Interleukin 1β Propeptide Is Detected Intracellularly and Extracellularly When Human Monocytes Are Stimulated with LPS In Vitro

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Summary

Human interleukin 1β (IL-1β) is synthesized as an inactive precursor that is cleaved by IL-1 converting enzyme (ICE) between Asp^{116} and Ala^{117} to form COOH-terminal mature IL-1β and NH_2-terminal IL-1β propeptide. Little is known about the fate of the NH_2-terminal cleavage product. In this study, human recombinant (hr)IL-1β propeptide (amino acids 2-116) was produced and used to prepare specific antibodies which do not recognize mature human IL-1β. These anti-propeptide antibodies were used for immunoprecipitation of biosynthetically labeled proteins from lipopolysaccharide-stimulated human monocytes. Analysis of immunoprecipitates by sodium dodecyl sulfate–polyacrylamide gel electrophoresis and fluorography revealed that these antibodies recognize precursor IL-1β and two unique proteins: one migrating at 17.5 kD and one at 14 kD. The larger of these two proteins has a migration nearly identical to that of the recombinant IL-1β propeptide, and most likely represents naturally derived propeptide. The protein migrating at 14 kD may result from a second cleavage by ICE, between Asp^{27} and Gly^{28}. These proteins accumulate intracellularly and extracellularly during pulse–chase experiments, and therefore represent stable products of precursor IL-1β cleavage.

Human $(hIL-1β)^1$ IL-1β is synthesized as a 35-kD inactive precursor protein (1–4) and is cleaved to release the COOH-terminal portion of the molecule, mature 17.5-kD IL-1β, which possesses all the known activities of IL-1β. Precursor IL-1β may be cleaved by a variety of proteases (3, 4) to give products with partial activity, but full activity is produced only by cleavage between Asp^{116} and Ala^{117} by the newly discovered IL-1β converting enzyme (ICE) (5, 6). Stimulated human monocytes synthesize large amounts of both precursor IL-1β and ICE (7, 8), and produce most of the circulating mature IL-1β present in humans.

Although mature IL-1β has been the subject of exhaustive research, surprisingly little attention has been paid to the NH_2-terminal product of precursor IL-1β cleavage, the IL-1β propeptide. One previous study using antibodies directed against short synthetic peptides from the propeptide region (9) failed to demonstrate the presence of intact propeptide in human monocytes induced to synthesize and process IL-1β. Other studies of IL-1β processing (10–14) have used antibodies made against mature IL-1β or peptides from the

COOH-terminal region, and therefore would not detect the propeptide. These results have led to the assumption that the NH_2-terminal propeptide is rapidly degraded after specific cleavage to precursor IL-1β by ICE. To the contrary, we show that intact IL-1β propeptide is present in significant amounts in LPS-stimulated human peripheral blood monocytes in vitro and, like mature IL-1β, is released into the culture medium.

Materials and Methods

Production of rIL-1β Propeptide. A cDNA clone encoding the complete amino acid sequence of precursor IL-1β was supplied by Upjohn Pharmaceuticals (Kalamazoo, MI). A DNA fragment encoding the IL-1β propeptide was produced by PCR (15) using the primers GCAGAAGTACCTGAGCT (corresponding to nucleotides 4-21 of the coding strand) and CGAATTCTTAATCGTGCACATAAGCCT (corresponding to nucleotides 331-348 of the noncoding strand, with a noncomplementary extension for a translation termination codon followed by an EcoRI site.) The PCR product was cut with EcoRI, isolated by electroelution from agarose gel electrophoresis, and ligated in the pMal-c expression vector (16) (New England Biolabs, Beverly, MA) between StuI and EcoRI sites in the polylinker region. Products of the ligation reaction were transformed into *Escherichia coli* TB1. A positive clone with a DNA sequence iden-

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tical to the published sequence (17) was obtained. This clone expressed a fusion protein consisting of the maltose binding protein (MBP) of *E. coli*, the factor Xa cleavage site, and IL-1β propeptide (amino acids 2-116). (The NH$_2$-terminal amino acid residue of natural precursor IL-1β is not known. We chose to omit the NH$_2$-terminal methionine, because the NH$_2$-termini of most cytosolic proteins with alanine as the second amino acid are posttranscriptionally modified by cleavage of methionine [18]. In any event, this decision is not relevant to the results of this paper.)

Synthesis of the fusion protein (MBP-IL-1β propeptide) was induced by treatment of bacterial suspension with 3 mM isopropylthiogalactoside (IPTG) for 2 h. The bacteria were collected by centrifugation, and bacterial extract was prepared by resuspension in lysis buffer (15 mM sodium phosphate, 30 mM NaCl, 25 mM benzamidine, 1 mM PMSF, 1 mM EDTA, 0.2% Tween-20, pH 7.3) containing 4 mg/ml lysozyme (Boehringer Mannheim Biochemicals, Indianapolis, IN), followed by sonication. The sonicated suspension was then brought to 0.5 M NaCl, and 5 μg/ml DNAase-free RNAase was added. After removal of particulate material by centrifugation at 50,000 g for 30 min at 4°C, the extract was applied to amylose resin (New England Biolabs). The resin was washed with 10 volumes of 20 mM sodium phosphate, 200 mM NaCl, 1 mM PMSF, pH 7.4, and fusion protein was eluted with 10 mM maltose in 20 mM Tris, 100 mM NaCl, pH 8.0. When indicated, fusion protein was digested overnight at 4°C with 1 μg of Factor Xa protease (New England Biolabs) per 100 μg of fusion protein, to produce MBP and IL-1β propeptide.

