Human pro-Tumor Necrosis Factor Is a Homotrimer

Ping Tang, Mien-Chie Hung, and Jim Klostergaard*

Department of Tumor Biology, The University of Texas, M. D. Anderson Cancer Center, Houston, Texas 77030

Received September 12, 1995; Revised Manuscript Received April 15, 1996[⊗]

ABSTRACT: The structure of human transmembrane pro-TNF-α was studied both in intact cell systems and in an *in vitro* translation system. In intact cell systems (LPS-induced THP-1 and TNF cDNA-transfected COS-7), a trimer of pro-TNF was detected after chemical cross-linking based on its molecular weight in Western blotting analysis. The trimer was shown to be a TNF-specific protein and could be partially cleaved to 26-kDa pro-TNF monomers by cleaving the cross-linkers. The trimeric structure was assembled intracellularly, because it could be detected in both the *in vitro* microsomal translation system and in THP-1 cells coincident with the appearance of pro-TNF in the cell lysate, prior to secretion of mature TNF. To further analyze the relationship between the trimeric structure and the biological activity of pro-TNF, we characterized several noncleavable pro-TNF deletion mutants. We observed a correlation between expression of TNF cytotoxicity in a juxtacrine fashion and detection of trimer. Thus, human pro-TNF-α, like the secreted mature TNF-α, has trimeric structure which is assembled intracellularly before transport to the cell surface and is apparently required for mediating its biologic activity.

Tumor necrosis factor (TNF)-α is primarily produced by activated monocytes/ macrophages. There are two forms of TNF-α, a 26-kDa pro-TNF and a 17-kDa mature TNF. Pro-TNF, the precursor of mature TNF, is a type II transmembrane protein with its N-terminus inside the cytoplasm and C-terminus outside the cell. Pro-TNF is first displayed on the plasma membrane and then proteolytically cleaved between alanine (−1) and valine (+1) in the extracellular domain to release the mature TNF (Kriegler et al., 1988). Several recent studies have suggested that the cleavage protease(s) belongs to the metalloprotease family (Mohler et al., 1994; Gearing et al., 1994; McGeehan et al., 1994). However, other studies have suggested that the cleavage enzyme(s) belongs to the serine protease family (Scuderi, 1989; Kim et al., 1993; Robecha-Gallea et al., 1995).

The active form of mature TNF-α has been determined to be a compact, bell-shaped trimer. Studies from analytical ultracentrifugation (Wingfield et al., 1987), gel filtration chromatography, and cross-linking (Smith & Baglioni, 1987) first showed that mature TNF-α was present as a trimer in solution. More detailed structural information from X-ray crystallographic studies of mature TNF-α and TNF-β have revealed that both types of native proteins exist as very similar, compact, bell-shaped homotrimers, with a height of 5.5 nm and a maximum breadth of 5.0 nm (Hakoshima & Tomito, 1988; Jones et al., 1989; Eck & Sprang, 1989; Eck et al., 1992). TNF-α and TNF-β can compete for the same TNF receptors. Each subunit contains essentially a β-sandwich formed by two antiparallel β-pleated sheets. This main-chain folding corresponds to the "jelly roll" motif observed in several viral coated proteins such as VP1, VP2, and VP3 of rhinovirus, and hemagglutinin (HA), the important surface protein of the influenza virus (Jones & Stuart, 1990). The C-terminus of each subunit is embedded in the base of the trimer, and the N-terminus, which has no β-sheet structure, is relatively free from the base of the trimer, does not participate in trimer interactions, and is not critical for its biological activity (Creasey et al., 1987).

The results from studies of substitutive mutations of mature TNF suggested that each TNF trimer has three receptor interaction sites located in the intersubunit grooves near the base of the trimer and that the integrity of the trimeric structure is essential for TNF activity (Van Ostade et al., 1991; Zhang et al., 1992). The most direct evidence came from Banner and co-workers, who studied the crystal structure of the complex between human TNF-β and the extracellular domain of the p55 TNF receptor (Banner et al., 1993). They showed that, indeed, each mature TNF trimer could interact with three receptor molecules at the interface between subunits. They proposed that receptor aggregation upon TNF binding may be the mechanism for receptor activation and subsequent initiation of signal transduction in the target cells. This proposal is supported by previous studies, which showed that TNF receptors cross-linked by anti-TNF receptor antibodies can mimic TNF-induced responses, while Fab fragments cannot (Englemann et al., 1990). All these studies have clearly demonstrated that the trimeric structure of mature TNF is very important for its action.

Little is known about the three-dimensional structure of pro-TNF and its structure−function relationship. Pro-TNF is not a silent precursor molecule for mature TNF, as it also can induce TNF responses in target cells. Previous studies have reported that paraformaldehyde-fixed mouse and human monocytes/macrophages, rendered incapable of TNF secretion, were able to kill TNF-sensitive target cells by cell-to-cell contact (Decker et al., 1987; Espevik & Nissen-Meyer, 1987). In addition, a noncleavable mutant of human pro-TNF expressed in NIH3T3 cells could kill TNF-sensitive L929 cells in a plaque assay (Perez et al., 1990). These results clearly demonstrated that the transmembrane form of pro-TNF is biologically active, but how it interacts with its receptor is unclear. From the studies with mature TNF, we know that the trimeric structure is important for its activity. In the present study, our goal was to elucidate the quaternary structure of pro-TNF and its relationship to the

* Author to whom correspondence and reprint requests should be addressed: Department of Tumor Biology, Box 108, The University of Texas M. D. Anderson Cancer Center, 1515 Holcombe Blvd., Houston, TX 77030. Telephone: 713-792-8962. Fax: 713-794-0209.

