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Formation of neutrophil-activating peptide 2 from platelet-derived connective-tissue-activating peptide III by different tissue proteinases

Bruce D. CAR, Marco BAGGIOLINI and Alfred WALZ*
Theodor-Kocher Institute, University of Bern, P.O. Box 99, CH-3000 Bern 9, Switzerland

Neutrophil-activating peptide 2 (NAP-2) is generated by cleavage of two inactive precursors, connective-tissue-activating peptide III (CTAP-III) and platelet basic protein (PBP), which are stored in the α-granules of blood platelets. Using highly purified CTAP-III as the substrate we studied the generation of NAP-2 by several neutral tissue proteinases. CTAP-III was rapidly cleaved by chymotrypsin, cathepsin G and trypsin, yielding products with neutrophil-stimulating activity. This activity remained unchanged for 24 h in the presence of chymotrypsin, decreased only slowly in the presence of cathepsin G, but was rapidly destroyed by trypsin. CTAP-III was also degraded by human neutrophil elastase and porcine pancreatic elastase, but no active fragments were obtained. By contrast, no degradation of CTAP-III was observed with thrombin, plasmin or 'granzymes' from cytolytic T-lymphocyte granules. Two active fragments of CTAP-III, generated by chymotrypsin or cathepsin G, were purified and partially sequenced, and were found to have the same *N*-terminal sequence as NAP-2. These results indicate that both proteinases cleave preferentially the bond between amino acids 15 (Tyr) and 16 (Ala) of CTAP-III. We conclude that chymotrypsin-like proteolytic activity in the vicinity of activated platelets may generate NAP-2 intravascularly. Due to its presence in the primary granules of neutrophils and monocytes, cathepsin G is likely to be involved in this process.

INTRODUCTION

Neutrophil-activating peptide 2 (NAP-2) is generated through the cleavage of two proteins, connective-tissue-activating peptide III (CTAP-III) and platelet basic protein (PBP), which are stored in the α-granules of blood platelets and are released by exocytosis upon platelet stimulation [1–3]. CTAP-III and PBP have no stimulatory activity with neutrophils and can thus be regarded as pro-forms of NAP-2 [3]. NAP-2 belongs to a family of structurally similar peptides, including NAP-1/interleukin-8 (IL-8) [4], the first to be discovered, and *gro*/melanoma-growth-stimulating activity (MGSA) [5], that act as chemotactic agonists on human neutrophils and induce migration, shape change, granule enzyme release and the respiratory burst [2,4–6].

In contrast to NAP-1/IL-8 [4,6–8] and *gro*/MGSA [9,10], whose production is induced by inflammatory stimuli in mononuclear phagocytes and a variety of tissue cells, NAP-2 is only generated when activated platelets release CTAP-III and PBP and proteinases suitable for their processing are present. It has been shown that neutral proteinases derived from monocytes cleave CTAP-III, yielding NAP-2 [3], but the properties of these enzymes have not been studied in detail. We therefore chose to examine a number of neutral tissue proteinases for their ability to generate neutrophil-stimulating activity from highly purified CTAP-III. In this paper we show that chymotrypsin-like proteinases selectively cleave CTAP-III between Tyr-15 and Ala-16 producing NAP-2, and we suggest that cathepsin G from neutrophils or monocytes is likely to be involved in the generation of neutrophil chemotactic activity in the vicinity of activated platelets.

MATERIALS AND METHODS

Materials

CTAP-III was purified from platelet release supernatants by heparin–Sepharose and CN-propyl reversed-phase chromatography [4], and its purity was assessed by silver-stained SDS/PAGE. Synthetic NAP-2 used as control was provided by Dr. I. Clark-Lewis, University of British Columbia, Vancouver, Canada. Enzymes used in the study were trypsin (Gibco, Paisley, Scotland), gel-bound bovine pancreatic chymotrypsin, bovine plasmin, porcine pancreatic elastase (all Boehringer, Mannheim, Germany), human neutrophil elastase (Serva, Heidelberg, Germany), bovine thrombin (Merck, Darmstadt, Germany), human leucocyte cathepsin G (Sigma, St. Louis, MO, U.S.A.), and T-cytolytic cell granzymes (generously provided by Dr. J. Tschopp, ISREC, Epalinges, Switzerland).

