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Endotoxin-Stimulated Monocytes Release Multiple Forms of IL-1β, Including a ProIL-1β Form Whose Detection Is Affected by Export

Mark D. Wewers, Alissa V. Winnard, and Heidi A. Dare

The processing and release of 31-kDa proIL-1β to the mature 17-kDa form of IL-1β are still poorly understood. To help elucidate the mechanisms involved in IL-1β processing and release, we measured IL-1β forms released from endotoxin-stimulated monocytes by immunoprecipitation of [35S]methionine-labeled protein, by Western blots, and by our recently developed ELISA specific for proIL-1β. Our studies demonstrate that in addition to the 17-kDa mature IL-1β, IL-1β is also released as 31-, 28-, and 3-kDa molecules. The 31-kDa released form of proIL-1β represented 20–40% of the total released IL-1β, as measured by SDS-PAGE with densitometry. This released proIL-1β was susceptible to ICE processing; however, this proIL-1β was not detectable by either a mature or proIL-1β-specific ELISA, suggesting that release induces a conformational change. The ELISA inability to detect proIL-1β was not due to inadequate sensitivity or subsequent degradation in the ELISA. Furthermore, while immunofluorescence-purified cytosolic proIL-1β could complex the type II IL-1R, released proIL-1β did not. Finally, the absence of a band shift in non-denaturing gel electrophoresis excluded proIL-1β binding to another protein. These findings imply that IL-1β is exported from monocytes as 3-, 17-, 28-, and 31-kDa forms and that the released 31-kDa form differs from cytosolic proIL-1β.

Interleukin-1β is a potent proinflammatory cytokine regulated at many sites in its induction, production, and function. Its induction by endotoxin is modified by the concentration of LPS-binding protein, soluble and surface CD14 expression, and factors that control endotoxin tolerance (1–5). Once induced, its gene expression is regulated by both transcriptional and translational controls (6–10). Once released, mature IL-1β is antagonized by IL-1ra and the soluble form of the type II IL-1R (11–15). However, it is most tightly regulated in processing and release (10, 16, 17). This aspect of regulation is possibly the least understood.

IL-1β is translated as a 31-kDa molecule that lacks a conventional leader sequence or membrane-spanning region (18). It is synthesized in the cytosol on free polyribosomes (19), and its conversion to mature IL-1β requires processing by a cysteine protease termed IL-1β-converting enzyme (ICE), or more recently caspase 1 (20–22). Like proIL-1β, ICE is also found in the cytosol as an inactive precursor protein (23). Two 45-kDa ICE precursor molecules must be processed at specific aspartic acid-X sites to form homodimers that condense to form the functional tetramer (24, 25). While ICE is assumed to be the IL-1β convertase, active ICE has yet to be convincingly identified in cells that process and release IL-1β (23, 26). Furthermore, the connection between proIL-1β conversion to the mature 17-kDa IL-1β and its release from the cytosol is uncertain.

It has been suggested that released proIL-1β may be subject to processing (and hence activation) extracellularly (27). Furthermore, using a synthetic ICE inhibitor, it has been shown that proIL-1β processing is not a prerequisite for IL-1β export from the cell (20). In this context, components of IL-1β distinct from the 17-kDa mature form are released from intact mononuclear phagocytes (28). The present study demonstrates that IL-1β is released in a number of different forms, one of which is proIL-1β, which differs significantly from cytosolic proIL-1β in its ability to be recognized by Abs and the type II IL-1R.

Materials and Methods

Purification of PBMC and cell culture conditions

Venous blood was obtained from healthy normal volunteers by venipuncture. The blood was anticoagulated with sodium heparin (Elkins-Shinn, Cherry Hill, NJ) at 15 U/ml and kept at 4°C during processing. PBMC were separated by polycryosaccharide diatrizoate (Histopaque; Sigma Diagnostics, St. Louis, MO) density-gradient centrifugation, washed three times with sterile saline, and then resuspended at 5 × 10^6 cells/ml in RPMI 1640 (BioWhittaker, Walkersville, MD) and supplemented with 5% FBS (HyClone, Logan, UT) and gentamicin (50 µg/ml), unless otherwise noted. PBMC were cultured in either 6- or 24-well tissue culture plates (Becton Dickinson, Lincoln Park, NJ) at 5 × 10^6 cells/ml and stimulated with LPS at 1 µg/ml (LPS W. Escherichia coli 102F:B8; Difco, Detroit, MI) at 37°C, 5% CO₂.