[⊗] Abstract published in *Advance ACS Abstracts,* June 1, 1996.

S0006-2960(95)02182-9 CCC: $12.00

molecule's biologic activities. We hypothesized that pro-TNF is also a trimer that interacts with TNF receptors much like mature TNF does and that this interaction is essential to its function.

MATERIALS AND METHODS

Stimulation of THP-1 Cells. The human monocytic cell line THP-1, obtained from ATCC (Rockville, MD), was maintained in RPMI 1640 (GIBCO BRL, Gaithersburg, MD) with 2×10^{-5} M 2-mercaptoethanol (Sigma, St. Louis) and 10% (v/v) fetal bovine serum (FBS; GIBCO BRL). LPS (Sigma) stock was made at 2 mg/mL in DPBS and stored at −20 °C in small aliquots. The THP-1 cells were stimulated with LPS (2 μg/mL)-containing culture medium at 37 °C for 1.5−2 h or as indicated otherwise, collected by centrifugation, washed with cold Dulbecco's phosphate-buffered saline (DPBS), and then used immediately or stored at −70 °C. One milliliter of the supernatant of each group was analyzed for secreted mature TNF both by Western blotting following immunoprecipitation and by the L929 cytotoxicity assay.

Transfection of COS-7 Cells. COS-7, an SV40-transformed African green monkey kidney cell line, obtained from ATCC, was maintained in DMEM/F12 medium (GIBCO BRL) which was supplemented with 10% (v/v) FBS. The COS-7 cells were trypsinized and plated into 100-mm dishes 1 day before the transfection so that they would reach about 50−70% confluence on the day of transfection. The transient transfections were conducted with LipofectAmine reagent (GIBCO BRL) following the instructions of the vendor. Briefly, for each 100-mm dish, 8 μg of plasmid DNA containing a 1.1 kb *Pst*I fragment of TNF cDNA (Utsumi et al., 1993) in one of two different vectors, pcDNAI or pMT2 (pcDNAI.TNF or pMTpTNF, respectively), was incubated with 24 μL of LipofectAmine reagent (2 mg/mL) in 800 μL of serum-free medium for 15 min at room temperature. The mixture was then added to the COS-7 cells with 3.2 mL of serum-free medium. Following incubation at 37 °C for 5 h, the plate contents were either split onto two plates or directly added to 4 mL of medium with 20% FBS. After incubation for another 24 h, the cells were washed once with DPBS, collected using a Cell Scraper (Costar, Cambridge, MA), and pelleted by centrifugation.

Cross-Linking of 17-kDa Recombinant TNF. Mature TNF (a generous gift of Dr. Gen-Ichiro Soma, Takano Hospital, Kumamoto, Japan) was diluted in DPBS to 200 ng/mL, and 20 μL aliquots were treated with homobifunctional cross-linkers. These included several primary amine-reactive *N*-hydroxysuccinimidyl (NHS) esters with different spans between functional groups: ethylene glycol bis(succinimidylsuccinate) (EGS, 1.61 nm), sulfo-EGS (1.61 nm), bis-[2-(succinimidooxycarbonyloxy)ethyl]sulfone (BSOCOES, 1.3 nm), sulfo-BSOCOES (1.3 nm), 3,3′-dithiobis(sulfosuccinimidylpropionate) (DTSSP, 1.2 nm), disuccinimidyl tartarate (DST, 0.64 nm), and one primary amine-reactive imidoester, dimethylsuberimidate hydrochloride (DMS, 1.1 nm). All cross-linkers were purchased from Pierce Chemical (Rockford, IL). They were dissolved in dimethyl sulfoxide (DMSO) at 100 mg/mL immediately before use. The final concentrations for cross-linking reactions were 10 mg/mL. The reactions were carried out at either room temperature or 4 °C for 2 h, and an aliquot from each group corresponding to 1 μg of 17-kDa TNF was analyzed by 12.5% SDS−PAGE and visualized by silver stain.

Cross-Linking of pro-TNF on Intact Cells. Two types of pro-TNF-producing cells, LPS-induced THP-1 cells and TNF cDNA-transfected COS-7 cells, were suspended in 100−200 μL of cold DPBS and cross-linked by EGS and/or BSOCOES. After testing the cross-linkers over a dose range from 5 to 15 mg/mL, we found that a final concentration of total cross-linker of 10 mg/mL was optimal for detection of pro-TNF dimer and trimer. While lower concentrations gave less cross-linked trimer, higher concentrations gave more nonspecific, higher molecular weight cross-linked products. The reaction mixtures were incubated at 4 °C with slow rotation for 2 h with Labquake shaker (Labindustries, Berkeley, CA). The cells were then lysed in RIPA buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS, and several protease inhibitors). We analyzed 75 μg of total cellular protein from each group on 12.5% SDS−PAGE and visualized the TNF proteins by ECL Western blotting reagent (Amersham, Arlington Heights, IL) with 0.5 μg/mL of primary goat anti-human TNF antibody (R&D Systems, Minneapolis, MN) and a 1:2000 dilution of rabbit anti-goat secondary antibody (1.5 mg/mL, Zymed, South San Francisco, CA) with 4% nonfat milk dissolved in 0.1% Tween 20 in DPBS.