Amino Acid Sequencing. Fusion protein was concentrated using Centricon-10 ultrafiltration device (Amicon Corp., Beverly, MA), digested with Factor Xa, electrophoresed on a 14% polyacrylamide SDS gel (19), and transferred to a PVDF membrane. The band corresponding to IL-1β propeptide was cut out and analyzed by NH$_2$-terminal amino acid sequencing using a pulse liquid-vapor phase protein sequencer (model 477A; Applied Biosystems Inc., Foster City, CA).

Antibodies. Rabbit anti–human rIL-1β (mature IL-1β), IgG fraction, was purchased from Upstate Biotechnology, Inc. (Lake Placid, NY). Antibodies against IL-1β propeptide were produced as follows: rabbits were first immunized with 500 μg of MBP-IL-1β propeptide fusion protein in CFA. For subsequent immunizations, factor Xa digests of fusion protein were separated by SDS-PAGE on 1.5-mm-thick gels. To rapidly visualize protein bands, gels were soaked in distilled water at 23°C for 15 min, and then in 2 M KCl at 4°C for 15 min. The band corresponding to IL-1β propeptide (seen as cloudy against a clear background) was cut out and electroeluted, and then emulsified in IFA. The antiserum produced in this manner recognized both MBP and IL-1β propeptide, but since there is no correlate of MBP in mammalian cells, this specificity is irrelevant. For production of anti-peptide antibodies, peptides corresponding to amino acids 1-27, 28-57, 58-87, and 88-116 of the IL-1β propeptide sequence were produced by the technique of Merrifield (20) using a peptide synthesizer from Applied Biosystems, Inc. (Model 430). Peptide-KLH conjugates were made by mixing peptide and KLH (Sigma Chemical Co., St. Louis, MO), both 0.5 mg/ml in 0.1 M NaHCO$_3$/Na$_2$CO$_3$ buffer, pH 8.5, with 1.25% glutaraldehyde at 23°C for 48 h, followed by exhaustive dialysis against water, and then against PBS. Rabbits were immunized with 200 μg of individual peptide–KLH conjugates in CFA, followed by two boosters of conjugate in IFA. Only immunization with peptides 28-57 and 88-116 resulted in production of antibodies that were usable for Western blot and immunoprecipitation. IgG from rabbit antisera was prepared by adsorption to recombinant protein A–Sepharose 4B (Zymed Laboratories, Inc., South San Francisco,

CA) and elution at neutral pH (ImmunoPure Gentle Ag/Ab Buffer System from Pierce, Rockford, IL), followed by exhaustive dialysis.

Western Blots. hrIL-1β was kindly donated by Upjohn Pharmaceuticals. Factor Xa digests of MBP-IL-1β propeptide were prepared as described above. Samples were electrophoresed on 14% acrylamide SDS gels and electrophoretically transferred to nitrocellulose membranes (Trans-blot System from Bio-Rad Laboratories, Hercules, CA). Prestained molecular weight markers (Bio-Rad Laboratories) were used. Blots were blocked overnight with 3% gelatin (Bio-Rad Laboratories) in TBS (20 mM Tris, 500 mM NaCl, pH 7.5), and then incubated with the indicated primary rabbit antibody in 1% gelatin-TTBS (TBS with 0.05% Tween-20), washed in TTBS, and incubated with 1/3,000 goat anti–rabbit IgG alkaline phosphatase conjugate (Bio-Rad Laboratories) in 1% gelatin-TTBS. Blots were then developed by adding substrate solution consisting of 132 μl of p-nitroblue tetrazolium chloride (50 mg/ml in 70% dimethylformamide) and 66 μl of 5-bromo-4-chloro-3-indolyl phosphate (50 mg/ml in dimethylformamide) per 20 ml of 100 mM Tris, 100 mM NaCl, 5 mM MgCl$_2$, pH 9.5.

