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Processing of Newly Synthesized Cachectin/Tumor Necrosis Factor in Endotoxin-Stimulated Macrophages†

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ABSTRACT: The biosynthesis and processing of cachetin/tumor necrosis factor (TNF) were examined in the murine macrophage-like cell line RAW 264.7. Lipopolysaccharide-stimulated cells secreted both glycosylated and nonglycosylated 17-kilodalton (kDa) mature cachectin/TNF into the culture medium. Secreted cachectin/TNF was derived from membrane-associated precursors that were precipitated by polyclonal antisera raised against either the mature protein or synthetic peptide fragments of the 79 amino acid cachectin/TNF prohormone sequence. About half of the precursors were N-glycosylated, apparently cotranslationally. The cachectin/TNF precursors were then proteolytically cleaved to release soluble mature cytokine into the medium, while the membrane-bound 14-kDa prosequence remained cell associated. During the period of LPS stimulation, the amount of macrophage cell surface cachectin/TNF remained at a low level, suggesting that both nonglycosylated and glycosylated precursors of cachectin/TNF are efficiently cleaved by these cells. These findings suggest the presence of a unique mechanism for the secretion of cachectin/TNF.

Microbial and parasitic infections and neoplastic diseases in mammals alter the physiological and metabolic state of the host and can advance to cachexia and septic shock (Beisel, 1975). Recently, macrophage-derived polypeptide cytokines (monokines), especially cachectin/tumor necrosis factor (TNF)[1] and interleukin 1 (IL-1), have been implicated as mediators of such metabolic changes in infected hosts [reviewed by Beutler and Cerami (1987) and Dinarello (1984)]. When stimulated by bacterial endotoxin, macrophages secrete large amounts of these cytokines. Cachectin/TNF has been found to mediate catabolic responses in septic animals and to be responsible for endotoxin-induced injury and death (Tracey et al., 1986). Cachectin/TNF has other known in vivo and in vitro effects on tumor cells (Carswell et al., 1975) and participates in host inflammatory responses to viral, bacterial, and parasitic stimuli (Beutler & Cerami, 1987).

Cloning of cDNA for full-length cachectin/TNF mRNA and comparison of its coding sequence with that of the mature, secreted, 17-kDa hormone had revealed that cachectin/TNF is synthesized as a prohormone, whose prosequence is so long that it has not been regarded as a typical "signal" sequence: 76 and 79 amino acid residues for human (Pennica et al., 1984; Shirai et al., 1985) and murine (Fransen et al., 1985; Pennica et al., 1985; Caput et al., 1986) cachectin/TNF's, respectively. The propeptide sequence is highly conserved (86% homologous) between human and mouse proteins, which raised the possibility that it serves a distinct biological function (Beutler & Cerami, 1987). The prosequence has a centrally located hydrophobic region and in this respect resembles other secretory signal sequences (Blobel et al., 1979). However, recent reports indicate unusual features in posttranslational processing of cachectin/TNF as a secretory protein (Muller et al., 1986; Decker et al., 1987; Kriegler et al., 1988). In human monocytes and cells transfected with a cachectin/TNF prohormone cDNA construct, the intact cachectin/TNF prohormone remains associated with the membrane fraction. It was postulated that this long form is clipped to release mature 17-kDa cachectin/TNF into the medium (Muller et al., 1986; Kriegler et al., 1988).

In the present study we investigated the processing of cachectin/TNF in an endotoxin-stimulated murine macrophage-like cell line. Cachectin/TNF was produced initially as a membrane-bound, cell-associated, 26-kDa precursor that was then cleaved to yield soluble, mature, 17-kDa protein, while 14-kDa prosequence peptide remained membrane bound.

Experimental Procedures

Cell Culture and in Vitro Cytotoxicity Assay. RAW 264.7 murine macrophage and L-929 mouse fibroblast lines were obtained from American Type Culture Collection (Rockville, MD). RAW 264.7 cells were grown in RPMI 1640 medium

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[1] Abbreviations: TNF, tumor necrosis factor; IL-1, interleukin 1; HEPES, *N*-(2-hydroxyethyl)piperazine-*N'*-2-ethanesulfonic acid; FBS, fetal bovine serum; DMEM, Dulbecco's modified Eagle's medium; LPS, lipopolysaccharide; D-PBS, Dulbecco's phosphate-buffered saline; EDTA, ethylenediaminetetraacetic acid; PMSF, phenylmethanesulfonyl fluoride; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis.

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(Gibco, Grand Island, NY) supplemented with 20 mM *N*-(2-hydroxyethyl)piperazine-*N'*-2-ethanesulfonic acid (HEPES, Research Organics, Cleveland, OH), 50 μg/mL gentamycin (Gibco), and 10% heat-inactivated (56 °C, 30 min) fetal bovine serum (FBS, Hyclone, Logan, UT) (RPMI/F10).