Cross-Linking of pro-TNF from in Vitro Translation. The *Pst*I fragment of TNF cDNA subcloned into pcDNAI (Invitrogen, San Diego, CA), termed pcDNAI.TNF, was used as the template. After linearization with *Hin*dIII, an Sp6 polymerase was used to obtain the TNF transcripts, which were then purified by repeated extraction with phenol/chloroform/isoamyl alcohol (25:24:1, Sigma). The canine pancreatic microsomal system with rabbit reticulocyte lysate kits deficient in cysteine (Promega, Madison, WI) and [^{35}S]-cysteine (Amersham) were used to produce a metabolically labeled pro-TNF protein in a total volume of 50 μL. After incubation at 37 °C for 1 h, this reaction mixture was cross-linked directly by EGS and BSOCOES under conditions similar to those used for intact cells. Following cross-linking, 10 μL of anti-TNF antibody (1 mg/mL, R&D Systems) and 40 μL of protein G Sepharose (Pharmacia Biotech, Piscataway, NJ) were used for immunoprecipitation in 500 μL of RIPA. The samples were analyzed by 12.5% SDS−PAGE, and the gels were fixed in 25% 2-propanol and 10% acetic acid, amplified in Amplify (Amersham), and exposed to X-ray film.

Electroelution of Cross-Linked pro-TNF Trimer. Cross-linked products from LPS-induced THP-1 cells and pMTpTNF-transfected COS-7 cells were separated on 10% SDS−PAGE. The bands of putative trimeric pro-TNFs were excised based on molecular weight (confirmed by Western blotting with the rest of the gel). The electroelution was carried out in 1 mL of 75 mM NH_4HCO_3, 0.1% SDS, 1 mM EDTA, and 1% β-mercaptoethanol in Molecularporous membrane tubing with a molecular weight cutoff of 6000−8000 Da (Spectrum Medical Industries, Los Angeles, CA) at 100 mA overnight. The eluted product was concentrated by lyophilization, dissolved in water, and prepared for cleavage.

The initial concentration of NH_4HCO_3 evaluted in electroelution buffer was 150 mM. However, we observed extensive spontaneous cleavage of the cross-linked pro-TNF trimer under this condition. After testing various concentra-

tions of NH_4HCO_3, we elected the 75 mM level since it gave the least spontaneous cleavage of the cross-linked pro-TNF without significant effect on the efficiency of electroelution.

Cleavage of Cross-Linked TNF. The samples of cross-linked mature TNF and pro-TNF obtained from cross-linking of intact cells were cleaved using 1 M hydroxylamine, pH 8.5, for 4 h at 37 °C. In pro-TNF samples, PMSF and aprotinin were added to prevent protein degradation. The electroeluted pro-TNF trimers were also cleaved under the same conditions. The cleaved products were analyzed either by Western blotting for pro-TNF or silver staining for mature TNF following SDS−PAGE.

Site-Directed Mutagenesis. Both oligonucleotide primers for mutagenesis were synthesized by the M. D. Anderson Cancer Center Macromolecular Synthesis Facility. The sequences were 5′-TCG AGA AGA TGA TCT GAC GAT CAC TCC AAA GTG CAG for mutant Δ −20- −1, and 5′-AGG GCT GAT TAG AGA GAG GAT CAC TCC AAA GTG CAG for mutant Δ −20- −11. One microgram of each primer was phosphorylated by T4 polynucleotide kinase (USB, Cleveland, OH) in a 20 μL total volume for 1 h at 37 °C. The enzyme was then inactivated by a 65 °C heat treatment for 10 min. The *Pst*I fragment of TNF cDNA subcloned into puc19M (Clontech, Palo Alto, CA) was used as the template, and Trans oligo *Nde*I/*Nco*I (Clontech) was used as the selective primer. Both mutants were constructed with the Transformer site-directed mutagenesis kit (Clontech) following the manufacturer's instructions. Briefly, 0.1 μg each of template, selective primer, and mutagenesis primer were mixed with annealing buffer in a 20 μL total volume per reaction. The DNA double helix was denatured by boiling at 100 °C for 3 min and annealed by immediately chilling in ice water for 5 min. The synthesis of the mutant DNA strand was carried out by addition of T4 DNA polymerase and T4 DNA ligase to the annealing mixture. After incubation at 37 °C for 2 h, the reaction was stopped by incubation at 70 °C for 5 min. Twenty units of restriction enzyme *Nde*I (20 units/μL, New England BioLabs, Beverly, MA) was added for primary selection, and then this mixture was used to transform competent BMH 71-18 *mut* S cells. The miniprep DNA from it was cut with *Nde*I again and transformed into competent XL-1 cells. The sequence of each mutant was confirmed by DNA sequencing with the primer 5′-CACGCCATTGGCCAGGAGGGC (spanning the codons for residues 35−41) at the M. D. Anderson Cancer Center DNA Core Sequencing Facility. The mutated DNA was cloned back into pcDNAI (Invitrogen) for expression and analysis in COS-7 cells.