Isolation and Biosynthetic Labeling of Human Monocytes. Leukocyte-rich plasma was prepared from peripheral blood of normal human volunteers by anticoagulation with 3 mM EDTA and removal of erythrocytes by 1 g sedimentation for 60 min in 0.6% dextran. Monocytes were then separated from the remainder of the leukocytes by density gradient centrifugation on 1-Step Monocytes (Accurate Chemical & Scientific Corp., Westbury, NY), according to the manufacturer's directions. The cells obtained by this method were >99% monocytes by Wright stain and >98% viable by exclusion of 0.1% trypan blue. Monocytes were washed twice and suspended at 10^6 cells/ml in culture medium (methionine-free RPMI-1640 plus 2 mM L-glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin, and 2% heat-inactivated autologous human serum). LPS (10 μg/ml) from *E. coli* serotype 055:B5 (Sigma Chemical Co.) and 100 μCi/ml [^{35}S]methionine and [^{35}S]cysteine (Expre^{35}S^{35}S protein labeling mix, >1,000 Ci/mmol, New England Nuclear-DuPont, Wilmington, DE) were added, and cells were cultured for 4 h at 37°C. Virtually all the monocytes adhered to the tissue culture vessel.

For pulse–chase experiments, LPS-stimulated human monocytes were biosynthetically labeled as above, washed with culture medium containing unlabeled methionine, and cultured for an additional 4 h in culture medium containing unlabeled methionine and 10 μg/ml LPS. Replicate cultures were harvested at various intervals.

Immunoprecipitation of Culture Supernatants and Cell Lysates. After 4 h, culture supernatant was collected and the adherent cells were washed once with fresh culture medium. The culture supernatant and wash were pooled and centrifuged at 600 g for 10 min at 4°C to remove any monocytes that may have become nonadherent. PMSF and benzamidine were added to achieve final concentrations of 1 and 25 mM, respectively, and the culture supernatant was then centrifuged at 30,000 g for 30 min at 4°C. Adherent cells were lysed by addition of ice-cold lysis buffer (50 mM Tris, 150 mM NaCl, 1 mM PMSF, 25 mM benzamidine, 1% NP-40, pH 7.5), followed by incubation at 4°C for 30 min. Cell lysate was centrifuged at 30,000 g for 30 min at 4°C. Samples were stored overnight at −70°C, quickly thawed at 37°C, and chilled on ice. All subsequent immunoprecipitation and washing operations were carried out at 4°C. In some experiments described but not shown, a mixture of five additional protease inhibitors was added to the culture supernatants and cell lysis buffer (final concentrations 1 mM EDTA, 10 mM N-ethylmaleimide, 1 μg/ml leupeptin, 1 μg/ml pepstatin, and 1 μg/ml aprotinin).

Culture supernatants and cell lysates were cleared of IgG from

the culture medium by tumbling with an excess amount (at least 100 μl per 5 ml of sample) of recombinant protein G–Sepharose 4B beads (PGS) (Zymed Laboratories, Inc.). Samples were centrifuged at 50 g for 5 min to remove the beads, and then divided into the desired number of equal aliquots. Supernatants and cell lysates representing equal numbers of cells, usually between 0.5 × 10⁶ and 1 × 10⁶, were used for immunoprecipitation with each different antibody. The IgG fractions of rabbit antisera, purchased or prepared as described above, were individually titered for use in immunoprecipitation.

For competition studies, a limiting amount of antibody was added. For all other studies, antibody was added in approximately fivefold excess of the amount needed to precipitate the maximal amount of radiolabeled IL-1β moieties. At fivefold excess, the efficiency of immunoprecipitation for all the antibodies, determined by repeating the same immunoprecipitation on the supernatant from the first immunoprecipitation, was between 80 and 85%. However, antibody against IL-1β propeptide consistently precipitated more precursor IL-1β, on both the first and second immunoprecipitations, than did antibody against mature IL-1β.

Each aliquot of culture supernatant or cell lysate was incubated for 1 h with the indicated antibody, and then tumbled for 90 min with 40 μl of fresh PGS beads. The beads were collected by centrifugation at 50 g for 3 min and were washed three times with wash buffer A (10 mM Tris, 500 mM NaCl, 2 mM EDTA, 1 mM PMSF, 0.2% NP-40, pH 7.5), two times with wash buffer B (same as A but with 150 mM NaCl), and once with 10 mM Tris, 1 mM PMSF, pH 7.5, taking care not to lose beads when aspirating the wash solutions. The PGS was eluted twice with 40 μl of sample buffer for SDS-PAGE (19) for 10 min at room temperature, and eluates were pooled and frozen overnight at −70°C.

SDS-PAGE and Detection of Labeled Proteins. The entire [³⁵S]-methionine-labeled eluate from each immunoprecipitation was applied to 14% polyacrylamide SDS gels for electrophoresis. Molecular weight standards from Bio-Rad Laboratories were used. Gels were stained with Coomassie Blue, impregnated with fluor (En³Hance; New England Nuclear-DuPont) according to manufacturer's directions, dried, and exposed to X-Omat AR film (Kodak, Rochester, NY) for the indicated times. For some experiments, gels were dried without impregnation with fluor and analyzed using a Molecular Dynamics PhosphorImager, which allowed quantitation of bands but did not produce images acceptable for reproduction.