Proteolytic processing and bioassay

CTAP-III (80 μg/ml) was incubated with cathepsin G (2.5 μg/ml), chymotrypsin (2.2 μg of protein/ml), trypsin (0.5 mg/ml), pancreatic elastase (200 μg/ml), neutrophil elastase (8 μg/ml), plasmin (1 unit/ml), granzymes A and B, and mixed granzymes (A–H) (all 25 μg/ml) in calcium-free phosphate buffered-saline (PBS) in a total volume of 50 μl for time periods ranging from 1 min to 24 h at pH 7.4 and 37 °C. The reactions were stopped either by quick freezing in ethanol precooled in solid CO_2 (for neutrophil enzyme release assay), or by the addition of sample buffer [0.06 M-Tris/HCl, pH 6.8, 2% SDS, 4 mM-EDTA, 20% glycerol, 0.5 mg of Bromphenol Blue/ml and 2% (v/v) 2-mercaptoethanol] for PAGE analysis.

To assay for neutrophil-stimulating activity, the sample was thawed with prewarmed PBS supplemented with 2.5 mg of BSA/ml, 0.9 mM-$CaCl_2$ and 0.5 mM-$MgCl_2$, and transferred to multiwell plates for elastase or glucosaminidase assay [11]. Neutrophils (10^6 in 100 μl of PBS/BSA) were pretreated with 5 μg of cytochalasin B/ml at 37 °C for 10 min before addition of the peptide stimulus (100 μl). Samples were incubated for 15 min (glucosaminidase assay) or 20 min (elastase assay) at 37 °C.

Abbreviations used: NAP-2, neutrophil-activating peptide 2; CTAP-III, connective-tissue-activating peptide III; PBP, platelet basic protein; IL-8, interleukin-8; MGSA, melanoma-growth-stimulating activity; PBS, phosphate-buffered saline (137 mM-NaCl, 2.7 mM-KCl, 8.1 mM-Na_2HPO_4, 1.5 mM-KH_2PO_4, pH 7.3).

* To whom reprint requests should be addressed.

Synthetic NAP-2 was treated similarly with trypsin, human neutrophil elastase, cathepsin G and chymotrypsin and assayed for residual neutrophil granule-releasing activity.

Electrophoresis

Samples were separated by SDS/PAGE on 18% gels containing 7.2 M-urea, and protein bands were visualized by silver staining. Aprotinin (6.5 kDa) and lysozyme (14.4 kDa) were used as molecular mass markers.

Amino acid sequence analysis

Active CTAP-III fragments were isolated by reversed-phase CN-propyl h.p.l.c. (4.6 mm × 250 mm; Baker Research Products, Philipsburg, NJ, U.S.A.), and *N*-terminal sequences were obtained by automated phenyl isothiocyanate degradation analysis on an Applied Biosystems gas phase sequencer Model 477 A [3].

RESULTS

Proteolysis of CTAP-III

As indicated by the effects on elastase release from human neutrophils (Fig. 1.), degradation of CTAP-III by chymotrypsin and cathepsin G resulted in the formation of neutrophil-stimulating activity. Activity reached a maximum between 30 min and 3 h and then declined slowly. After 24 h the activity had decreased to about 80% of the maximum with chymotrypsin and to about 40% with cathepsin G. Digestion with trypsin yielded some neutrophil-stimulating activity within 30 min that was rapidly lost, presumably as a consequence of cleavage of NAP-2 into smaller peptides. No activity, by contrast, was observed upon incubation of CTAP-III with plasmin (Fig. 1), human leucocyte elastase, porcine pancreatic elastase or cytolytic T-cell granzymes (results not shown). Neither CTAP-III nor the proteinases alone, at the concentrations used in the assays, exhibited any neutrophil-stimulating effects, as detected by elastase or *N*-acetyl-β-glucosaminidase release.

The formation of neutrophil-stimulating activity was correlated, on SDS/PAGE, with the disappearance of the CTAP-III band at 9 kDa and the appearance of a single, distinct band at approx. 6 kDa. This is shown in lanes 3, 4 and 6 of Fig. 2(*a*), representing the conversion of CTAP-III into its active fragment by a 20 min incubation with cathepsin G, chymotrypsin and trypsin, respectively. Upon prolonged incubation (Fig. 2*b*, 3 h) CTAP-III was degraded to undetectable fragments by trypsin (lane 6), porcine elastase (lane 7) and human neutrophil elastase (lane 8). Lane 9 documents that CTAP-III is resistant to plasmin. Similarly, no substrate degradation was observed with purified granzymes A or B or with a mixture of granzymes A–H on incubation for up to 6 h (results not shown). None of the proteinases studied cleaved CTAP-III to β-thromboglobulin, as evidenced by failure to identify h.p.l.c. protein peaks other than NAP-2 and CTAP-III, and the failure in a kinetic examination of CTAP-III degradation between 1 min and 24 h to observe intermediate-molecular-mass proteins (between CTAP-III and NAP-2) for any enzyme tested (results not shown). All proteinases, on the other hand, were proteolytically active, as confirmed by SDS/PAGE analysis of the degradation of established protein substrates under conditions identical to those in the CTAP-III degradation experiments.