^{51}Cr Release Assay. L929 cells (ATCC) in log-phase growth were trypsinized and counted, and the desired number of cells was labeled with 100 μCi of [^{51}Cr]Na_2CrO_4 (ICN Radiochemicals, Irvine, CA) in normal saline solution in 200 μL total volume for 1 h. After being washed with warm medium and incubated with ^{51}Cr-free medium for another hour, 2.5×10^4 labeled L929 cells were added to each well of a 96-well plate that contained either 2.5×10^4 TNF cDNA-transfected COS-7 cells or a 1:1 dilution of the supernatant harvested from each transfected group 24 h after transfection. Following incubation for 24 h, a fraction of the total volume was collected and counted in a gamma counter. Each group was assayed in triplicate, and each experiment was repeated 3−5 times. The percent cytotoxicity was calculated by

$$\% \text{ cytotoxicity} = \frac{\text{ER} - \text{SR}}{\text{TR} - \text{SR}} \times 100$$

ER refers to the cpm from each TNF cDNA transfected group, SR refers to the cpm from the vector-alone transfected group which is similar to those from non-transfected group, and TR refers to the cpm of total labeled cells added to each well.

RESULTS

The Trimer of Human Recombinant Mature TNF-a Can Be Cross-Linked by either EGS or BSOCOES. To choose the most efficient chemical cross-linker(s) of the pro-TNF trimer, we first used a panel of homobifunctional, primary amine-reactive cross-linkers to cross-link human recombinant mature TNF. The products were then analyzed by SDS−PAGE and silver staining. The results (Figure 1A,B) showed that only EGS (1.6 nm) and BSOCOES (1.3 nm) could efficiently cross-link the mature TNF trimer; their sulfo-forms and the shorter cross-linkers (0.6−1.2 nm) could not. The efficiency of cross-linking was similar at either room temperature (Figure 1A) or 4 °C (Figure 1B). Thus, the subsequent cross-linking reactions were done with EGS and/or BSOCOES at 4 °C to minimize any possible protein degradation.

To confirm that the higher molecular weight products detected as dimers (34 kDa) and trimers (51 kDa) of mature TNF were indeed due to cross-linking of a native homotrimer of 17-kDa subunits, a specific cleavage reagent, hydroxylamine, was used to regenerate the lower molecular weight monomer. After cross-linking with the combination of EGS and BSOCOES, the mature TNF trimer and dimer could be partially cleaved to dimer and monomer by treatment with 1 M hydroxylamine for 2−24 h at 37 °C (Figure 1C). Therefore, the cross-linked 34-kDa and 51-kDa proteins detected in our system were indeed the mature TNF dimer and trimer, respectively. Note that increasing incubation time for the cleavage reaction did not significantly affect the efficiency of cleavage.

Pro-TNF of Intact Cells Exists as a Trimeric Structure. Chemical cross-linking of proteins is an inefficient reaction. Even when concentrated solutions of homogeneous populations of oligomeric molecules are used as substrates, the cross-linking reaction does not procede to completion (Figure 1A,B; Davies & Start, 1970). Gething et al. (1986) also demonstrated that although hemagglutinin (HA) is a trimer, the majority of HA was detected as monomers and dimers based on the analysis of cross-linked cell extracts of HA cDNA transfected cells. So, to minimize artificial cross-linking between TNF molecules themselves or between TNF and other membrane molecules due to high concentration of pro-TNF protein in either cell extracts or membrane preparations, here we used a more physiological condition, intact cell systems producing pro-TNF, to exam whether pro-TNF exists as a trimer.

We conducted cross-linking experiments on intact TNF cDNA-transfected COS-7 cells and LPS-induced THP-1 cells. The expression levels of pro-TNF stayed fairly constant in COS-7 cells 24−72 h after transfection, while the level of secreted mature TNF continued to rise for up to 72 h (data not shown). Maximal production of pro-TNF occurred between 1 and 1.5 h after LPS induction in THP-1 cells, and secretion of mature TNF peaked at 4 h (data not shown).