For the in vitro cytotoxicity assay, L-929 cells were grown in Dulbecco's modified Eagle's medium (DMEM, Gibco) supplemented with 50 μg/mL gentamycin and 10% calf serum (Hyclone) (DMEM/C10). The assay was performed as described previously (Ostrove & Gifford, 1979) with slight modifications. L-929 cells at a density of 30000 cells per well in 96-well plates were incubated at 37 °C for 24 h before the addition of cachectin/TNF and 1 μg of actinomycin D/mL. After 16 h, viable cells were stained with a chromogen, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (Sigma Chemical Co., St. Louis, MO) and optical densities at 570/690 nm were measured by using an automated enzyme-linked immunosorbent assay (ELISA) plate reader (Bio-Tek Instruments, Burlington, VT) (Mosmann, 1983; Wolpe et al., 1988).

Antisera. Polyclonal antisera to murine cachectin/TNF were prepared by immunizing rabbits with purified 17-kDa cachectin/TNF (B. Sherry, D.-M. Jue, A. Zentella, and A. Cerami, unpublished result). Rabbit antibodies to portions of the prosequence of cachectin/TNF were raised against three synthetic peptides that correspond to different portions of the murine prosequence (residues 1–11, 47–67, and 68–77 in procachectin/TNF). Each peptide was conjugated to bovine thyroglobulin (Sigma) by using glutaraldehyde (Sigma) (molar ratio of peptide to carrier protein, 10:1) and 250 μg of peptide–thyroglobulin conjugate was injected into rabbits for immunization (B. Sherry, D.-M. Jue, A. Zentella, and A. Cerami, unpublished result). All antisera against prosequence peptides gave qualitatively similar results in immunoprecipitations and a mixture of equal volumes of these antiprosequence antisera was used for immunoprecipitations.

Cell Stimulation and Metabolic Labeling. RAW 264.7 cells grown to confluence in six-well plates (Linbro, Flow Laboratories, McLean, VA) were treated with 1 μg of lipopolysaccharide (LPS, LPS W, *E. coli* 0127:B8, Difco Laboratories, Inc., Detroit, MI) per milliliter in RPMI/F10. After incubation for 2 h at 37 °C, the cells were washed twice with warm methionine-free DMEM supplemented with 2 mM L-glutamine (Gibco) (labeling medium) and incubated for 30 min in the same medium containing the stimulants. The medium was then replaced with 0.5 mL of labeling medium containing 50 μCi [^{35}S]methionine (New England Nuclear, Boston, MA) and the cells were incubated for another 30 min. In some samples tunicamycin (Sigma) was added to the cells at a concentration of 10 μg/mL in both stimulation and labeling media. Tunicamycin was dissolved in RPMI/F10 adjusted to pH 9.0. After the supernatant was harvested, the cells were washed with ice-cold Dulbecco's phosphate-buffered saline (D-PBS) three times and then lysed in 0.5 mL of 50 mM Tris-HCl (pH 7.4), 0.5% Nonidet P-40 (NP40), 0.15 M NaCl, 0.02% sodium azide, 5 mM ethylenediaminetetraacetic acid (EDTA), 0.1 mM phenylmethanesulfonyl fluoride (PMSF), soybean trypsin inhibitor (20 μg/mL) (Sigma), 5 μM leupeptin (Sigma), and 5 μM antipain (Sigma) (lysis buffer). After 10 min, cell lysate was removed, vortexed, and clarified by centrifugation. Reserved culture medium was centrifuged and 50 μL of 10-fold-concentrated lysis buffer was added. For pulse-chase experiments, cells were labeled for various times and the medium was changed with complete DMEM. At various times after the medium change, cells and culture medium were harvested and treated as described above. The culture media of the remaining samples were also changed with fresh ones in order to measure the secretion of cachectin/TNF during certain periods of time.

Immunoprecipitation and Gel Electrophoresis. Cell lysates and culture supernatants were incubated with 10 μL of 50% protein A Sepharose (Pharmacia, Piscataway, NJ) suspension at 22 °C for 1 h. The Sepharose beads were removed by centrifugation and 40 μL of rabbit antisera against mature cachectin/TNF or prosequence fragment conjugates was added. After incubation for 16 h at 4 °C, 80 μL of 50% protein A Sepharose bead suspension was added and the mixture was incubated for another 2 h at 4 °C. All incubations were performed with gentle agitation. The Sepharose beads were washed twice each with lysis buffer, D-PBS containing 0.05% sodium azide, and then with 0.1% sodium dodecyl sulfate (SDS). The washed beads were combined with 50 μL of 2X sample buffer (final concentration, 62.5 mM Tris-HCl, pH 6.9, 2% SDS, 10% glycerol, 5% 2-mercaptoethanol) and heated at 90 °C for 5 min. The samples were analyzed in denaturing 12.5–18% gradient polyacrylamide gels as described previously (Laemmli, 1970). Gels were fixed with 35% methanol/10% acetic acid, treated with Autofluor (National Diagnostics, Manville, NJ), dried, and used to expose Kodak XAR-5 film at −70 °C.

Digestion with Glycosidic Enzymes. [^{35}S]Methionine-labeled cells were immunoprecipitated and the beads were washed as described above. Fifty microliters of 0.2 M sodium phosphate buffer (pH 6.0), 0.2% SDS, and 2% 2-mercaptoethanol were added and the samples were heated for 5 min at 95 °C. After the addition of 10 μL of 10% NP40 and 10 mM PMSF, the samples were incubated at 37 °C for 12 h with 250 milliunits of endoglycosidase F (endo-β-*N*-acetylglucosaminidase F, Boehringer Mannheim Biochemica, Indianapolis, IN), 100 milliunits of neuraminidase (Sigma, type II), and/or 3 milliunits of *O*-glycanase (endo-α-*N*-acetylgalactosaminidase, Boehringer Mannheim Biochemica). Digestions were stopped by the addition of 100 μL of 2X electrophoresis sample buffer before analysis by gel electrophoresis.