Characterization of active fragments

Since digestion with chymotrypsin and cathepsin G resulted in the long-lived generation of neutrophil-stimulating activity, the fragments obtained with these proteinases were separated by reversed-phase CN-propyl h.p.l.c. Unprocessed CTAP-III, which is inactive toward neutrophils, had a retention time of 56 min. As shown in Fig. 3, peaks with neutrophil-stimulating activity were detected at 38 and 46 min. After 3 h of incubation with chymotrypsin, the ratio of the peak areas was consistently 3:1. Following cathepsin G proteolysis under the same conditions, the 46 min peak was quantitatively less prominent and its area was only one tenth of that of the 38-min peak. Both peaks were partially sequenced and found to share the *N*-terminal sequence with NAP-2, i.e. Ala-Glu-Leu-Arg-Cys-Met-. The difference in h.p.l.c. mobility is thus likely to depend on *C*-terminal processing.

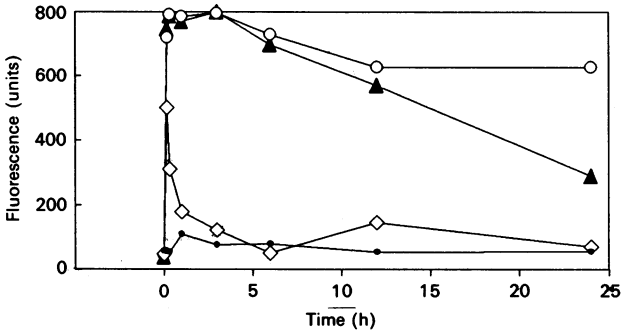

Fig. 1. Formation of neutrophil-stimulating activity from CTAP-III by proteolytic enzymes

Highly purified CTAP-III was incubated for various times with cathepsin G (▲, 2.5 μg/ml), chymotrypsin (○, 2.2 μg/ml), trypsin (◇, 0.5 mg/ml) or plasmin (●, 1 unit/ml). NAP-2 activity was then determined at the time points indicated by measuring elastase release from cytochalasin B-treated human neutrophils.

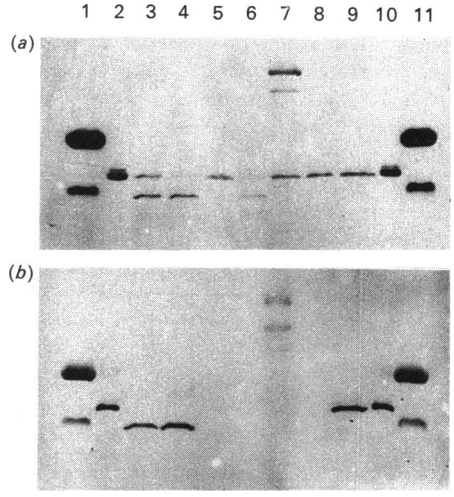

Fig. 2. Conversion of CTAP-III into NAP-2

CTAP-III was incubated for various times with different proteinases and the formation of cleavage products was determined on silver-stained PAGE (18% polyacrylamide containing 7.2 M-urea). Cleavage products after 20 min (*a*) and 2 h (*b*) of incubation are shown. Digestions of CTAP-III with cathepsin G (lane 3), chymotrypsin (lane 4), gel-bound trypsin (lane 5), trypsin (lane 6), porcine pancreatic elastase (lane 7), neutrophil elastase (lane 8) and plasmin (lane 9) are shown. Lanes 2 and 10 show untreated CTAP-III (9 kDa) and lanes 1 and 11 contain the molecular mass markers aprotinin (6.5 kDa) and lysozyme (14.4 kDa). The higher-molecular-mass band in lane 7 probably represents the porcine elastase protein. The same samples were analysed for neutrophil-stimulating activity as described in the legend to Fig. 1. Experiments of this type (Figs. 1 and 2) were performed 3–5 times with essentially the same results.

