Volume 247, number 2, 386-390 FEB 07069 April 1989

Activation of interleukin-1β by a co-induced protease

Roy A. Black, Shirley R. Kronheim and Paul R. Sleath

Department of Protein Chemistry, Immunex Corporation, 51 University Street, Seattle, WA 98101, USA

Received 8 March 1989

The proteolytic generation of mature interleukin-1β (IL-1β) from its inactive precursor does not proceed by a conventional pathway for hormonal processing. Pro-IL-1β is found dispersed in the cytoplasm, and there are no basic amino acid residues or other commonly recognized processing sites adjoining the mature N-terminus. Processing appears to occur during release of the hormone. In the present study, we have identified a specific protease that generates mature IL-1β from the precursor. This enzyme is co-induced with the hormone, and it differs in its cleavage specificity and inhibitor sensitivity from all known proteases.

Interleukin-1β; Prohormone processing; Hormone activation; Protein processing; Proteolytic enzyme

1. INTRODUCTION

Interleukin-1β is a key hormone of the immune system, with roles in hematopoiesis, inflammation and wound healing [1]. Mature IL-1β consists of the C-terminal 153 residues of an inactive 33 kDa precursor, which is produced primarily by macrophages [2]. The maturation and release of this hormone do not proceed by conventional mechanisms. Unlike the case with most secretory proteins, the precursor lacks a signal sequence [2] and is not associated with membrane-bound compartments [3]. No mature IL-1β is found in the cytoplasm [4,5], suggesting that processing occurs during or following release of the precursor. Further indications of an unusual maturation pathway are the absence of any typical processing sites near the mature N-terminus (-Tyr-Val-His-Asp precedes the N-terminal Ala-Pro-) [2] and the inability of fibroblasts to process the prohormone [6]. Pulse-chase studies indicate that mature IL-1β is generated by a single cleavage of the precursor ([5], our unpublished results), although a 26 kDa form has also been observed [6]. The monocytic cell line THP-1 can be induced to produce the mature hormone [7]. Here, we used THP-1 cells to investigate whether there is a specific protease that generates mature IL-1β from the precursor.

Correspondence address: R.A. Black, Department of Protein Chemistry, Immunex Corporation, 51 University Street, Seattle, WA 98101, USA

Abbreviations: IL-1β, interleukin-1β; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; PMSF, phenylmethanesulfonyl fluoride

2. MATERIALS AND METHODS

2.1. *Cell culture*

THP-1 cells were obtained from the American Type Culture Collection. The cells were propagated and stimulated (with lipopolysaccharide, hydroxyurea, and silica) as described by Oppenheim [7] for 16 h or for shorter lengths of time as indicated.

2.2. *Cell lysates*

Cells were harvested by centrifugation at 2000 × *g* for 5 min, and then washed once by resuspension in Hank's balanced salt solution and recentrifugation. The cells were then resuspended to 1×10^8/ml in a solution of 10 mM Tris-HCl (pH 8.1), 5 mM dithiothreitol, frozen on dry ice, and thawed in a 37°C water bath. For the experiment shown in fig.1, the lysis buffer also contained 1 mM PMSF, 1 μg/ml leupeptin, and 1 μg/ml pepstatin.

2.3. *Protease assay, inhibitor study, N-terminal sequencing, and bioassay*

These procedures were carried out as previously described [8,9].

Published by Elsevier Science Publishers B.V. (Biomedical Division)

2.4. *Purification*

Lysates were freeze-thawed four times and then centrifuged for 20 min at 36590 × *g*. This procedure released over 50% of the processing activity to the supernatant fraction. The solubilized activity was applied to a column of DEAE-Sephacel equilibrated with 10 mM Tris-HCl (pH 8.1), 5 mM dithiothreitol. It was eluted with 80–140 mM NaCl. (Fractions were desalted by centrifugation through Biogel P6-DG prior to assay, because as little as 50 mM NaCl was found to inhibit the protease.) The eluted material was diluted 1:5 with 10 mM Tris-HCl (pH 8.1), 5 mM dithiothreitol and then applied to a column of procion red agarose (Bethesda Research Laboratories). The activity was eluted with 0.5–0.8 M NaCl, concentrated 20-fold in a Centriprep-10 concentrator, and then subjected to gel filtration with Sephadex G-75.

2.5. *Peptide analysis*

The peptide synthesis and analysis of cleavage products were carried out as in [9], except that the digestions were terminated by freezing rather than by addition of trichloroacetic acid.