Fractionation of RAW 264.7 Cells into Cytosol and Membrane/Particulate Fractions. LPS-stimulated RAW 264.7 cells were labeled with [^{35}S]methionine as described above. The cells were washed three times with ice-cold D-PBS, harvested with a cell scraper (Gibco), and collected by centrifugation. Hypotonic swelling and homogenization of the cells were performed as described by Matsushima et al. (1986) except that soybean trypsin inhibitor (20 μg/mL), 5 μM leupeptin, and 5 μM antipain were added to the hypotonic buffer. The nuclei and undisrupted cells were removed by centrifugation at 200*g* for 10 min and the supernatant was centrifuged again at 100000*g* for 60 min at 4 °C. After centrifugation the supernatant was used as the cytosol fraction and the pellet as the membrane/particulate fraction. Each was combined with lysis buffer and analyzed by immunoprecipitation and gel electrophoresis.

Determination of Cell Surface Cachectin/TNF. RAW 264.7 cells were grown to confluence in six-well plates and stimulated with LPS. At 1, 2, 4, 8, and 16 h after LPS addition, culture media were harvested and the cells were washed with warm D-PBS three times. The cells were fixed by addition of 1 mL of 1% paraformaldehyde (Sigma) in D-PBS. The plate was incubated for 30 min at 4 °C with gentle shaking. Fixed cells were washed three times with D-PBS and 2 mL of fresh RPMI/F10 was added. After

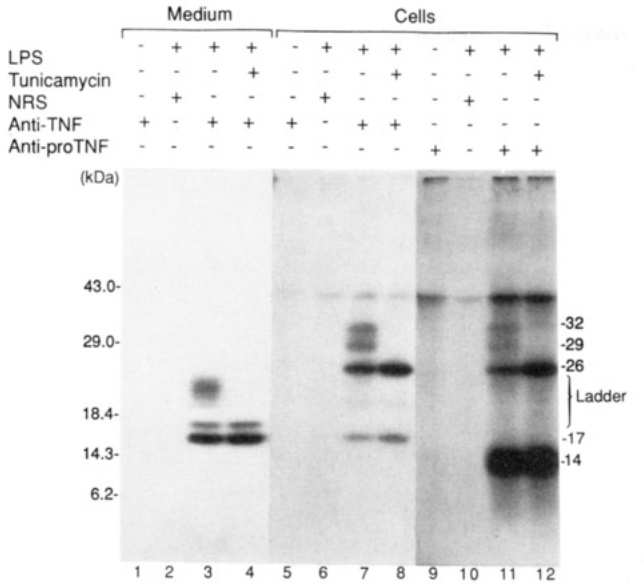

FIGURE 1: Immunoprecipitation analysis of cachectin/TNF produced by RAW 264.7 cells. Cells were incubated in the presence (lanes 2–4, 6–8, and 10–12) or absence (lanes 1, 5, and 9) of LPS for 2.5 h and then labeled with [^{35}S]methionine for 30 min. Culture media (lanes 1–4) and cell lysates (lanes 5–12) were immunoprecipitated with anti-mature cachectin/TNF antisera (lanes 1, 3–5, 7, and 8), anti-propeptide fragment antisera (lanes 9, 11, and 12), or normal rabbit sera (NRS) (lanes 2, 6, and 10) and analyzed by electrophoresis. Lanes 4, 8, and 12 are from cells treated with tunicamycin during incubation with LPS and labeling. The positions of molecular weight markers are shown on the left, and cachectin/TNF immunoreactive bands are identified by their apparent molecular weights at right.

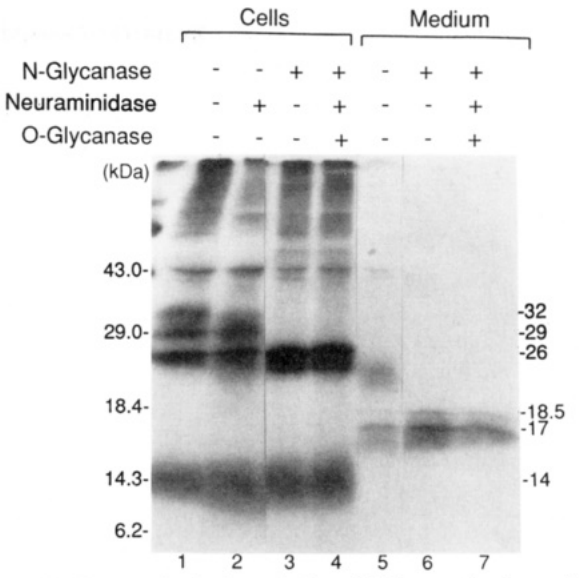

FIGURE 2: Enzymatic deglycosylation of higher molecular weight precursor and mature cachectin/TNF's. [^{35}S]Methionine-labeled cell lysates (lanes 1–4) and culture media (lanes 5–7) from LPS-stimulated RAW 264.7 cells were immunoprecipitated with antiprocachectin/TNF and anti-mature cachectin/TNF antisera, respectively. Immunoprecipitates were treated with digestion buffer alone (lanes 1 and 5), neuraminidase (lane 2), *N*-glycanase (lanes 3 and 6), or neuraminidase, *O*-glycanase, and *N*-glycanase (lanes 4 and 7) and were analyzed by SDS–PAGE and fluorography. The positions of molecular weight markers are shown on the left, and cachectin/TNF immunoreactive bands are identified by their apparent molecular weights on the right.