Results

Production and Enzymatic Cleavage of Fusion Protein. The recombinant fusion protein MBP-IL-1β propeptide was expressed in large amounts in induced *E. coli* and rendered substantially free of contaminating bacterial proteins or degradation products by amylose affinity chromatography. A typical preparation, analyzed by SDS-PAGE and Coomassie Blue staining, is shown in Fig. 1 *A*. The heavy band migrating at approximately 60 kD in SDS-solubilized induced *E. coli* (lane 2) is the fusion protein, which is absent from uninduced bacteria (lane 1). This fusion protein was partially purified from bacterial extracts by selectively binding to amylose and eluting with maltose (lanes 3–10). Typical yields from this procedure were 3 to 6 mg of fusion protein per liter of induced *E. coli* suspension. As shown in Fig. 1 *B*, digestion of this product with Factor Xa produced maltose binding protein, migrating at ~38 kD, IL-1β propeptide, migrating at ~17.5 kD, and some residual undigested fusion protein. Although the apparent molecular weight of the IL-1β propeptide was larger than expected, its identity was verified by DNA sequencing of the expression vector (21) and amino acid sequencing of the first 10 NH₂-terminal amino acid residues of the 17.5-kD band.

Specificity of Antibodies. The specificity of rabbit polyclonal antibodies produced against hrIL-1β propeptide (amino acids 2-116), and against smaller synthetic peptides of this protein, was checked by Western blotting, as described in Materials and Methods and shown in Fig. 2. Commercially obtained rabbit polyclonal antibody against hrIL-1β (mature IL-1β, amino acids 117-269) detected mature IL-1β (lane 1), but not hrIL-1β propeptide prepared by digestion of MBP-IL-1β propeptide with Factor Xa (lane 2). Antibody prepared against MBP-IL-1β propeptide did not detect mature IL-1β (lane 3), but did detect IL-1β propeptide, maltose binding protein, and undigested fusion protein (lane 4). A mixture of antibodies against IL-1β synthetic peptides 28-57 and 88-116 detected only hrIL-1β propeptide and undigested fusion protein (lane 6). This blot also demonstrates that the migration of hrIL-1β propeptide is only slightly faster than that of the
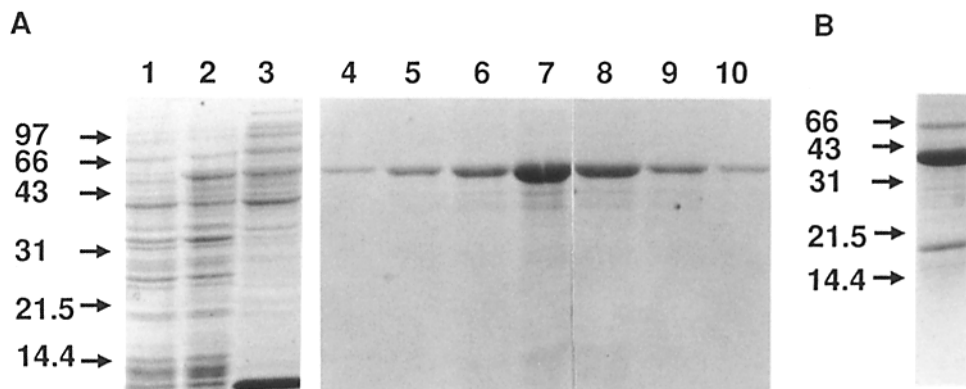

Figure 1. SDS-PAGE of MBP-IL-1β propeptide fusion protein preparation and digestion with Factor Xa. (*A*) Lanes *1* and *2* show the induction of MBP-IL-1β fusion protein in transformed *E. coli* by IPTG. Lane *1* is uninduced, and lane *2* is IPTG-induced whole bacteria solubilized by boiling in SDS. The bacterial extract of induced *E. coli* (lane *3*) prepared by lysozyme treatment and detergent and hypotonic lysis, was subjected to amylose affinity chromatography. Lanes *4–10* show sequential fractions obtained by elution of the amylose column with maltose. (*B*) Treatment with factor Xa overnight at 4°C resulted in almost complete digestion of fusion protein to give MBP (38 kD) and IL-1β propeptide (17.5 kD). Gels are: *A*, 12% acrylamide; and *B*, 15% acrylamide, stained with Coomassie Blue.
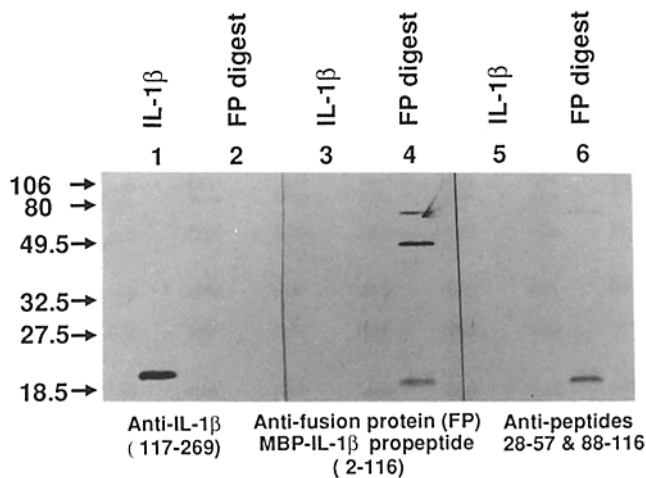
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Figure 2. Western blot analysis of antibodies specific for portions of the IL-1β precursor molecule. Rabbit polyclonal antibodies against either mature IL-1β (lanes *1* and *2*), MBP-IL-1β propeptide fusion protein (lanes *3* and *4*), or IL-1β synthetic peptides (amino acids 28-57 and 88-116) (lanes *5* and *6*) at 1/500 dilutions were used to probe this blot. The antigen in lanes *1*, *3*, and *5* was 100 ng of commercially obtained hrIL-1β (mature, amino acids 117-269) and in lanes *2*, *4*, and *6*, 300 ng of Factor Xa digest of fusion protein (MBP-IL-1β propeptide amino acids 2-116) containing ∼100 ng of propeptide.