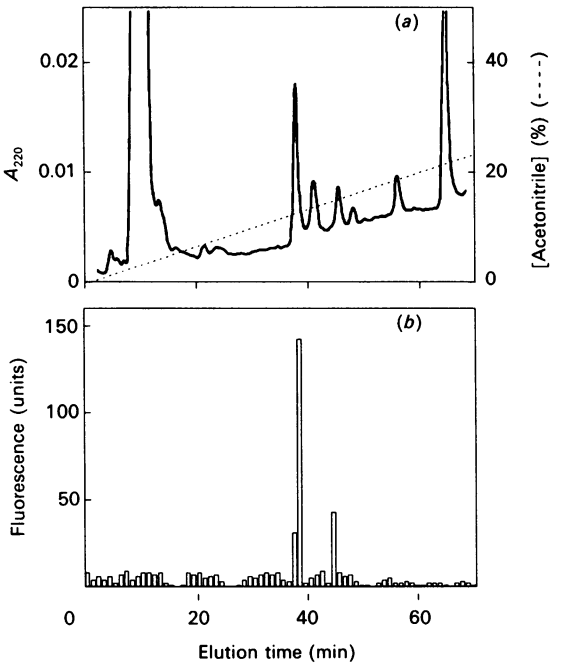

Fig. 3. Purification of active CTAP-III fragments

Fragments obtained from chymotrypsin digestion of CTAP-III (4 h at 37 °C) were separated on an h.p.l.c. reversed-phase CN-propyl column (*a*). Fractions of 1 mm were collected and tested for elastase release from cytochalasin B-treated human neutrophils (*b*). Fractions from the two activity peaks (retention times 38 and 46 min) were then used for *N*-terminal sequencing.

N-Terminal amino acid sequences of NAP-2, its precursor proteins and NAP-1/IL-8 are shown in Fig. 4.

Proteolysis of NAP-2

To ascertain the possibility of a proteolytic degradation of NAP-2, as suggested by the loss of neutrophil-stimulating activity arising from CTAP-III processing, synthetic NAP-2 was incubated with human neutrophil elastase, trypsin, cathepsin G or chymotrypsin, and the neutrophil-stimulating activity was monitored after various time intervals up to 24 h. As shown in Fig. 5, digestion with trypsin resulted in virtually complete inactivation of NAP-2 within 1 h. NAP-2 was also degraded by neutrophil elastase and cathepsin G, which inactivated the preparation within 3–6 h and 24 h respectively. By contrast, NAP-2 was completely resistant to chymotrypsin.

DISCUSSION

The platelet α-granule protein CTAP-III was rapidly degraded by most of the tissue proteinases used in this study, except for plasmin and the granzymes from cytolytic T-lymphocyte granules. With cathepsin G and chymotrypsin, two serine proteinases with similar selectivity, the two main cleavage products had neutrophil-stimulating activity and an *N*-terminal sequence identical to that of NAP-2 [1,3]. These products were apparently resistant to chymotrypsin, and were cleaved and inactivated only slowly by cathepsin G. The generation of neutrophil-stimulating activity was transient with neutrophil elastase and trypsin, which rapidly degraded NAP-2. Our study could not confirm the recently reported apparent conversion of

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NAP-2                                          AELRCMC....
β-TG                                    GKEESLDSDLYAELRCMC....
CTAP-III                          NLAKGKEESLDSDLYAELRCMC....
PBP          SSTKGQTKRNLAKGKEESLDSDLYAELRCMCIKTTS-GIHPKNIQSLE
NAP-1                                          SAKELRCQCIKTYSKPFHPKFIKELR

PBP/NAP-2    VIGKGTHCNQVEVIATLKDGRKICLDPDAPRIKKIVQKKLAGDESAD
NAP-1        VIESGPHCANTEIIVKLSDGRELCLDPKENWVQRVVEKFLKRAENS
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Fig. 4. Amino acid sequences of NAP-2, its precursors and NAP-1/IL-8

Abbreviation: β-TG, β-thromboglobulin.