incubation at 37 °C for 24 h, the cells were scraped off the plate, washed again with fresh media, and resuspended in 0.5 mL of RPMI/F10. Serial dilutions of these cells were added to monolayers of L-929 cells to measure cytotoxic activity.

Results

Glycosylation of Cachectin/TNF. RAW 264.7 cells were stimulated for 2.5 h in LPS-containing medium and metabolically labeled with [^{35}S]methionine for another 30 min. When the medium was analyzed by immunoprecipitation with anticachectin/TNF antiserum and electrophoresis, mature 17-kDa cachectin/TNF and related cachectin/TNF "ladder" proteins were observed (Figure 1, lane 3). As previously observed in this laboratory (B. Sherry, D.-M. Jue, A. Zentella, and A. Cerami, unpublished results), the synthesis of "ladder" proteins was blocked when the cells were cultured in the presence of tunicamycin, an inhibitor of asparagine (N)-glycosylation (Takatsuki & Tamura, 1971). Tunicamycin treatment did not significantly affect the total amount of secreted cachectin/TNF. When the mixture of secreted cachectin/TNF was treated with *N*-glycanase to remove N-linked carbohydrate groups (Elder & Alexander, 1982), most of the cachectin/TNF "ladder" proteins disappeared and the intensity of the 17-kDa band increased (Figure 2, lane 6). However, one of the "ladder" proteins with apparent molecular weight of 18.5 kDa was found to be resistant to both treatments (Figures 1 and 2), indicating that it has a structure other than that from N-glycosylation of the mature protein. Incubation with neuraminidase and *O*-glycanase also did not alter the 18.5-kDa protein and the nature of its structure is still unknown (Figure 2, lane 7). Since these studies were completed, Cseh and Beutler have reported an 18.5-kDa secreted species of cachectin/TNF that carries a 10 amino acid N-terminal extension corresponding exactly to the propeptide sequence, apparently the result of alternate prohormone processing (Cseh & Beutler, 1989).

In lysates of endotoxin-stimulated RAW 264.7 cells, a 26-kDa protein was immunoprecipitated either by antibodies against prosequence or antibodies against the 17-kDa mature form of cachectin/TNF (Figure 1). Considering its apparent molecular weight, its reactivity with both antibodies, and its resistance to tunicamycin treatment, we propose that the 26-kDa protein represents the nonglycosylated 235 amino acid prohormone precursor of mature 156 amino acid 17-kDa cachectin/TNF. We also detected two bands of higher molecular mass, 29 and 32 kDa, which were also precipitated by either antiprosequence or anti-mature cachectin/TNF antibodies (Figure 1, lanes 7 and 11). Synthesis of 29- and 32-kDa species was inhibited by treatment of cells with tunicamycin, and the two bands were no longer detectable in samples digested with *N*-glycanase (Figure 2, lanes 3 and 4). The results suggested that they were N-glycosylated forms of the 26-kDa prohormone. In a sample treated with neuraminidase, the 32-kDa band disappeared and another protein of slightly higher mobility on sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) was observed (Figure 2, lane 2). Treatment of the immunoprecipitate with *O*-glycanase (alone or along with other glycosidases) had no effect on the mobilities of cachectin/TNF-related proteins on gel electrophoresis (Figure 2 and data not shown). Densitometric measurement of intensities of the bands shown in Figure 1 indicated that, as with secreted cachectin/TNF, about half of the precursors were glycosylated (data not shown). The 29-kDa glycosylated precursor was present in the lysate of RAW 264.7 cells that were labeled for 2.5 min (Figure 3a), suggesting that glycosylation occurs cotranslationally.

Proteolytic Cleavage of Precursor Cachectin/TNF. As shown in Figure 1 (lanes 11 and 12), a broad band of protein centered at about 14 kDa was precipitated from RAW 264.7 cell lysates by antiprosequence fragment antibodies. This band