Preparation of [35S]methionine-labeled released and cytosolic IL-1β

IL-1β preparations were metabolically labeled with [35S]methionine by culture in methionine-free RPMI 1640 and the addition of 0.5 µCi/ml [35S]methionine (Amersham, Arlington Heights, IL) with the endotoxin. After 18 h, culture supernatants and cells were harvested and prepared for immunoprecipitation. Cells were lysed in a buffer containing 10 mM Tris, pH 7.4, 1% Nonidet P-40 (Sigma), 1 µg/ml leupeptin, 1 µg/ml pepstatin, 1 mM EDTA, and methoxy succinylala-ala-da-da-pro-val chloromethyl ketone. Lysates were cleared of nuclei and cell particulate by centrifugation at 10,000 × g.

Supernatants and lysates were buffered to pH 8 with 1 M Tris and then incubated with either carbonyl terminus-specific (Rc) or amino terminus-specific (Rn) rabbit antiserum to human IL-1β at 1/100 dilution at 4°C for

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3 Abbreviation used in this paper: ICE, IL-1β-converting enzyme.

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Proteolysis was stopped by the addition of 1 mM iodoacetate. Processed for proIL-1

immunoprecipitate proIL-1

The first tested the ability of the soluble recombinant type II IL-1R to

been generated with [35 S]methionine, as outlined above. After incubation
cytosol or supernatant samples of endotoxin-stimulated PBMC, which had

HEPES, pH 7.5, 10% sucrose, 0.1% CHAPS, 2 mM DTT) for 1 h at 30°C.

samples by both the mature IL-1

ICE processing was to quantify

macrophages released what appeared to represent 31-kDa IL-1

m.w. protein release paralleled the release

High m.w. protein coprecipitates with IL-1β in mononuclear phagocyte supernatants

While characterizing the processing and release of mature IL-1β by mononuclear phagocytes, we noted that endotoxin-stimulated monocyte supernatants also contain a 31-kDa protein that coincides with the size of intracellular proIL-1β. Metabolic labeling with [35 S]methionine, followed by immunoprecipitation and SDS-PAGE, revealed that both human blood monocytes and alveolar macrophages released what appeared to represent 31-kDa IL-1β. The kinetics of the high m.w. protein release paralleled the release of 17-kDa mature IL-1β

Demonstration that the high m.w. factor is proIL-1β

To determine whether the 31-kDa protein was proIL-1β, selective immunoprecipitation experiments were performed to further characterize this protein. Fig. 1 compares the relative ability to immuno-precipitate this protein by a rabbit antibody specific to the amino terminus of proIL-1β (Rn) and a carboxyl terminus-specific rabbit antibody (Rc). Both the Rn and Rc antisera were able to immunoprecipitate the 31-kDa molecule, as would be expected for proIL-1β. Furthermore, the Rn antisera also immunoprecipitated a 3-kDa fragment of IL-1β (the expected product of cleavage at Asp28) (30), while the Rc antisera immunoprecipitated the carboxyl-terminal fragment of this processing, 28-kDa IL-1β. Consistent with this observation, the Rn antisera did not detect the 28- or 17-kDa fragments, and the Rc antisera did not detect the 3-kDa fragment.

To confirm that the 31-kDa protein immunoprecipitated by Rn was proIL-1β, the immunoprecipitation was repeated in the presence or absence of the peptide that had been used to generate the Rn antisera. Fig. 2 documents the specificity of the 31-kDa protein as IL-1β by demonstrating that the immunizing peptide blocked the Rn antisera detection of the 31-kDa protein, but not the Rc antisera detection of either the 31- or 17-kDa proteins. The immunizing peptide also blocked the detection of the 3-kDa fragment (data not shown), confirming its identity as a precursor fragment.