Figure 3. SDS-PAGE and fluorography of biosynthetically labeled proteins from LPS-stimulated human monocytes, immunoprecipitated with antibodies specific for different portions of the IL-1β precursor molecule. Culture supernatant (lanes *1–3*) and cell lysate (lanes *4–6*) were immunoprecipitated with rabbit antibody against IL-1β synthetic peptides (amino acids 28-57 and 88-116) in lanes *1* and *4*, against MBP-IL-1β propeptide (amino acids 2-116) in lanes *2* and *5*, and against mature IL-1β (amino acids 117-269) in lanes *3* and *6*. This gel was exposed to film for 3 wk.

17.5-kD hrIL-1β. The lowest prestained marker, lysozyme, is said to migrate at 18.5 kD (Bio-Rad Laboratories, package insert), but its migration is anomalous with respect to the known migration of mature IL-1β, and with respect to unmodified molecular weight markers (not shown).

Detection of IL-1β Propeptide from LPS-stimulated Human Monocytes. Human peripheral blood monocytes were stimulated with LPS and biosynthetically labeled with [^{35}S]methionine-cysteine mix. Proteins recognized by antibody against mature IL-1β, MBP-hrIL-1β propeptide, and IL-1β synthetic peptides were isolated by immunoprecipitation. The entire sample from each immunoprecipitation was analyzed by SDS-PAGE and fluorography. As shown in Fig. 3, the major intracellular precursor form of IL-1β migrated at ∼35 kD. Multiple smaller bands between 29-35 kD were seen, comparable to other reports (11, 13). Precursor Il-1β was also found, in smaller amounts, in culture media, mainly as the 35-kD form (lanes *1-3*). In subsequent experiments (not shown), the amount of precursor in the culture media could be reduced, but not entirely eliminated, by more thorough removal of cells from the media before the second, high speed, centrifugation. Others have also demonstrated the apparent secretion of precursor IL-1β (9, 11, 13, 14). Mature 17.5-kD IL-1β was present largely as a secreted product (lane *3*), although some was observed in cell lysates (lane *6*). The apparent retention of a small amount of mature IL-1β intracellularly by human monocytes has been shown in previous reports (11, 13, 14). An artifact, such as continued activity of ICE after cell lysis, or sticking of processed IL-1β to the plasma membrane before lysis, has not been entirely ruled out.

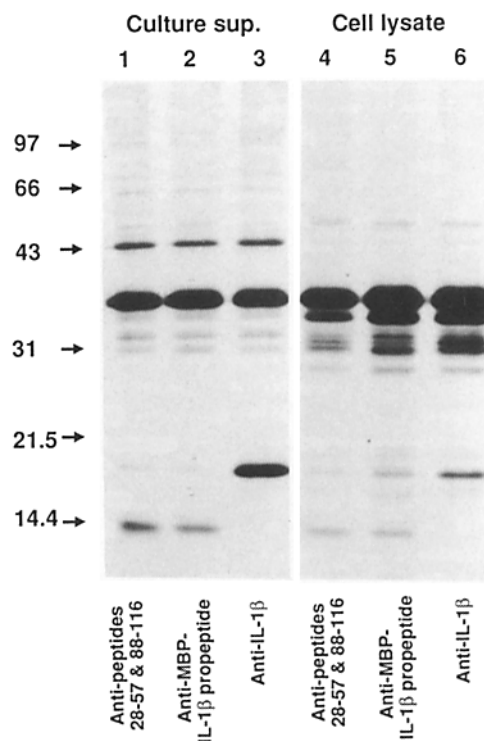The observation unique to this report is that two proteins are recognized by specific antibodies against IL-1β propep-

tide and its smaller synthetic peptides (Fig. 3, lanes *1, 2, 4,* and *5*). Based on the unusually slow migration of the 115–amino acid recombinant propeptide (Fig. 2), the band with mobility virtually identical to that of mature 17.5-kD IL-1β (Figs. 2 and 3) appears to be the naturally produced IL-1β propeptide. A second band migrating at ∼14 kD, also immunoprecipitated by the anti-propeptide antibodies, is evident in Figs. 3 and 4. As shown in Fig. 4, antibody against amino acids 28-57, and antibody against amino acids 88-116 of the propeptide sequence, both precipitate this band in approximately equal amounts. The presence of epitopes from these two noncontiguous portions of the precursor suggests that this protein may be a result of cleavage near the amino terminus of the 17.5-kD propeptide. This 14-kD protein may result from further processing of the propeptide at a second site, Asp^{27}-Gly^{28}, characterized previously using highly purified ICE and synthetic peptides (22).