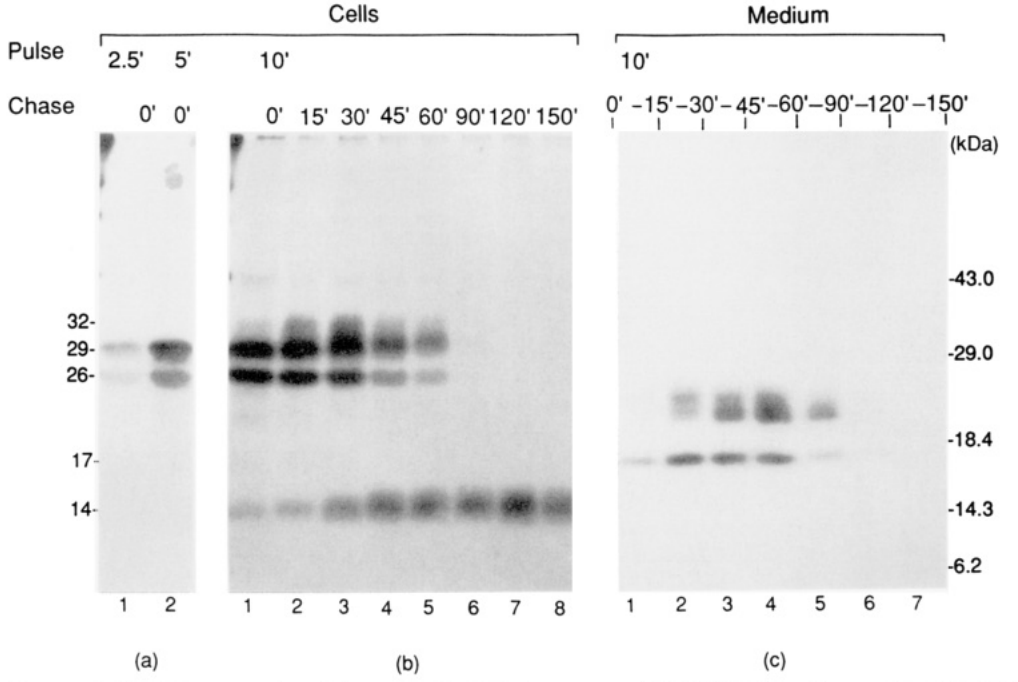

FIGURE 3: Pulse-chase analysis of the processing of the cachectin/TNF precursor. (a) RAW 264.7 cells were labeled with [^{35}S]methionine for 2.5 (lane 1) or 5 min (lane 2), washed with D-PBS, and then lysed. The lysates were immunoprecipitated with antiprocachectin/TNF antisera. (b) Cells were pulse-labeled for 10 min and subsequently chased with a medium containing cold methionine. The cell lysates were prepared at the indicated times after the labeling and immunoprecipitated with antiprocachectin/TNF antisera. (c) Culture media of the cells in (b) were collected at the indicated times and immunoprecipitated with anti-mature cachectin/TNF antisera. Medium from each culture was replaced with fresh medium at the indicated times. Note, then, that time points during the chase of pulse-labeled cell lysates show the differential accumulation of radiolabel in distinct electrophoretic pools of cachectin/TNF from $t = 0$ until the indicated time point (i.e., 0–15 min, 0–30 min, 0–45 min, etc.), whereas time points during the chase of pulse-labeled culture media show the differential proportioning of radiolabel into distinct electrophoretic pools of secreted cachectin/TNF from one time point to the next (i.e., 0–15 min, 15–30 min, 30–45 min, etc.). All samples were analyzed by SDS–PAGE; the positions of molecular weight markers are indicated on the right, and cachectin/TNF immunoreactive bands are identified by their apparent molecular weights on the left.

was precipitated only from lysates of LPS-stimulated cells and did not react with antibodies to mature cachectin/TNF. These results suggest that the 14-kDa band may represent the prosequence peptide cleaved from its cachectin/TNF prohormone precursor. Treatment of cells with tunicamycin or incubation of immunoprecipitates with *N*-glycanase, neuraminidase, and *O*-glycanase did not alter the intensity or mobility of the 14-kDa band in SDS–PAGE (Figures 1 and 2). Relative to the large amounts of mature 17-kDa cachectin/TNF released into the culture medium, only small amounts of mature 17-kDa cachectin/TNF could be detected in the cell lysate (Figure 1, lanes 7 and 8).

To test whether the 17- and 14-kDa bands might be derived from a common 26-kDa precursor, LPS-treated cells were briefly pulsed with [^{35}S]methionine and then chased for various times with cold methionine. Culture media and cell lysates were harvested at intervals up to 150 min and were analyzed by immunoprecipitation with antibodies to mature 156 amino acid cachectin/TNF or to synthetic prosequence fragments (Figure 3). Twenty-six- and twenty-nine-kilodalton proteins were precipitated by antipropeptide antibodies at 10 min of pulse. Another protein of even higher molecular mass (32 kDa) appeared after 15 min of chase and the relative proportions of cachectin/TNF-like proteins above 26 kDa seemed to change gradually over time: at 60 min a 30-kDa species became a predominant protein among the 29–32-kDa proteins. As described above, these proteins are likely to represent N-glycosylated prohormone species of cachectin/TNF undergoing further processing in their carbohydrate groups. These proteins and the "nonglycosylated" 26-kDa precursor decreased gradually with longer chase times and had virtually disappeared after 90 min. A 14-kDa band could be detected in the cell lysate at 10 min of pulse, increased to maximal level at 45 and 60 min of chase, and maintained a similar level for up to 2.5 h (Figure 3b). In similar experiments with a longer chase period, the 14-kDa protein disappeared completely after 3 h (data not shown).