3. RESULTS

3.1. *Generation of mature IL-1β by THP-1 lysates*

Lysates of THP-1 cells were incubated with recombinant pro-IL-1β, and processed forms of the protein were then detected by Western blot analysis with a C-terminus-specific antibody [8]. We found that lysates of unstimulated cells converted little if any pro-IL-1β to the 17.5 kDa mature form of the hormone (fig.1, 0 h, +), but a lysate of cells that had been stimulated to produce IL-1β clearly generated a fragment that co-migrated with the mature hormone (fig.1, 6 h, +). This product was purified [10], and its N-terminal 15 amino acids were sequenced and found to be identical to the corresponding residues of mature IL-1β [2]. The purified product was also found to have the level of biological activity expected for the corresponding amount of authentic IL-1β.

3.2. *Co-induction of the processing activity and IL-1β*

We then determined whether the expression of the induced proteolytic activity correlated with the appearance of active IL-1β in THP-1 cultures. Lysates were prepared at various times following stimulation and tested for their ability to generate mature IL-1β from the recombinant precursor. The processing activity of the lysates started to increase, above a low background level, between 2 and 3 h after stimulation (fig.1, + lanes). To measure the production of mature IL-1B by the cultures, aliquots of the culture medium were withdrawn at various times and assayed for IL-1 activity (little IL-1α is produced by THP-1 cells [7]). We found a large increase in the level of active IL-1 during the same interval in which the processing activity began to rise: there was no activity at 1

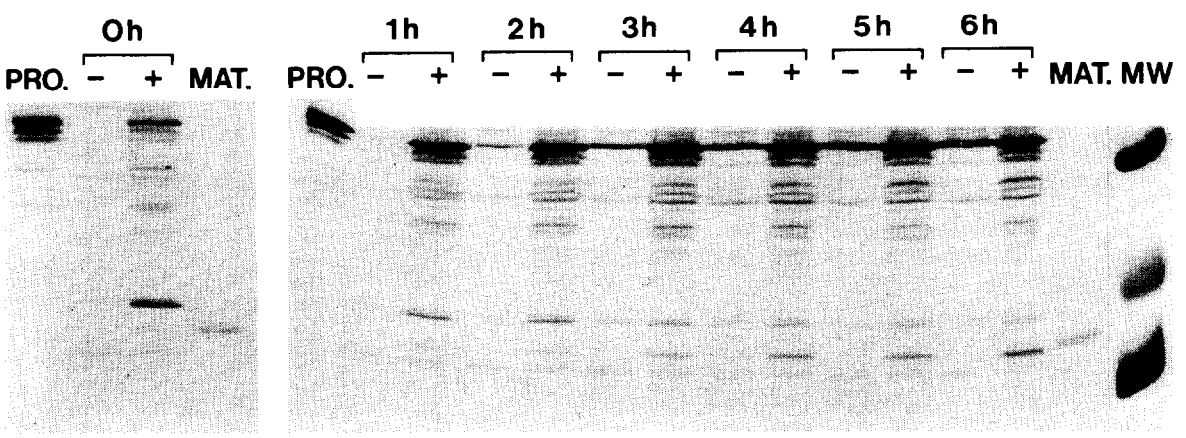

Fig.1. Western blot analysis showing time course of expression of mature-IL-1β-generating activity. THP-1 cells were lysed at the indicated number of hours following stimulation, and the lysates were prepared immediately for Western blot analysis (−) or first incubated for 40 min with recombinant pro-IL-1β (+). (PRO.) PRO-IL-1β incubated with lysis buffer, (MAT.) mature IL-1β, (MW) molecular mass standards - 14.3, 18.4, and 25.7 kDa. The immunoreactive proteins of less than 33 kDa in the pro-IL-1β preparation were generated during fermentation, and we have been unable to separate them from the full-length precursor [8]. In addition to endogenous pro-IL-1β, the various stained bands in the lysates themselves may be due to fragments of the precursor generated during cell lysis or to nonspecific staining of highly abundant proteins.