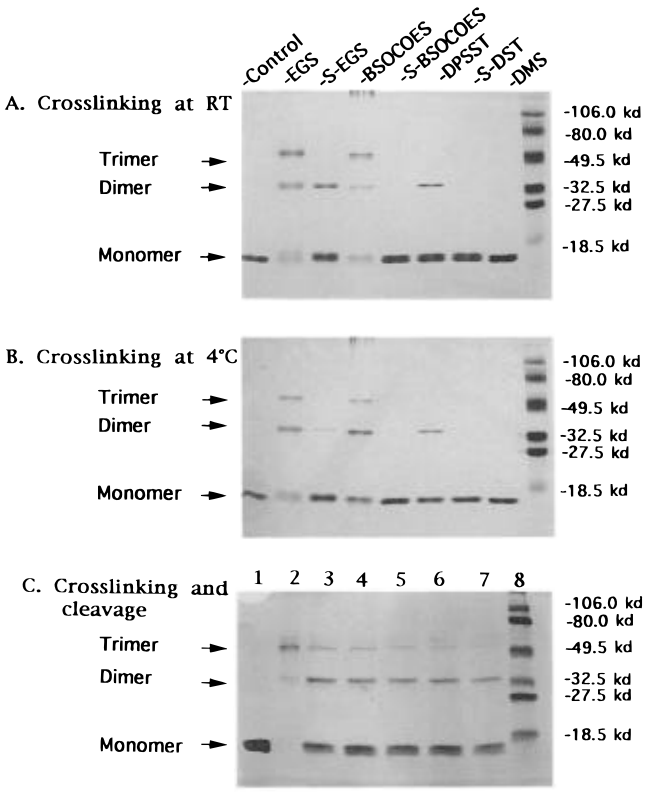

FIGURE 1: Cross-linking of mature TNF-α (A,B). Four micrograms of recombinant mature TNF was cross-linked using the indicated cross-linker at room temperature (A) or 4 °C (B) for 2 h, and 1 μg of TNF from each group was analyzed on 12.5% SDS−PAGE and visualized by silver staining. Cleavage of cross-linked mature TNF (C). Four micrograms of mature TNF was cross-linked by the combination of EGS and BSOCOES in a 20 μL total volume for 2 h at 4 °C, and cleaved by 1 M hydroxylamine, pH 8.5, for 2−24 h at 37 °C. One microgram of TNF from each group was analyzed as above. Lanes 1−8 are un-cross-linked (1), cross-linked (2), cleaved at 2 h (3), 4 h (4), 6 h (5), 7 h (6), 24 h (7), and molecular markers (8), respectively.

The results of cross-linking pro-TNF in intact pcD-NAI.TNF transfected COS-7 cells and LPS-induced THP-1 cells cell systems are shown in Figure 2, panels A and B, respectively. While vector-only transfectants expressed no detectable TNF protein (lane 2), pro-TNF cDNA transfectants expressed TNF-specfic bands at 26- and 24-kDa, corresponding to full-length and truncated pro-TNFs, respectively (lane 4). The treatment with the combination of EGS and BSOCOES of intact cells was able to cross-link pro-TNF and produced dimer (52 kDa) and trimer (78 kDa) bands based on their calculated molecular weights (lane 5). Similar results were also observed with LPS-induced THP-1 cells (Figure 2B). After LPS induction, THP-1 cells produced mainly the 17- and 26-kDa bands, corresponding to the mature TNF monomer and pro-TNF monomer, respectively (lane 4), while noninduced THP-1 cells produced no detectable TNF bands (lane 2). Cross-linking with EGS and BSOCOES again yielded pro-TNF dimer and trimer bands.

The slightly higher molecular weight of the pro-TNF monomer observed in cross-linked groups may be due to the attachment of several cross-linkers to the molecule. Their molecular weight (the cross-linked groups of Figure 3A) decreased slightly after cleavage (the cleaved groups of Figure 3A), in agreement with this suggestion. The source of the high molecular weight aggregates in the cross-linked groups is less clear. Since they appeared as a smear rather than as distinct bands, they may result from nonspecific interactions between TNF molecules themselves, or, more likely, TNF and other membrane proteins. So these aggregates are unlikely to play any significant role under physiological conditions.

In cross-linked groups from both cell systems (lane 5 of Figure 2A,B), we also observed two minor bands, one just below the 51-kDa band at 43 kDa (perhaps due to cross-linking between 17-kDa mature TNF monomer and 26-pro-TNF monomer) and the other just above the 26 kDa band at 34-kDa (perhaps due to cross-linking between two 17-kDa mature TNF monomers). All bands detected in the cross-linked groups were TNF-specific because they were not present in the vector only COS-7 transfectants (Figure2 A, lane 5) or in unstimulated THP-1 cells (Figure 2B, lane 5), and they could be competed by 2 ug/mL of free TNF in the Western analysis (data not shown). Thus, in both the human TNF cDNA transfected COS-7 cells and LPS-induced human monocytic cell line, THP-1, the 26-kDa pro-TNF apparently existed as a trimer.

To verify that these higher molecular weight, TNF-specific cross-linked products were indeed cross-linked dimers or trimers of pro-TNF, we conducted the cross-linking procedures on intact cells as described earlier, followed by cleavage experiments on either total cell lysates or on the putative cross-linked trimer previously purified by electroelution; the results are shown in Figure 3. Pro-TNF in three cell systems, including LPS-induced THP-1 cells, pcD-NAI.TNF-transfected COS-7 cells, and pMTpTNF-transfected COS-7 cells, could be cross-linked and detected as trimers, and these cross-linked products in total cell lysates could be cleaved to lower molecular weight monomers and dimers by hydroxylamine (Figure 3A), to about the same extent as mature TNF (Figure 1). Similar results were obtained from purified pro-TNF trimer from both LPS-induced THP-1 cells and pMTpTNF-transfected COS-7 cells (Figure 3B); that is, the pro-TNF dimer and monomer could be generated by cleavage of the purified, cross-linked pro-TNF trimer. These results strongly suggested that pro-TNF exists as a homotrimer.