Ability of IL-1-converting enzyme to process released proIL-1β

One of the characteristics of proIL-1β is its ability to be processed by ICE to functional 17-kDa IL-1β. The ability of the released proIL-1β to undergo activation by ICE is particularly relevant to understanding the significance of the released proIL-1β. To test this question, released and cytosolic proIL-1β were labeled metabolically with [35 S]methionine in the same individuals and purified by affinity chromatography. The released and cytosolic

IEC processing of proIL-1β

The ability of proIL-1β to be processed by ICE was evaluated by incubating immunofluorinity-purified [35 S]methionine-labeled proIL-1β with 5 U functional rICE (gift of Nancy Thornberry and Doug Miller, Merck Research Laboratories, Rahway, NJ) in 25 μl vol of processing buffer (50 mM HEPES, pH 7.5, 10% sucrose, 0.1% CHAPS, 2 mM DTT) for 2 h at 30°C. Proteolysis was stopped by the addition of 1 mM iodoacetate. Processed proIL-1β was evaluated by subsequent SDS-PAGE and autoradiography for generation of the appropriately sized proIL-1β fragments.

Another approach to document proIL-1β processing was to quantify samples by both the mature IL-1β ELISA (B1/Rc) and the proIL-1β ELISA (Rn/G). ICE processing of proIL-1β was characterized by gain of B1/Rc detection and loss of Rn/G detection.

Binding of proIL-1β to type II IL-1R

Binding of proIL-1β to the type II IL-1R was evaluated by two techniques. The first tested the ability of the soluble recombinant type II IL-1R to immunoprecipitate proIL-1β, and the second utilized the type II IL-1R in a capture ELISA format.

Three distinct IL-1β ELISA formats were utilized. Mature IL-1β was detected using a sandwich ELISA format that we have previously described (10). This ELISA, termed B1/Rc, utilizes a mouse mAb to mature human IL-1β, B1 (gift of Ann Berger, Upjohn, Kalamazoo, MI), as the capture, a rabbit polyclonal antisera to mature human IL-1β (R&D Systems, Minneapolis, MN) to complete the sandwich. A horseradish peroxidase-conjugated rabbit anti-goat Ab, followed by o-phenylenediamine, was used to detect the bound goat Ab. This ELISA, termed B1/Rn, has documented specificity for proIL-1β and a sensitivity to 100 pg/ml (29).

A second proIL-1β-specific ELISA was developed that did not require the availability of the functional B1 epitope. This ELISA utilizes the amino terminus-specific antisera, Rn, as the capture Ab, and a goat anti-human IL-1β Ab (R&D Systems, Minneapolis, MN) to complete the sandwich. Recovered lavage fluid was passed through one lobe of sterile surgical gauze to remove mucus and particulate. Cells were counted by hemocytometer, pelleted, and resuspended in RPMI 1640 for in vitro studies.

Western blot analysis

Immunoblots of cell supernatants (10⁶ cells/ml) and lysates (10⁷ cells/ml) were used to assay mononuclear phagocytes for endogenous cytokine ex-pression. Samples were boiled for 3 min in equal volume of Laemmli sample buffer and electrophoresed into a 15% SDS-polyacrylamide gel, transferred to Immobilon P membranes (Millipore, Bedford, MA) in Tris/ 10% methanol/0.01% SDS buffer, air dried, and then blocked and assayed by a lumiphos technique per manufacturer’s recommendation (Amersham). ProIL-1β was detected using a 1/100 dilution of rabbit antisera to proIL-1β (Rn).