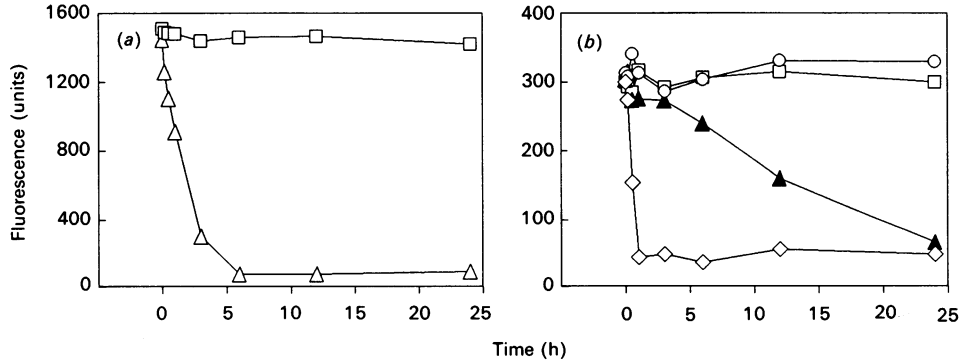

Fig. 5. Proteolysis of NAP-2

Synthetic NAP-2 (0.2 μM) was incubated with various proteinases and tested for residual activity at the time points indicated. (*a*) Incubation with leucocyte elastase (△, 8 μg/ml). (*b*) Incubation with cathepsin G (▲, 2.5 μg/ml), chymotrypsin (○, 2.2 μg/ml) and trypsin (◇, 0.5 mg/ml). Biological activity was tested by measuring *N*-acetyl-β-glucosaminidase (*a*) or elastase (*b*) release from cytochalasin B-treated human neutrophils. Controls in both panels are indicated by □.

CTAP-III to NAP-2 by elastase [12]. In addition, we found no evidence for the formation of β-thromboglobulin.

From the present data, the bond in the CTAP-III sequence that is most susceptible to chymotrypsin and cathepsin G appears to lie between amino acids 15 (tyrosine) and 16 (alanine), as indicated by the generation of NAP-2. Preferred cleavage sites for both enzymes are around the *C*-terminal side of aromatic amino acids of substrate proteins [13]. Such sites exist one, four and five amino acids from the *C*-terminus of CTAP-III, and it is possible that their cleavage led to the generation of NAP-2 variants which were separated on h.p.l.c. Ongoing structure–activity relationship studies with NAP-1/IL-8 (B. Moser, personal communication) show that small truncations at the *C*-terminus have little effect on the neutrophil-stimulating potency of the molecule. We therefore assume that a similar situation applies to NAP-2, in view of its considerable structural similarity to NAP-1/IL-8.

Whereas chymotrypsin is confined to the digestive tract, cathepsin G is stored in the primary granules of neutrophils [14] and monocytes [15]. It is thus readily available to convert CTAP-III that is released from activated platelets into chemotactically active NAP-2. This potential role of cathepsin G is made even more probable by the recent observation that this enzyme binds to the platelet surface [16]. Since it was demonstrated that neutrophil elastase degrades NAP-2, the potential for simultaneous production and degradation of NAP-2 by these azurophil granule enzymes exists; however, in contrast with the rapidity with which the activity is generated, the degradative process is quite slow and would be unlikely to interfere with net generation to activity. Other leucocyte enzymes are less suited to the generation of chemotactic activity: the serine proteinases from the granules of cytolytic T-lymphocytes [17] did not degrade CTAP-III, whereas neutrophil elastase degraded CTAP-III without producing NAP-2.

Concentrations of plasma inhibitors normally greatly exceed the concentrations of serine proteinases. Mechanisms to overcome this inhibitory environment have been proposed, such as the association of proteinases with binding sites that sterically prevent inhibitor, but not substrate, interactions [18]. A scenario whereby released α-granule proteins are processed in a protected microenvironment (e.g. by platelet-bound cathepsin G [16]), with the generation of high local concentrations of NAP-2, may potentially be invoked as a mechanism for neutrophil recruitment into platelet microthrombi. A positive feedback loop may be postulated whereby released cathepsin G or monocyte proteinases [10] convert CTAP-III into NAP-2, resulting in additional neutrophil activation. Furthermore, cathepsin G may contribute to platelet activation [19]. A pathophysiological role for NAP-2 has yet to be described. Given its ability to recruit and activate neutrophils at the sites of thrombosis, NAP-2 may have a role in neutrophil-dependent generation of plasmin and in fibrinolysis through the release of urokinase plasminogen activator [20]. Alternatively, NAP-2 could enhance anti-bacterial defence in the vicinity of fibrin-platelet clots, restricting bacterial colonization, particularly during infectious endocarditis and episodes of septic thromboembolism.

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