The intact cell systems we used here have the advantage that they are closely related to the physiological situation. However, we should note that this is a mixed system with the presence of both 17-kDa mature TNF and 26-kDa pro-TNF. Since both pro-TNF and mature TNF can be cross-linked, there are some overlapping TNF proteins in the cross-linked groups (17, 34, and 51 kDa of mature TNF vs 26, 52, and 78 kDa of pro-TNF). For example, there may be some mature TNF trimer contributing to the 52-kDa pro-TNF dimer band. This is in agreement with our observation in Figure 3A. That is, the 17-kDa mature TNF monomer band decreased in the cross-linked groups compared to the non-cross-linked groups; in the cleaved groups, while the intensity of the pro-TNF trimer and dimer bands decreased, both pro-TNF and mature TNF monomer bands increased. Nevertheless, any overlap of 51- and 52-kDa bands did not affect visualization of the 78-kDa pro-TNF trimer band. Furthermore, the generation of pro-TNF dimer and monomer from purified cross-linked pro-TNF trimer (Figure 3B) strongly suggests that pro-TNF exists as a homotrimeric structure, as does the mature TNF.

Note that some cleavage occurred during the elution process itself. This is apparently due to the presence of NH_4-

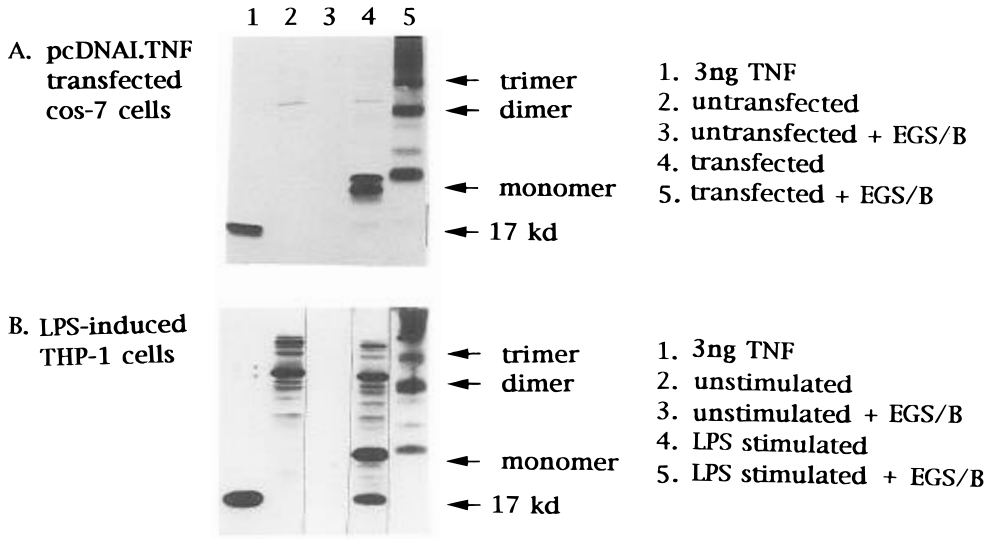

FIGURE 2: Cross-linking of pro-TNF on intact cells. (5−10) × 10^6 TNF cDNA-transfected COS-7 cells or LPS-induced THP-1 cells were resuspended in 100−200 μL of cold DPBS, and freshly prepared cross-linkers were added to the cells at a final concentration 10 mg/mL. After incubation at 4 °C for 2 h, the cells were lysed, and 75 μg of total protein was analyzed by Western blotting. A major 26 kDa pro-TNF monomer band is found in lane 4. The arrows indicate the locations of major trimer, dimer, and monomer bands of pro-TNF; also, faint 34-kDa and 43-kDa bands are found in lane 5. EGS/B indicates the combination treatment of EGS and BSOCOES.