Bronchoalveolar lavage

Bronchoscopic with bronchoalveolar lavage was performed, as we have previously described (10). Briefly, subjects underwent standard bronchoco-opy. The bronchoalveolar lavage consisted of instilling sequentially aspirating sterile saline in five 20-ml aliquots into the right middle or lingular bronchus from the wedged position. Recovered lavage fluid was passed through one lobe of sterile surgical gauze to remove mucus and particulate. Cells were counted by hemocytometer, pelleted, and resuspended in RPMI 1640 for in vitro studies.
documents the identity of the proIL-1β and 17-kDa forms. This characteristic cleavage pattern further documents the release of proIL-1β to mature IL-1β or the Rn (amino terminus-specific polyclonal antiserum to amino acids 3–21 of proIL-1β) to recognize the various forms is denoted by a (+). The right side of the graphic shows a representative autoradiogram after 12% SDS-PAGE. Immunoprecipitates are from supernatants of mononuclear cells stimulated overnight with 1 μg/ml of LPS in the presence of [35S]methionine. From left to right are protein A-Sepharose immunoprecipitations with rabbit preimmune serum (pre), with rabbit carboxyl terminus-specific IL-1β antiserum (Rc), and with rabbit amino terminus-specific IL-1β antiserum (Rn). It should be noted that four distinct forms can be detected: a 31-kDa form by both Rc and Rn, a 28-kDa form by Rc only, a 17-kDa form by Rc only, and a 3-kDa form by Rn only. This is typical of three separate immunoprecipitations.

Released proIL-1β present at about 25% of the amount in lysates (i.e., 1 ng/ml range).

Since it was conceivable that proIL-1β might undergo partial processing during the ELISA assay (and therefore prevent detection by the loss of the Rn epitope), purified [35S]methionine-labeled cytosolic proIL-1β was tested for its stability in the ELISA format. The ELISA-immobilized proIL-1β was not cleaved, as documented by SDS-PAGE and autoradiography (data not shown).

Finally, since the B1/Rn ELISA format utilizes mAb B1 as the capture Ab, it was conceivable that the B1 epitope is unavailable in the released form of proIL-1β, but available in the recombinant proIL-1β standard and cytosolic proIL-1β. Consistent with this hypothesis, a unique proIL-1β ELISA format (Rn/G), which utilizes the rabbit proIL-1β-specific antiserum (Rn) as the capture Ab and a polyclonal goat Ab (G) to mature IL-1β to complete the sandwich, was able to detect released proIL-1β. Table I compares the detection of released proIL-1β by the two ELISA formats.
FIGURE 4. Western blot of released proIL-1β. After overnight culture in the presence of 1 μg/ml LPS, normal human mononuclear cell supernatants and lysates were harvested as described in Materials and Methods and analyzed after 12% SDS-PAGE and electrophoretic transfer to nylon membranes. Shown is a representative blot from three different donors and decreasing amounts of standard rproIL-1β. From left to right are rproIL-1β, 1, 0.3, 0.1, and 0.03 ng/lane, followed by the lysate and supernatant, respectively, from donors A, B, and C. Lysates represent 10^6 monocytes, and supernatants represent 0.2 × 10^6 monocytes. By extrapolation to the standard curve, the released proIL-1β is similar in amount to the remaining intracellular proIL-1β.

again demonstrating that capture by the B1 Ab-specific epitope is impaired.

**Binding of released proIL-1β to type II IL-1R.** We have demonstrated recently that cytosolic proIL-1β can bind to the type II IL-1R (31). To determine whether released proIL-1β could also bind the type II IL-1R, [35S]methionine-labeled cytosolic and released proIL-1β forms were incubated with recombinant type II IL-1R and immunoprecipitated with a nonneutralizing mAb to the type II IL-1R, M2. M2 complexes were retrieved by protein A-Sepharose beads, eluted into Laemmli sample buffer by boiling, and analyzed by SDS-PAGE and autoradiography. Fig. 5 demonstrates that released mature IL-1β and cytosolic proIL-1β could be immunoprecipitated by this procedure, but that released proIL-1β could not. Furthermore, the released proIL-1β was not complexed to another protein since denatured and nondenatured samples of released proIL-1β ran identically to the recombinant proIL-1β standard (data not shown).