Figure 3c shows the variety of murine cachectin/TNF species secreted into the culture medium at various times after a 10-min [^{35}S]methionine pulse. Culture medium of each dish was harvested and replaced with fresh medium at each time point to determine the amount of cachectin/TNF produced between indicated time points. When the samples were precipitated by anticachectin/TNF antibodies, mature cachectin/TNF (17 kDa) and glycosylated "ladder" proteins were detected at the first time point (0–15 min). Maximal secretion occurred from 15 to 60 min and amounts of secreted cachectin/TNF decreased thereafter. After 90 min no more labeled cachectin/TNF was secreted into the medium, which temporally coincided with the depletion of pulse-labeled cellular cachectin/TNF precursors (Figure 3b). At 15 min of chase, the nonglycosylated 17-kDa protein was the predominant species among secreted cachectin/TNF's. During later stages of the chase, glycosylated forms became more prevalent in cells and medium. The formation of the 14-kDa protein in the cell lysate was also temporally coincident with formation of mature cachectin/TNF's and the results suggest that these proteins are derived from the 26-kDa and higher molecular mass glycosylated precursors.

Association of Precursors and the 14-kDa Prosequence of Cachectin/TNF with the Membrane/Particulate Fraction. To localize various cachectin/TNF-related proteins in the cell, [^{35}S]methionine-labeled RAW 264.7 cells were homogenized in hypotonic buffer and the cytosol and membrane fractions

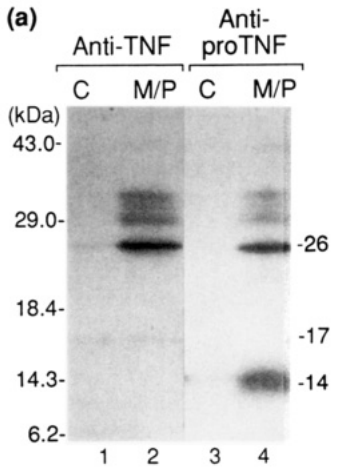

FIGURE 4: Immunoprecipitation analysis of cellular fractions. RAW 264.7 cells were stimulated with LPS and labeled with [^{35}S]methionine for 30 min. The cells were homogenized in hypotonic buffer and centrifuged to provide cytosol (lanes 1 and 3) and membrane/particulate (lanes 2 and 4) fractions. Samples were immunoprecipitated with anti-mature cachectin/TNF (lanes 1 and 2) or antiprocachectin/TNF (lanes 3 and 4) antisera and analyzed on SDS–PAGE.

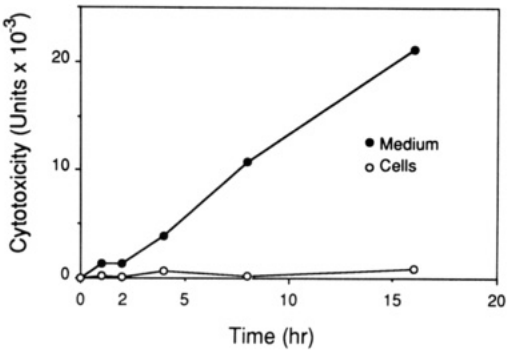

FIGURE 5: Production of cell-associated and supernatant cachectin/TNF's by stimulated RAW 264.7 cells. Cells at a density of (8 × 10^6)/well were incubated with LPS (1 μg/mL) for indicated times. After harvesting the culture media, cells were washed with D-PBS and fixed with paraformaldehyde. The cytotoxic activities of culture supernatant and fixed cells were measured with L-929 cells as target cells in the presence of actinomycin D.

were separated by centrifugation. Each fraction was analyzed by immunoprecipitation with antisera either against mature cachectin/TNF or against synthetic fragments of propeptide sequence. As shown in Figure 4, the majority of the 26-kDa precursor, N-glycosylated precursors, and 14-kDa cleaved propeptide were associated with the membrane/particulate fraction, while relatively small amounts of 17-kDa mature cachectin/TNF were present in both fractions.

To investigate the time course of appearance of cachectin/TNF as membrane-bound precursors and in soluble, mature forms, we measured cytotoxic activities of macrophage cells fixed with paraformaldehyde and of their corresponding culture supernatants. RAW 264.7 cells were incubated in medium containing LPS for up to 16 h and the cells and their culture media were harvested at various times. Paraformaldehyde-fixed cells or harvested media were then added to L-929 cells to measure cytotoxicity. Cell-associated cachectin/TNF activity could be detected 1 h after LPS addition (Figure 5). The levels of activity varied at different times but remained relatively constant: 240, 85, 650, 240, and 860 units per 8 × 10^6 macrophage cells at 1, 2, 4, 8, and 16 h, respectively. The secreted cachectin/TNF increased continuously to 21200 units per 8 × 10^6 cells at 16 h. The proportions of cytotoxicity associated with cells were 18%, 7%, 17%, 2%, and 4% of total cachectin/TNF activity detectable in fixed cells and culture medium at 1, 2, 4, 8, and 16 h of incubation, respectively. When culture medium containing cachectin/TNF was treated with paraformaldehyde and then dialyzed against D-PBS, no alteration in its cytotoxic activity was observed (data not shown). Release of cachectin/TNF from the fixed cells during the cytotoxicity assay period was negligible.