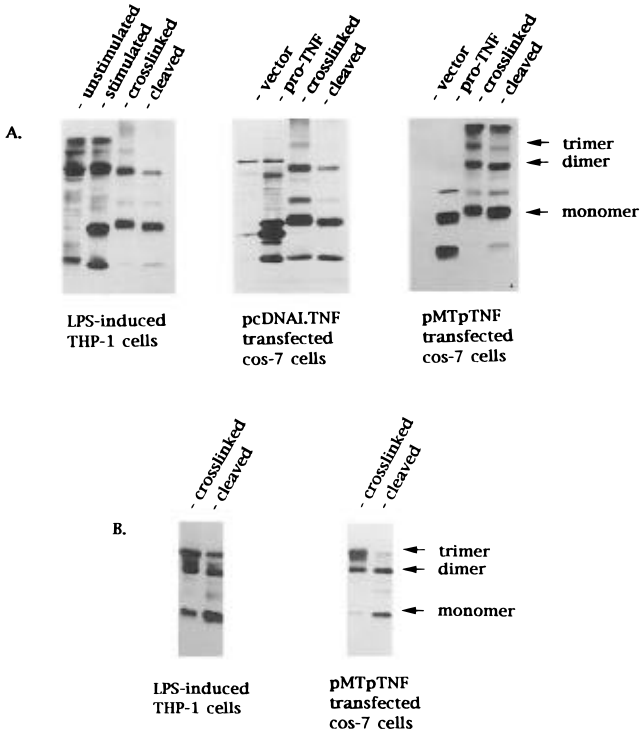

FIGURE 3: Cleavage of cross-linked pro-TNF. The cells were cross-linked and lysed as described in Figure 2. (A) Seventy-five micrograms of total cell lysate from cross-linked groups was cleaved by 1 M hydroxylamine, pH 8.5, at 37 °C for 3 h. (B) The electroeluted pro-TNF trimer was cleaved under the same conditions described for panel A. The samples were then analyzed by Western blotting.

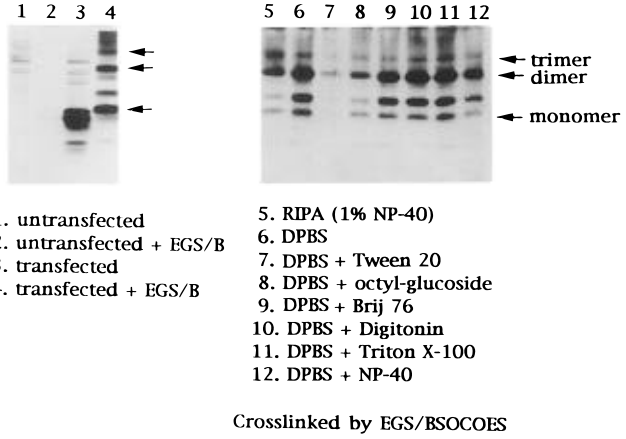

FIGURE 4: Cross-linking of pro-TNF from pcDNAI.TNF-transfected COS-7 cells in the presence of detergents. The cells were treated with indicated detergents on ice for 30 min and then subjected to the cross-linking reaction. The cell lysates were analyzed by Western blotting.

HCO_3 in the electroelution buffer, since we observed that a greater extent of spontaneous cleavage correlated with higher concentrations of NH_4HCO_3. A 34-kDa TNF-specific protein was consistently observed in COS-7 cells transfected with pMTpTNF. This protein may be derived from the longer transcripts we observed with this transfectant (data not shown).

The pro-TNF trimer was fairly stable and could retain its trimeric structure in the presence of many nonionic detergents (Figure 4), which included 0.1% NP-40, 0.1% Tween 20, 80 mM octyl-glucoside, 0.1% digitonin, and 0.1% Triton X-100. The mature TNF trimer had similar properties (data not shown).

Trimer Formation of pro-TNF Occurs Rapidly after Its Synthesis. To test whether trimer formation occurred intracellularly, we used an endoplasmic reticulum (ER) equivalent cell-free system, the *in vitro* translation system with rabbit reticulocyte lysate, and canine pancreatic microsomal membrane. In this system, we could detect a weak signal of TNF trimer and dimer in cross-linked groups, and, with increasing amounts of crosslinkers, the cross-linked product signals increased slightly (data not shown).

To confirm this observation, we reexamined the LPS-induced human THP-1 cell system. Here, we took advantage of the facts that (1) the transcription and translation of TNF is usually silent in THP-1 cells and can be turned on only after appropriate stimulation, and (2) 1−2 h is generally needed to transport the newly synthesized proteins from the ER to the plasma membrane. Therefore, if we could detect the pro-TNF trimer as early as pro-TNF protein and before the detection of mature TNF activity in the supernatant, it