Finally, to demonstrate that [35S]methionine labeling did not interfere with the ability of released proIL-1β to bind to the type II receptor, an ELISA format was devised to test for released proIL-1β binding to the type II IL-1R. In this format, functional type II IL-1R are captured by the nonneutralizing M2 Ab, and receptor-bound IL-1 can be detected with antisera specific to IL-1. While [35S]methionine-labeled cytosolic proIL-1β and released mature IL-1β complexed the immobilized type II IL-1R (as detected by Rn and Rc antisera, respectively), released proIL-1β did not. These data imply that receptor binding sites in released proIL-1β are altered significantly from those on cytosolic proIL-1β.

**Discussion**

The processing and release of proIL-1β by mononuclear phagocytes are an important regulatory point for IL-1β. It has been long recognized that abundant quantities of IL-1β remain intracellular in stimulated monocytes and macrophages (10, 16). In some cell types such as keratinocytes and to a lesser extent tissue macrophages, the predominant form of IL-1β after stimulation is intracellular proIL-1β (10, 16, 32). In this context, metabolic labeling studies of newly synthesized IL-1β demonstrate that precursor proIL-1β can be released intact from stimulated mononuclear phagocytes. In the present study, utilizing both a specific proIL-1β Ab and a traditional mature IL-1β-specific Ab, we show that IL-1β is released in a number of forms other than 17 kDa. The prevalent 31-kDa form of released IL-1β is truly proIL-1β since immunoprecipitation by the proIL-1β-specific Ab is blocked by the immunizing peptide, while its immunoprecipitation by the mature specific antisera is not. A 28-kDa form of released IL-1β is detected by the mature specific Ab, but not by the proIL-1β-specific Ab. Importantly, a 3-kDa piece is also recognized by the proIL-1β-specific antisera, but not by the mature specific antisera. The 3-kDa fragment detection is also inhibited by the 3–21 immunizing peptide.

This is the first direct demonstration that ICE cleavage at Asp^28 occurs during proIL-1β processing by monocytes and, furthermore, the first to show that this peptide is released from stimulated mononuclear phagocytes. It confirms the suggestion by Higgins et al. (28) that monocytes may clip proIL-1β at Asp^28, as evidenced

### Table I. Comparison of proIL-1β release by two ELISA formats

<table>
<thead>
<tr>
<th>Source</th>
<th>B1/Rn Format (pg/ml)</th>
<th>Ro/Go Format (pg/ml)</th>
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*Normal human monocytes and alveolar macrophages (10^6/ml) from five different individuals (1–5) were cultured overnight in RPMI 1640, 5% FBS, and 1 μg/ml LPS. Supernatants were harvested and assayed for proIL-1β by two ELISA formats (B1/Rn and Ro/Go, as described in Materials and Methods).
by a 14-kDa band seen in their experiments. The significance of this 3-kDa fragment is not known. However, the previous observation that the Asp\(^{28}\) processing site is not required for ICE to cleave at the activation site of IL-1\(\beta\), Asp\(^{116}\) (30), coupled with our observation that this 3-kDa peptide is present extracellularly, suggests the possibility of a unique function. Since the precursor component of proIL-1\(\beta\) is highly conserved between IL-1\(\alpha\) and IL-1\(\beta\), a functional role for the 3- and 11-kDa fragments should be considered, as March et al. have previously suggested (18). It is particularly noteworthy that 16-kDa N-terminal propiece of IL-1\(\alpha\) has been confirmed to be a nuclear oncoprotein (33). Although we have not attempted to demonstrate function for the released 3-kDa IL-1\(\beta\) propiece, the release of this molecule does have implications for the relationship of ICE to the pathway. It implies that the release pathway is intimately associated with the processing machinery. It is consistent with the possibility that the ICE cleavage event occurs on the outer membrane of monocytes and macrophages, as has been suggested by the demonstration of constitutively active ICE on monocyte plasma membranes by Singer et al. (34).