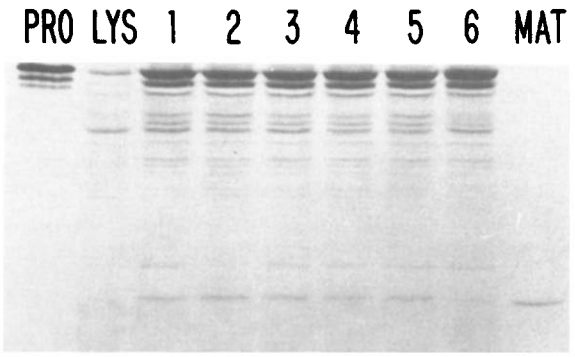

Fig.2. Inhibitor sensitivity of mature-IL-1β-generating activity in lysates. (1) No inhibitor, (2) 1 mM PMSF, (3) 1 μg/ml pepstatin, (4) 2 mM EDTA, (5) 0.1 mM E-64, (6) 1 mM iodoacetate. Aliquots of a lysate of THP-1 cells were incubated with 10 mM Tris-HCl buffer (pH 8.1) or with the inhibitors for 10 min prior to the incubation with pro-IL-1β and subsequent Western blot analysis. (PRO) pro-IL-1β, (LYS) unincubated lysate without added pro-IL-1β or any inhibitors, (MAT) mature IL-1β. The small amount of mature IL-1β in the unincubated lysate was generated from endogenous pro-IL-1β during the preparation of the lysate. The E-64 solution did inhibit papain (not shown).

h, 261 ± 62 U/ml at 2 h, and 3020 ± 140 U/ml at 3 h.

3.3. *Inhibitor sensitivity of the crude processing activity*

To determine whether the induced proteolytic activity could be due to a single protease, an inhibitor-sensitivity study was carried out. We found that one inhibitor, iodoacetate, completely inhibited the activity, while PMSF, pepstatin, EDTA, and E-64 had little or no effect (fig.2).

3.4. *Inhibitor sensitivity and substrate specificity of the purified processing activity*

The activity was purified about 500-fold as described in section 2. This preparation converted virtually all of the precursor to either mature IL-1β or a 26 kDa product (fig.3A). (A 26 kDa form may be produced by monocyte cultures as well [6].) The purified protease showed the same pattern of inhibitor sensitivity as had been found with the processing activity of the lysates (fig.3B). *N*-Ethylmaleimide was also tested (in the absence of dithiothreitol) and found to inhibit the enzyme completely (not shown). The 26 kDa product was subjected to N-terminal sequence analysis and found to result from a cleavage very similar to that which releases the mature hormone (see section 1): after Asp-27 of the precursor and preceding the sequence Gly-Pro. To test further the substrate specificity of the enzyme, we incubated the purified protease with a synthetic peptide representing the sequence from Ala-112 to Ser-121 of pro-IL-1β. Only two fragments were generated (fig.4), and analysis of their composition showed that they resulted from a single cleavage following Asp-116, the same cleavage that releases mature IL-1β from

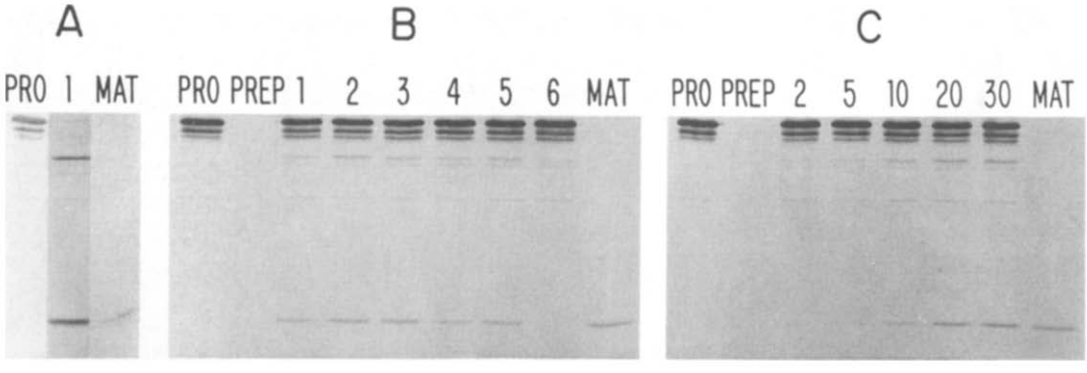

Fig.3. Studies with purified protease. (A) Products generated: (1) products resulting from a 60 min incubation of pro-IL-1β with a concentrated preparation of the purified protease, (PRO) pro-IL-1β, (MAT) mature IL-1β. (B) Inhibitor sensitivity: (1) no inhibitor, (2) 1 mM PMSF, (3) 1 μg/ml pepstatin, (4) 1 mM EDTA, (5) 0.1 mM E-64, (6) 1 mM iodoacetate. Aliquots of the purified protease preparation were incubated with 10 mM Tris-HCl buffer (pH 8.1) or with the inhibitors for 10 min prior to the incubation with pro-IL-1β: (PRO) pro-IL-1β, (PREP) purified protease preparation without added pro-IL-1β or any inhibitors, (MAT) mature IL-1β. (C) Time course of reaction. The purified protease preparation was incubated with pro-IL-1β for the indicated number of minutes: (PRO) pro-IL-1β, (PREP) purified protease preparation without added pro-IL-1β, (MAT) mature IL-1β. All samples (for A–C) were analyzed by Western blot.