As shown in Fig. 3, the IL-1β propeptide moieties were found both extracellularly (lanes *1* and *2*) and intracellularly (lanes *4* and *5*). The relative proportions of the 17.5- and 14-kD propeptide moieties varied somewhat among monocytes from several individual donors, but was consistent for each individual in separate experiments. In general, however,
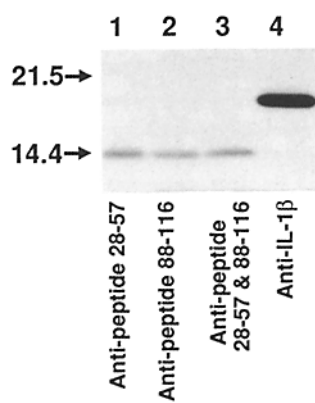
Figure 4. Immunoprecipitation of the 14-kD secreted propeptide moiety by antibodies against synthetic peptides representing two different portions of the IL-1β propeptide amino acid sequence. Culture supernatant from LPS-stimulated human monocytes, biosynthetically labeled with [³⁵S]methionine, was immunoprecipitated with antibody against IL-1β synthetic peptides or mature IL-1β. The immunoprecipitated material was analyzed by SDS-PAGE and fluorography. Antibodies were as follows: anti-synthetic peptide 28-57 (lane *1*), anti-synthetic peptide 88-116 (lane *2*), equal amounts of these two antibodies combined (lane *3*), and anti-mature IL-1β (amino acids 117-269) (lane *4*). This gel was exposed to film for 1 wk.

the 14-kD propeptide moiety predominated in culture supernatants, while the 17.5- and 14-kD propeptide moieties were usually present in approximately equal amounts in cell lysates.
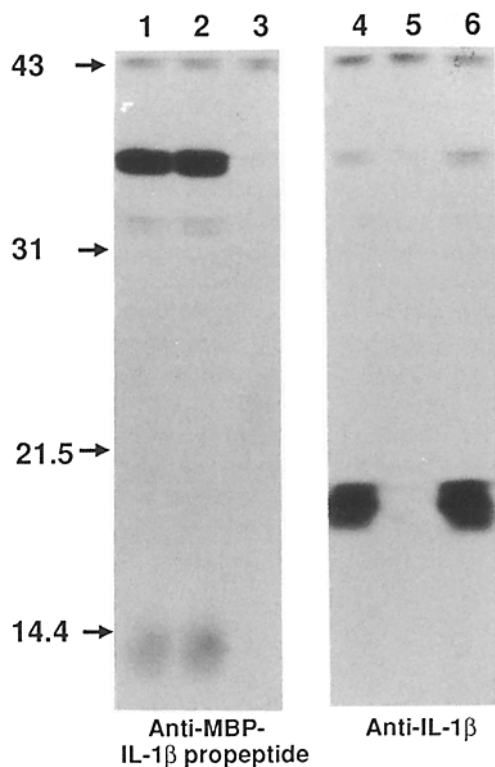
Competition by Unlabeled Mature IL-1β and MBP-IL-1β Propeptide. Although specificity of the antibodies used for these experiments was demonstrated by Western blot, we wished to demonstrate that they were specific for the relevant native, as well as denatured, proteins. For the experiment shown in Fig. 5, biosynthetically labeled culture supernatant from LPS-stimulated monocytes was divided into equal aliquots, which then received either no addition, unlabeled hrIL-1β (mature IL-1β), or unlabeled MBP-IL-1β propeptide. Limiting quantities of antibody against either mature Il-1β or Il-1β propeptide were added, and immunoprecipitation was carried out as usual. As expected, unlabeled mature IL-1β (lane *5*), but not unlabeled MBP-IL-1β propeptide (lane *6*), competed for binding of labeled mature IL-1β and precursor IL-1β to anti-mature Il-1β. Conversely, unlabeled MBP-IL-1β propeptide (lane *3*) but not unlabeled mature IL-1β (lane *2*) competed for binding of labeled IL-1β propeptide and precursor IL-1β to antibody against IL-1β propeptide. In experiments not shown, unlabeled recombinant MBP did not compete out any of the IL-1β bands. Therefore, the 17.5- and 14-kD bands immunoprecipitated with antibodies against IL-1β propeptide are not related to mature IL-1β, and represent authentic IL-1β propeptide moieties. In the experiment shown, mainly the 14-kD IL-1β propeptide was found in the culture supernatant. Longer exposure (not shown) was required to detect the 17.5-kD propeptide, which was competed out as described above.