Discussion

When RAW 264.7 macrophages are stimulated with LPS, they secrete a large amount of cachectin/TNF into the medium. The results shown in Figures 1 and 3 suggest that in these cells cachectin/TNF was first synthesized as a 26-kDa precursor. A similar 26-kDa protein was obtained by cell-free translation of human cachectin/TNF cDNA (Muller et al., 1986) and observed in stimulated human monocytes and NIH 3T3 cells transfected with human cachectin/TNF cDNA construct (Kriegler et al., 1988). About half of the precursor proteins were N-glycosylated upon synthesis and migrated to the 29-kDa position on SDS–PAGE (Figure 3). Some of the N-glycosylated oligosaccharide of precursor cachectin/TNF underwent further modification before secretion. In the pulse-labeled cells, a 32-kDa protein appeared at 15 min of chase and the predominant glycosylated precursor at a later stage of processing had a molecular weight of about 30000. Results from a separate experiment showed that among the secreted "ladder" proteins only the 20-kDa protein, which has a molecular size corresponding to the cleavage product of the 29-kDa precursor protein, had significant affinity for concanavalin A (unpublished observation). This suggests that, like other secretory proteins, the 29-kDa cachectin/TNF precursor has a high mannose N-linked oligosaccharide, which is then modified to a complex type in the 32-kDa protein, containing galactose, sialic acid, and fucose (Kornfeld & Kornfeld, 1985). In fact, neuraminidase treatment reduced the apparent molecular weight of the 32-kDa protein (Figure 2). Such modifications have been reported to occur in rough endoplasmic reticulum and the Golgi complex (Kornfeld & Kornfeld, 1985).

There were at least seven or eight proteins in the "ladder" when the conditioned media of RAW 264.7 cells were analyzed by immunoprecipitation and one-dimensional gel electrophoresis, including one that migrates to the position of the primary glycosylation product (20 kDa) (Figure 1). Nonglycosylated cachectin/TNF was secreted along with the glycosylated forms and tunicamycin treatment did not affect the total amount of secreted cachectin/TNF (Figure 1; B. Sherry, D.-M. Jue, A. Zentella, and A. Cerami, unpublished results). Therefore it seems unlikely that glycosylation of cachectin/TNF greatly affects the secretion of this protein from activated macrophages. Similar findings have been reported in the secretion of serum proteins from tunicamycin-treated hepatocytes (Struck et al., 1978) and viral protein from cells infected with vesicular stomatitis virus (Gibson et al., 1979). However, it was also noted that in pulse-labeled cells the majority of secreted cachectin/TNF was nonglycosylated very early in the chase, while glycosylated cachectin/TNF's were released at later times (Figure 3c). This could mean that nonglycosylated cachectin/TNF is cleaved and released faster than glycosylated forms. Since the ratio of glycosylated to nonglycosylated cachectin/TNF's does not seem to vary greatly between newly synthesized precursor and secreted mature cachectin/TNF, posttranslational conversion of nonglycosylated prohormone into the glycosylated form seems to occur minimally, if at all.

A 14-kDa protein was found only in immunoprecipitations

of cell lysates of stimulated macrophages with antibodies to cachectin/TNF prosequence and not in immunoprecipitations with anti-mature cachectin/TNF antibodies (Figure 1). We postulate that this 14-kDa protein is the prosequence of cachectin/TNF remaining after cleavage of the mature 156 amino acid protein from the 235 amino acid precursor. The results from pulse-chase experiments further supported this suggestion (Figure 3). The molecular weight of mouse cachectin/TNF propeptides is 8660, when calculated from its amino acid sequence. Although it is not clear why the calculated (8660) and apparent molecular weights (14 000) of prosequence differ to such a degree, the diffuseness of the 14-kDa band in gel electrophoresis suggests the possibility of differential posttranslational modification. However, treatment with various glycosidases did not change the pattern or mobility of the diffuse 14-kDa band (Figure 2). The possibility of posttranslational modification other than glycosylation, e.g., acylation with palmitate or myristate (Wold, 1986), should be considered.

It has been suggested that the cell surface cachectin/TNF precursor plays a role in cell–cell communication and in confining the action of cachectin/TNF to local tissues that are in close contact with activated macrophages (Decker et al., 1987; Kriegler et al., 1988). When we measured cell surface cachectin/TNF by adding paraformaldehyde-fixed RAW 264.7 cells to L-929 cells, a substantial amount of cytotoxic activity could be detected in cells stimulated with LPS for 1 h (Figure 5). However, there was no further accumulation of cytotoxic activity, which persisted at a relatively low level up to 16 h, while the level of secreted cachectin/TNF increased continuously. This result suggests that in activated macrophages membrane-bound cachectin/TNF precursor is an intermediate in the generation of mature cachectin/TNF. In fact, when we measured the band intensities of the proteins shown in Figure 1 by densitometry, the 26-kDa and glycosylated precursors constituted 14% of the total cachectin/TNF-related proteins (data not shown). If it is assumed that macrophages actively produce cachectin/TNF for 6 h and the amount of precursors displayed on the cell surface remains constant during this period, it can be estimated that at most 1% of produced cachectin/TNF is present as membrane-bound precursor at any point in time. However, it is obvious that a certain amount of cachectin/TNF precursor is present on the surface of activated macrophages and it might be used in cell–cell interactions.

