occur when the rECEF species were left in the presence of the maltose binding protein (data not shown). Therefore, preparations of cleaved but unseparated fusion proteins were used for most experiments, with controls for possible effects of the maltose-binding protein and factor Xa.

In the assay of eosinophil cytotoxic function, concentrations of 160 and 800 nM rECEF-104 had a slight, if any, stimulatory effect (Fig. 2). In separate experiments, higher concentrations of rECEF-104 did not have a greater effect on cytotoxic function (data not shown). By comparison, rECEF-80 and rECEF-84 enhanced eosinophil cytotoxic function substantially, at concentrations from 10 PM to 800 nM (Fig. 2). The effect of rECEF-80 was significant in three experiments at concentrations of 10 PM and higher; the effect of rECEF-84 was similar in two experiments and significant at 1.2 nM and higher in a third (p < 0.05 by the two-tailed t test for independent samples).

None of the rECEF preparations had direct toxicity to schistosomula targets. In parallel, two control samples (the maltose-binding protein as produced by the pMAL-c plasmid without an insert, treated with factor Xa and buffer alone treated with factor Xa, prepared by the same methods as the fusion proteins, and tested at the three highest concentrations) had no effect on eosinophil cytotoxic function (Fig. 2).

The rECEF preparations were tested for dithiol reductase catalytic activity (the ability to catalyze the reduction of the intrachain disulfide bonds of insulin in the presence of a suboptimal concentration (1 mM) of dithiothreitol). Of the three rECEF species, only rECEF-104 had the catalytic activity. This property was detectable at concentrations as low as 6.4 nM at the left to 82 fm at the right).

Fig. 3. Dithiol reductase activity of rECEF species. At a suboptimal concentration of dithiothreitol (DTT, 1 mM), the reduction of the intrachain disulfide bonds of insulin was monitored spectrophotometrically. A, the effects of rECEF species (concentrations from 4 μM to 6.4 nM) at 60 min of incubation are shown. B, the effects of rECEF-104 and rECEF-80 (concentrations of 4 μM and 800 nM) over time are shown.
160 nM (Fig. 3A) and was time-dependent (Fig. 3B). Control preparations of factor Xa-treated maltose-binding protein and Xa-treated buffer had no enzymatic activity (Fig. 3A).

Two further experiments were carried out to address the possible contribution of maltose-binding protein or factor Xa to the activities that were observed. The first of these involved the separation of rECEF-80 from maltose-binding protein and factor Xa by preparative SDS-PAGE. Material prepared in this manner enhanced eosinophil cytotoxic function over a range of concentrations from approximately 2 to 200 nM, whereas mock-eluted material from a different region of the gel did not (Fig. 4). However, as mentioned above, rECEF-80 prepared in this manner tended to precipitate from solution, an occurrence that interfered with accurate measurement of the effective dose.

The activity of factor Xa in these preparations was addressed specifically by assay with the chromogenic substrate Chromo-X. It was found that substantial factor Xa activity does specifically by assay with the chromogenic substrate Chromo-X. It was found that substantial factor Xa activity does exist in these preparations for over a month at 4 °C. However, this activity was almost completely destroyed by boiling of the sample for 5 min. Eosinophil-stimulating and dithiol reductase activities were not affected by boiling and, thus, were not dependent on the proteolytic activity of factor Xa (Fig. 5).

**FIG. 4.** Eosinophil-stimulating activity of rECEF-80 purified by SDS-PAGE. 40 µg of rECEF-80 was subjected to SDS-PAGE. Contents of the gel slice containing rECEF-80 and another slice from a blank region of the gel (mock-preparation) were recovered by electroelution and tested at dilutions of 10-10,000 (concentrations of 200 nM to 200 pM, assuming 100% recovery) in the assay of eosinophil cytotoxic function.

**FIG. 5.** Differential sensitivity to heating of rECEF and factor Xa activities. Aliquots of rECEF preparations were boiled for 5 min and assayed as described previously. Factor Xa activity was measured using a 20 µM concentration of rECEF-104 for heated and unheated samples, with spectrophotometric values (405 nm, background subtracted) to be read on the left axis. Dithiol reductase activity was measured using a 640 nM concentration of rECEF-104, with the spectrophotometric values (630 nm, background subtracted) to be read on the left axis. Eosinophil cytotoxic function was measured using a 1.2 nM concentration of rECEF-80, with the percentage of targets killed to be read on the right axis (compared with 28% with no ECEF).

**DISCUSSION**

The preparations of the rECEFs contained an equimolar concentration *E. coli* maltose-binding protein and 1% (in protein mass) of factor Xa. Neither maltose-binding protein, factor Xa, nor possible other undetected contaminants from *E. coli* or the isolation procedure had any independent activity in the assays of ECEF function. Taken together with the differential activities of the rECEF species, the heat sensitivity of factor Xa as compared with ECEF, and the activity of SDS-PAGE-purified ECEF, this information argues that the observed effects were due only to the rECEF species, not to other components of the preparation.

The activity of rECEF-80 and -84 was similar to that determined for purified natural 10-kDa ECEF (half-maximal between 16 and 400 pM), as evaluated by the same biological assay (3). This finding demonstrates the potential to generate an active 10-kDa ECEF species by proteolytic cleavage near the COOH terminus of 14-kDa ECEF. To date, it has not been possible either to demonstrate a precursor-product relationship between 14- and 10-kDa ECEF species by pulse-chase-type experiments (5) or to determine the COOH-terminal amino acid sequence of 10-kDa ECEF. The chief difficulty in these studies has been that 10-kDa ECEF is produced only in trace quantities, such that hours of metabolic radiolabeling are required to detect it (5).

The loss of dithiol reductase activity by the truncation of rECEF shows that at least part of the sequence from amino acid position 85 to the COOH terminus is essential to the dithiol reductase catalytic activity in solution, even though the conserved catalytic moiety (amino acids 31–34) was present in all rECEF species.

Thus, truncation of the 14-kDa ECEF COOH terminus increases the molecule's eosinophil-stimulating activity but abolishes the dithiol reductase activity. This evidence appears to argue against the involvement of the enzymatic activity in the mechanism of signal transduction in the eosinophil. Clearly, the dithiol reductase activity is not required for the initial interaction of rECEF-80 or -84 with the eosinophils. However, it is not possible to rule out a subsequent role of the dithiol reductase, because its activity might be reconstituted by a
conformational change or association with other molecules after the initial interaction with the eosinophil.

Studies involving the full-length ECEF (thioredoxin/adult T cell leukemia-derived factor) molecule suggest that it is pleiotropic in its activities, with documented effects on several cell types. In all of these studies, the effective dose was 100 nM or higher, a concentration uncharacteristically high for a cytokine. Our finding that rECEF-80 or -84 has stronger activity at doses as low as 10 pM suggests that a truncated form of ECEF may be the physiologically active molecule and that rECEF-80 or -84 should be used to study the biology of this regulatory factor.

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