A recent observation has suggested that the ability to detect this released form of proIL-1\(\beta\) is highly dependent upon the affinity of the Abs used (28). In this context, we and others have confirmed recently that intracellular concentrations of proIL-1\(\beta\) may be greatly underestimated by ELISAs designed to detect mature IL-1\(\beta\) (16, 35). However, in the process of analyzing our newly described proIL-1\(\beta\) ELISA, we observed a unique deficiency in the detection of released proIL-1\(\beta\). The proIL-1\(\beta\)-specific ELISA that readily detected cytosolic proIL-1\(\beta\) was virtually blind to the released forms of proIL-1\(\beta\) that could be detected by Western blotting or immunoprecipitation techniques.

To further characterize the released form of proIL-1\(\beta\), a number of experiments were performed to compare this form with the cytosolic form of proIL-1\(\beta\). As mentioned, while the B1/Rn ELISA format could not detect the released form of proIL-1\(\beta\), it could readily detect intracellular proIL-1\(\beta\), as referenced to Western blot detection (33). This inability to detect the released form of proIL-1\(\beta\) was not due to degradation within the ELISA format since \(^{[35}\text{S}]\)methionine-labeled proIL-1\(\beta\) remained intact when captured and incubated in the ELISA system. Furthermore, a unique ELISA format (Ru/G) was able to detect the released proIL-1\(\beta\).

Since the B1 Ab blocks mature IL-1\(\beta\) function, but does not recognize released proIL-1\(\beta\), released proIL-1\(\beta\) was tested for its ability to complex the type II IL-1R, another assay for the availability of functional epitopes. We have demonstrated recently that cytosolic and recombinant proIL-1\(\beta\) can complex the type II receptor. However, in agreement with the masking of the B1 epitope, released proIL-1\(\beta\) was also not detected by the type II IL-1R.

To explain the differences between these two forms of proIL-1\(\beta\) to date have been unsuccessful. It is possible that the released proIL-1\(\beta\) is part of a multimeric protein complex. However, in labeling studies, no \(^{[35}\text{S}]\)methionine-labeled protein co-purificates with the released proIL-1\(\beta\). Likewise, attempts to immunoprecipitate released proIL-1\(\beta\) with antisera to ICE or the type II IL-1R were also unsuccessful (data not shown). This does not exclude the possibility that the released form of proIL-1\(\beta\) elutes with constitutive proteins that are not labeled with methionine. However, non-denaturing polyacrylamide electrophoresis with immunoblotting reveals released proIL-1\(\beta\) migrates identically with the rproIL-1\(\beta\) standard. Thus, although it remains possible that the released form of proIL-1\(\beta\) is part of a unique protein complex, experiments to date do not support this explanation.

An alternative explanation is that proIL-1\(\beta\) is induced to undergo a conformational change in the process of secretion. Hayazda et al. have demonstrated that the carboxyl terminus of proIL-1\(\beta\) undergoes a conformational change with processing, as evidenced by proteinase K processing patterns (36). It was suggested that cytosolic proIL-1\(\beta\) exists in a loosely folded conformation that changes to a tightly folded conformation with processing. Applying this paradigm to the present data, one could postulate that the change from loose to tight folding may happen as a consequence of export from the cytosol. A change to a tight folding pattern when the amino terminus remains intact could produce a molecule whose receptor-binding epitopes are hidden. This would imply that the conformational change is critical to export. It also suggests that ICE processing is not required for this change. Knowledge of the three-dimensional structure of proIL-1\(\beta\) will undoubtedly be helpful in understanding these events. The significance of the difference in the detectability of cytosolic vs released proIL-1\(\beta\) is unknown at present. The change has implications from a measurement perspective and most likely is related to the poorly understood processes that are necessary to release this molecule, which lacks a leader sequence and is released via a non-Golgi pathway.

These studies demonstrate that the release of IL-1\(\beta\) is not dependent on processing, that released proIL-1\(\beta\) is not readily detectable by capture techniques that recognize receptor binding, and that with processing, precursor fragments of 28 and 3 kDa are also produced. These studies suggest that a conformational change in proIL-1\(\beta\) is part of the release process.

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References