The decreased ability of anti-mature IL-1β to immunoprecipitate the 35-kD precursor IL-1β was especially notice-

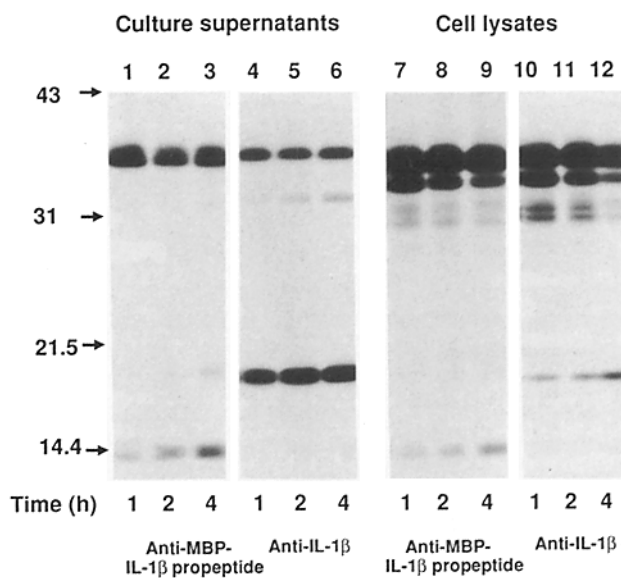

Figure 5. Specificity of immunoprecipitation of IL-1β moieties. Immunoprecipitation of biosynthetically labeled proteins in culture supernatant of LPS-stimulated human monocytes was carried out in the presence of no addition (lanes *1* and *4*), 6 μg of unlabeled mature IL-1β (amino acids 117-269) (lanes *2* and *5*), or 18 μg of unlabeled MBP-IL-1β propeptide (amino acids 2-116, containing ~6 μg of propeptide). The antibodies used were rabbit anti-MBP-IL-1β propeptide (lanes *1-3*) and anti-mature IL-1β (lanes *4-6*). Samples were analyzed by SDS-PAGE and fluorography. This gel was exposed to film for 3 wk.

Figure 6. Processing of biosynthetically labeled precursor IL-1β. Culture supernatants (lanes *1-6*) and cell lysates (lanes *7-12*) of biosynthetically labeled LPS-stimulated human monocytes were collected at 1 h (lanes *1, 4, 7,* and *10*), 2 h (lanes *2, 5, 8,* and *11*), and 4 h (lanes *3, 6, 9,* and *12*) after changing into unlabeled medium. The samples were immunoprecipitated with either anti-MBP-IL-1β propeptide (lanes *1-3* and *7-9*) or anti-mature IL-1β (lanes *4-6* and *10-12*). Immunoprecipitates were analyzed by SDS-PAGE and fluorography. This gel was exposed to film for 1 wk.

able at limiting antibody dilutions (in Fig. 5, compare lanes *1* and *2* to lanes *4* and *6*). This may reflect a low affinity of anti-mature IL-1β antibodies for precursor IL-1β, as has been noted previously (12, 13).

A band of ~43 kD was found in immunoprecipitates of culture supernatants and, to a much lesser extent, cell lysates. This band appeared with all the antibodies used, and was not competed out by unlabeled mature IL-1β or MBP-IL-1β propeptide (Fig. 5), suggesting that this protein binds nonspecifically to antibodies. This unexplained 43-kD band is also evident in previous reports of IL-1β processing (9, 10, 14).

Accumulation of IL-1β Propeptide in Culture Medium and Cell Lysates. To follow the course of precursor IL-1β processing and production of propeptide moieties, pulse–chase experiments were conducted. As shown in Fig. 6, both the predominant 14-kD IL-1β propeptide and the 17.5-kD propeptide accumulate in culture medium over time (lanes *1–3*). As demonstrated by others (11, 13, 14), mature IL-1β also accumulates in culture medium (lanes *4–6*). Smaller amounts of propeptides and mature IL-1β are also seen in cell lysates. Intracellularly, the 35-kD precursor appears to remain relatively constant (better seen on shorter exposures) but precursors of ~29- and 31-kD decrease. The results do not distinguish which size of precursor IL-1β gives rise to mature IL-1β and IL-1β propeptide, and may reflect a complex relationship between precursors and products.

Discussion

We report here the synthesis of recombinant human IL-1β propeptide (Ala[2]-Asp[116]), the production of antibodies which specifically react with this propeptide, and the immunoprecipitation by these antibodies of two unique proteins from human monocytes stimulated to synthesize and process precursor IL-1β. The larger of these two proteins migrates on SDS-PAGE with a mobility almost identical to that of mature 17.5-kD IL-1β, but is clearly not mature IL-1β because the specific antibody does not bind mature IL-1β. This protein is almost certainly bona fide IL-1β propeptide, the NH2-terminal product of the ICE which cleaves proIL-1β between Asp[116] and Ala[117]. To our knowledge, this is the first demonstration of the existence of the intact IL-1β propeptide in vitro.