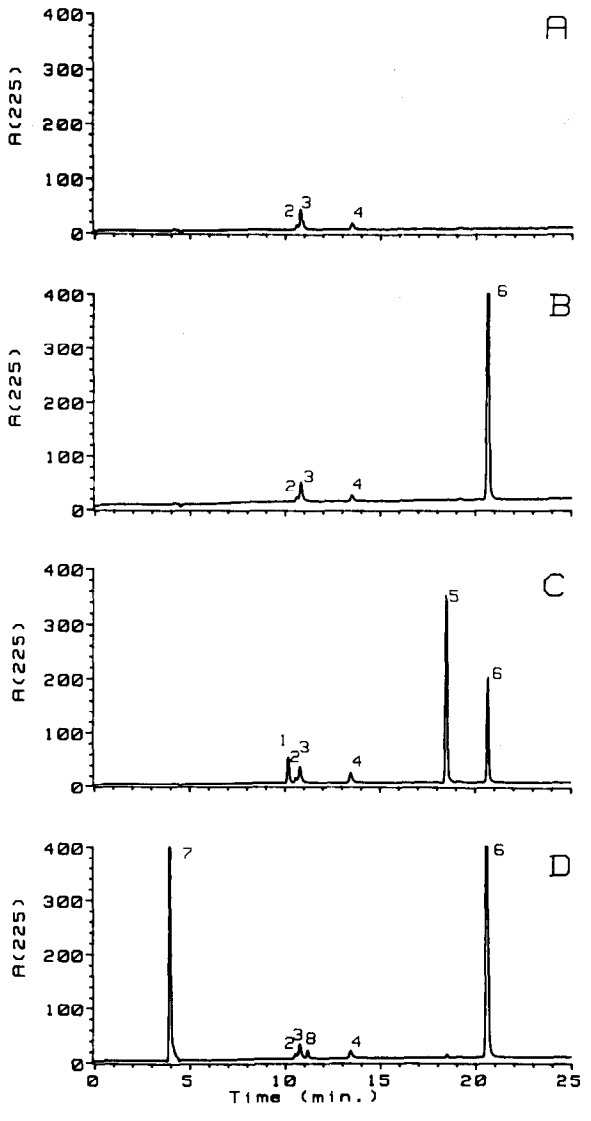

Fig.4. Digestion of a synthetic peptide representing Ala-112 to Ser-121 of pro-IL-1β (acetyl-Ala-Tyr-Val-His-Asp-Ala-Pro-Val-Arg-Ser-NH_2). (A) Purified protease preparation. (B) Peptide mixed with the purified protease, prior to incubation. (C) Peptide mixed with the purified protease, after 4 h incubation at 37°C. (D) The same as (C), except that 1 mM iodoacetate was added to the reaction mixture prior to incubation. Samples were applied to a Vydac C_{18} column, and material was eluted with a gradient of acetonitrile. Peak assignments: 2–4, impurities from the protease preparation; 6, peptide representing Ala-112 to Ser-121 of pro-IL-1β; 1, the fragment composed of Ala-117 to Ser-121; 5, the fragment composed of Ala-112 to Asp-116; 7, iodoacetate; 8, iodoacetate impurity. The amino acid analyses of the two generated fragments showed that they were present in equimolar amounts, and that the difference in peak height was due to a difference in extinction coefficients at 225 nm.

the precursor. The reaction with the peptide, as with the precursor protein, was completely inhibited by iodoacetate. As a further demonstration that the processing activity did not entail any intermediate cleavages, the time course of the reaction with pro-IL-1β was followed; we found that the two products (mature IL-1β and the 26 kDa form) were generated directly, without the appearance of any intermediate forms (fig.3C).