Kriegler et al. (1988) suggested that cachectin/TNF precursor is transported to the cell surface and then cleaved by a proteolytic enzyme outside the cell. Our result with paraformaldehyde-fixed macrophages also showed the presence of cachectin/TNF on the surface of activated cells (Figure 5). However, it is not certain whether the plasma membrane is the only site for the release of cachectin/TNF or if the cleavage occurs also in other intracellular organelles, such as the endoplasmic reticulum or the Golgi apparatus. The presence of the mature form of cachectin/TNF in the cell lysate might suggest that the precursor is also cleaved inside the cell (Figures 1 and 4). Cachectin/TNF produced in these organelles might then be secreted like other secretory proteins. The intracellular 17-kDa mature protein does not seem to come from receptor-mediated uptake of secreted cachectin/TNF, since we could not see any detectible amount of radiolabeled 17-kDa cachectin/TNF in the lysate of macrophages that were incubated with [^{35}S]methionine-labeled mature cachectin/TNF (unpublished observation). It is possible, however, that the mature protein is generated by endogenous protease during lysis of cells, although we added several protease inhibitors with different specificities to the lysis buffer.

Previous reports (Muller et al., 1986; Decker et al., 1987; Kriegler et al., 1988) and our results show that cachectin/TNF is produced as a membrane-associated precursor that is then proteolytically cleaved to release mature cytokine into the medium. Recently, a number of cytokines produced by macrophages (IL-1) (Kurt-Jones et al., 1985; Matsushima et al., 1986), tumor cells (transforming growth factor α) (Bringman et al., 1987; Teixido et al., 1987) and mesenchymal cells (colony stimulating factor 1) (Rettenmier et al., 1987; Rettenmier & Roussel, 1988) have been shown or suggested to be secreted by similar mechanisms. Although the biological significance of membrane-bound precursors of these cytokines has not yet been clarified, it is obvious that the mode of secretion differs from that common to other secretory proteins. The cleavage of the signal peptide of other secretory proteins by signal peptidase is known to occur cotranslationally during the translocation of the nascent polypeptide chain across the endoplasmic reticulum (Blobel et al., 1979). The difference suggests that these cytokines are secreted by mechanisms that include proteolysis by enzymes distinctive from the well-known signal peptidase. By characterizing the enzymes involved, it should be possible to understand the mechanism and significance of this unusual mode of protein secretion.

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Estrogen Receptor Binding to a DNA Response Element in Vitro Is Not Dependent upon Estradiol†

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ABSTRACT: Gel shift assays were employed to distinguish between the contribution of 17β-estradiol (E_2) and a short heating step to the ability of the rat uterine cytosolic estrogen receptor (ER) to bind to the estrogen response element (ERE) from the vitellogenin A2 gene (vitERE). Despite the popularity of models in which the ER is a ligand-activated DNA-binding protein, these studies find that estrogen does not significantly contribute to receptor–DNA complex formation. An avidin–biotin complex with DNA (ABCD) assay was utilized to obtain quantitative measurement of the affinities of the ER for the vitERE and a mutant sequence. Scatchard analysis gave a dissociation constant of 390 ± 40 pM for the E_2-occupied, heated ER to the vitERE. The data fit a one-site model and evidence for cooperativity was not observed. A dissociation constant of 450 ± 170 pM was obtained for the unoccupied, heated ER, leading to the conclusion that estrogen was not necessary for specific binding to DNA. The percentage of ER capable of binding vitERE varied with each cytosol preparation, ranging from 60 to 100% and estrogen did not appear to affect this variation. Competition against the vitERE with a 2-bp mutant sequence showed a 250-fold lower relative binding affinity of the receptor for the mutant over the vitERE sequence. This ability of the ER to discriminate between target and nonspecific DNA sequences was also not dependent on the presence of estrogen.

It has been widely proposed that estrogens effect gene transcription by inducing their receptor protein to bind to specific DNA sequences in target genes. This attractive model of steroid receptors as ligand-activated DNA-binding proteins has been commonly presented as established consensus in the literature (Baulieu, 1989; Evans, 1988; Hunt, 1989; Picard et al., 1988, 1990) and even in a recent textbook (Alberts et al., 1989). This model traces its origin to the fact that a characteristic of the in vitro transformed estrogen receptor (ER) is its ability to bind to DNA–cellulose, albeit with only micromolar affinity and a modest increase in affinity induced by steroid (Skafar & Notides, 1985). A number of treatments, including heating or high salt, lead to receptor transformation and this process was generally agreed to also require the presence of hormone (Grody et al., 1982). The receptor would be expected to display a 1000- to 10000-fold increase in binding affinity to target DNA sequences in response to hormone according to theoretical calculations, if, indeed, hormone confers the ability of the receptor to select target sequences over the mass of DNA in the nucleus (Lin & Riggs, 1975; Ptashne, 1984; Travers, 1983, 1984; von Hippel & Berg, 1989).

One such target sequence for estrogen action has been identified in the 5′-flanking region of the vitellogenin genes of *Xenopus* and chicken (Klein-Hitpass et al., 1986; Walker et al., 1984). Transcription of these genes in vivo is strongly dependent on the presence of estrogen (Wahli, 1981). The

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