Schmidt and Bomford (9) did identify an 18-kD biosynthetically labeled protein from stimulated monocytes, using antibodies against amino acids 1-15 of precursor IL-1β. However, since antibodies against other short synthetic peptides from the NH2-terminal region (amino acids 17-32, 43-54, and 89-100) failed to detect this protein, they concluded that it was not derived from the NH2 terminus of precursor IL-1β. It is possible that antibodies against most of the 15–amino acid synthetic peptides failed to react with intact IL-1β propeptide because of conformational differences. The peptides used successfully in the present study for production of anti-IL-1β propeptide antibodies were nearly twice as long as those described above. Other studies (10–14) of IL-1β processing

have not been designed to address the fate of the IL-1β propeptide because only antibodies against mature IL-1β were used.

A second protein of ~14 kD is also detected by antibodies against recombinant IL-1β propeptide, and by antibodies against synthetic peptide fragments 28-57 or 88-116. The simplest explanation for this smaller propeptide moiety is that it lacks some of the NH2-terminal residues of the 17.5-kD propeptide. This is an especially attractive explanation, since precursor IL-1β contains a second site for cleavage by ICE, at Asp[27]-Gly[28] (22). Thus, the 14-kD moiety may represent the Gly[28]-Asp[116] fragment of the IL-1β propeptide. Since immunization with the synthetic peptide 1-27 did not result in the production of antibodies useful for immunoprecipitation, we have not further tested this hypothesis.

The recombinant IL-1β propeptide (amino acids 2-116) migrates more slowly on SDS-PAGE than would be expected for a 115–amino acid protein. The monocyte-derived propeptide, demonstrated by biosynthetic labeling and immunoprecipitation, migrates slightly more slowly than its recombinant counterpart, which could possibly be accounted for by post-translational modifications. The NH2-terminal portion of the intact precursor IL-1β is myristoylated (23), but is not glycosylated (11).

The 17.5- and 14-kD propeptide moieties do not appear to be the result of nonspecific or artifactual cleavage of precursor IL-1β by proteases, other than ICE, found in the culture medium or released during lysis of cells. Inclusion of five additional protease inhibitors (see Materials and Methods) in the harvested culture medium and the cell lysis buffer did not result in immunoprecipitation of less mature IL-1β or propeptide moieties (experiments not shown). It is also unlikely that the 17.5- and 14-kD propeptide moieties are a result of toxic cell damage from the high concentration of LPS used in these experiments. In separate experiments (not shown), both mature IL-1β and IL-1β propeptide increased in culture supernatants with increasing concentrations of LPS from 1 ng/ml through 10 μg/ml, while the amount of intracellular precursor was already maximum at 1 ng/ml LPS. Furthermore, increasing concentrations of LPS did not result in increasing amounts of precursor IL-1β being released into the supernatant, which could be a marker for cell damage.

The band intensities of IL-1β propeptide moieties which are detected at the end of 4 h of biosynthetic labeling, or which accumulate during a chase, seem to be less than would be expected if the propeptide(s) persists in a 1:1 relationship with mature IL-1β. The fluorographic technique used here tends to underestimate proteins which contain less radiolabel or are present in smaller amounts (24). Therefore, separate experiments were performed for the purpose of estimating the relative amounts of precursors and products. Using a PhosphorImager, the relative band volumes of intracellular 35-kD precursor to extracellular mature IL-1β to extracellular 14-kD propeptide were roughly 100:10:1. Correcting for methionine content (11:6:3, respectively) results in an approximate molar ratio of 27:5:1. Since IL-1β propeptide(s) should be initially produced in 1:1 relationship with mature IL-β, it appears that the propeptide moieties are degraded faster

than mature IL-1β. However, the observation that radiolabeled 17.5 kD and 14 kD IL-1β propeptide moieties do accumulate in pulse–chase experiments does suggest that a pool of IL-1β propeptide may exist in vivo.

These results lead us to speculate that the NH$_2$-terminal portion of precursor IL-1β serves functions other than rendering the precursor inactive. The precedent is established in vertebrate biology for two or more portions of a cleaved precursor protein to serve different roles. For example, complement components C3 and C5 are cleaved to form C3a and C3b, C5a and C5b, which have different effector roles in the complement cascade (25); procollagen is cleaved to form structural collagen, and noncollagenous carboxypeptide and aminopeptide fragments which modulate collagen synthesis (26, 27); and pro-opiomelanocortin is cleaved to form several peptide hormones (28). The apparent distribution of IL-1β propeptide moieties in both the intracellular and extracellular compartments raises interesting hypotheses concerning possible roles in regulating the processing of IL-1β, modulating the activity of mature IL-1β, or functioning as an independent cytokine. The recent discovery that overexpression of ICE induces apoptosis in fibroblast cell line (29) suggests the exciting possibility that the IL-1β propeptide could play a role in programmed death of monocytes. The ability to produce reasonable quantities of purified recombinant propeptide should allow investigation of these possibilities.

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