4. DISCUSSION

We have found that stimulation of THP-1 cells to produce IL-1β also induces the expression of a proteolytic activity that correctly processes pro-IL-1β in vitro. Our study of this activity suggests that it is due to a single protease: (i) iodoacetate inhibited all of the activity in cell lysates while other common inhibitors had no effect; (ii) the activity was purified 500-fold; (iii) the purified activity cleaved a synthetic peptide at only one site; and (iv) the purified preparation cleaved the precursor directly between Asp and Ala-Pro and between Asp and Gly-Pro without the prior generation of intermediates (our SDS-PAGE system resolves Asp-IL-1β from IL-1β [9]). Our study also demonstrates that this enzyme has a highly restricted and unusual substrate specificity: (i) while the precursor is susceptible to cleavage at many points [8,9], the purified protease cleaved it at only these two, very similar sites; (ii) the one cleavage site in the synthetic peptide corresponded to one of the sites where the precursor was cleaved; and (iii) no known enzyme, other than a general degradative protease, perhaps, would be expected to effect this cleavage [11], and none that we have tested does so [8,9]. The enzyme's sensitivity to iodoacetate and *N*-ethylmaleimide but not to the other class-specific inhibitors tested suggests that it is a cysteine protease, while its resistance to E-64 (an inhibitor of most cysteine proteases [12]) is consistent with its unusual substrate specificity.

Evidence that this protease is required for the activation of the prohormone by monocytic cells includes the correlation of its appearance with that of mature IL-1β following stimulation of the cells, and the generation of the mature hormone and a 26 kDa form by the enzyme without intermediates, as appears to be the case in cultures of monocytes [5,6]. Our results thus indicate that the maturation

of IL-1β is carried out by a specific protease, and that it may be possible to control IL-1β levels by inhibiting this unusual proteolytic activity.

Acknowledgements: We thank Teresa Metzger for the Western blot analyses and for assistance in all aspects of this work, Paula Glackin for assistance with the purification, Victoria Batler and Teresa Tough for assistance with cell cultures, Diana Boivin and Kerri Biggs for performing the IL-1 bioassays, Kirk Van Ness for the N-terminal sequence analyses, Janet Merriam for the amino acid analyses, Ronald Hendrickson for assistance with the peptide digests, and Linda Troup for assistance with the manuscript.

REFERENCES

[1] Oppenheim, J.J., Kovacs, E.J., Matsushima, K. and Durum, S.K. (1986) Immunol. Today 7, 45–56.
[2] March, C.J., Mosley, B., Larsen, A., Cerretti, D.P., Braedt, G., Price, V., Gillis, S., Henney, C.S., Kronheim, S.R., Grabstein, K., Conlon, P.J., Hopp, T.P. and Cosman, D. (1985) Nature 315, 641–647.
[3] Singer, I.I., Scott, S., Hall, G.L., Limjuco, G., Chin, J. and Schmidt, J.A. (1988) J. Exp. Med. 167, 389–407.
[4] Bayne, E.K., Rupp, E.A., Limjuco, G., Chin, J. and Schmidt, J.A. (1986) J. Exp. Med. 163, 1267–1280.
[5] Hazuda, D.J., Lee, J.C. and Young, P.R. (1988) J. Biol. Chem. 263, 8473–8479.
[6] Young, P.R., Hazuda, D.J. and Simon, P.L. (1988) J. Cell Biol. 107, 447–456.
[7] Matsushima, K., Copeland, T.D., Onozaki, K. and Oppenheim, J.J. (1986) Biochemistry 25, 3424–3429.
[8] Black, R.A., Kronheim, S.R., Cantrell, M., Deeley, M.C., March, C.J., Prickett, K.S., Wignall, J., Conlon, P.J., Cosman, D., Hopp, T.P. and Mochizuki, D.Y. (1988) J. Biol. Chem. 263, 9437–9442.
[9] Black, R.A., Kronheim, S.R., Merriam, J.E., March, C.J. and Hopp, T.P. (1989) J. Biol. Chem., in press.
[10] Kronheim, S.R., March, C.J., Erb, S.K., Conlon, P.J., Mochizuki, D.Y. and Hopp, T.P. (1985) J. Exp. Med. 161, 490–502.
[11] Barrett, A.J. (1977) Proteinases in Mammalian Cells and Tissues, Elsevier/North-Holland, Amsterdam.
[12] Barrett, A.J., Kembhavi, A.A., Brown, M.A., Kirschke, H., Knight, C.G., Tamai, M. and Hanada, K. (1982) Biochem. J. 201, 189–198.