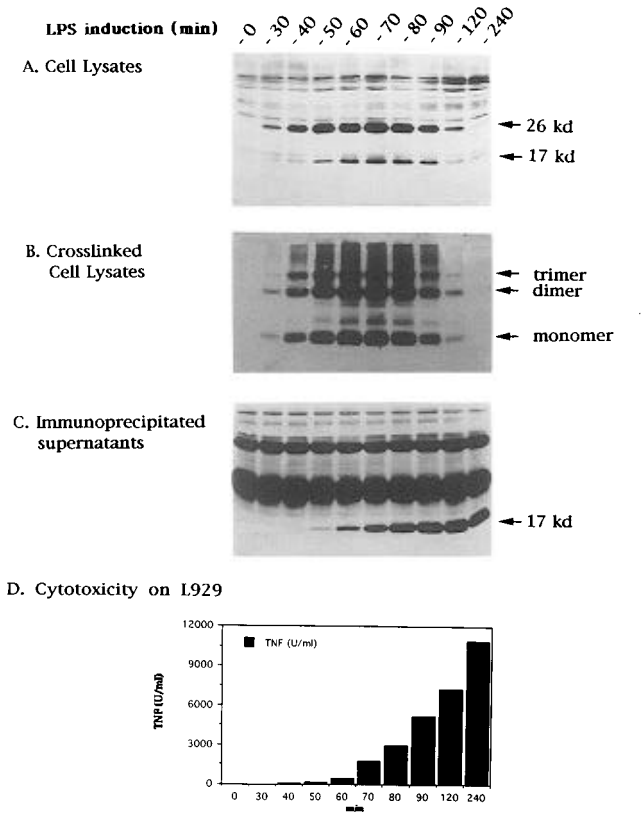

FIGURE 5: Cross-linking of LPS-induced THP-1 cells at different times after induction. THP-1 cells were stimulated by 2 μg/mL LPS, and the cells were collected at the indicated times. The cells were lysed in RIPA buffer directly (A) or following cross-linking (B). Seventy-five micrograms of total protein from each group was analyzed by Western blotting. The supernatant from each group was analyzed by immunoprecipitation followed by Western blotting (C) and by the TNF cytotoxicity assay (D). The prominent bands in panel C correspond to heavy and light chains of the primary antibody.

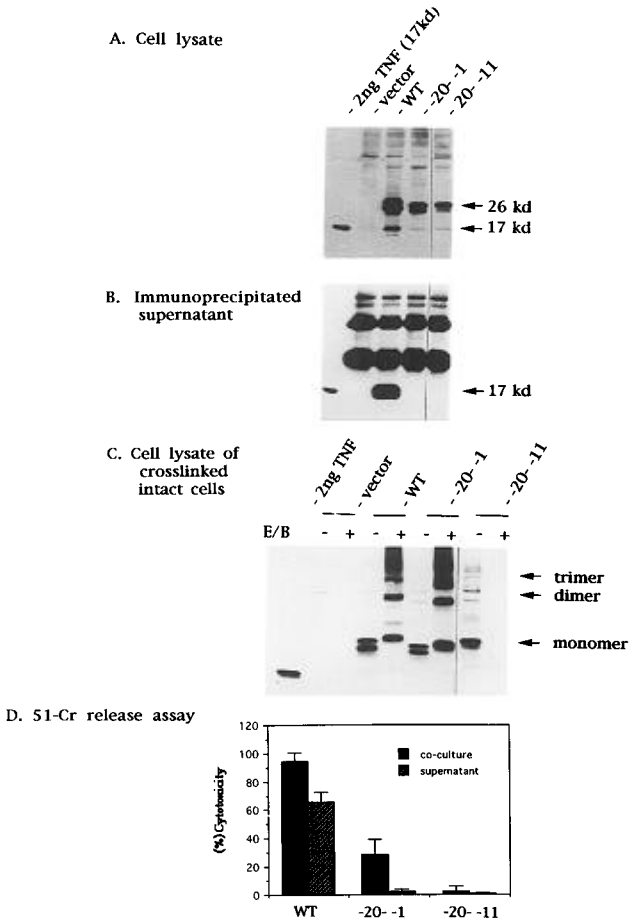

FIGURE 6: (A) Expression of pro-TNF in transfected COS-7 cells. (B) Immunoprecipitation of the supernatants of transfected COS-7 cells. (C) Trimer formation of pro-TNF. (D) ^{51}Cr release assay of transfected COS-7 cells and its supernatants.

would provide strong evidence that trimer formation took place in the ER.

We performed the following experiments to study the kinetic relationship between pro-TNF protein expression, its trimer formation, and the appearance of mature TNF in the supernatant. LPS-induced THP-1 cells were collected at different times (from 30 min to 4 h) after induction and analyzed as follows: the cells from each group either were lysed directly (Figure 5A) or were lysed after cross-linking and analyzed by Western blotting (Figure 5B). The supernatants were analyzed by Western blotting following immunoprecipitation (Figure 5C) and by TNF cytotoxicity assay on L929 cells (Figure 5D). Pro-TNF started to appear as early as 30 min after LPS induction, reached peak levels by 1−1.5 h, and disappeared by 4 h (Figure 5A). Most importantly, cross-linked pro-TNF trimer appeared in a very similar time frame (Figure 5B). At the 30 min time point, all of the ∼52 kDa dimer band is indeed attributable to dimeric pro-TNF rather than trimeric mature TNF, since no mature TNF is detectable. At later time points at which mature TNF is evident, the latter may contribute as a cross-linked trimer to the "dimer" pro-TNF band. As we expected, the appearance of pro-TNF protein and its trimer was first detected about 30 min earlier than was mature TNF (Figure 5C,D), suggesting that mature TNF may actually be a trimer before its release from pro-TNF. The prominent bands at ∼50 and ∼25 kDa in Figure 5C correspond to the heavy and light chains, respectively, of the primary goat IgG used in the immunoprecipitation. So pro-TNF trimer formation occurs rapidly after its synthesis and prior to its reaching the plasma membrane. Thus, these results strongly suggested that trimer formation of pro-TNF occurs in the ER.

The Trimeric Structure of pro-TNF Is Required for Its Biological Activity. Previous studies have suggested that the linking domain of pro-TNF, residues −20 to −1, may be involved in the processing of pro-TNF to ultimately release the mature TNF into its surroundings (Perez et al., 1990; Muller et al., 1986). Small deletions around the native cleavage site (−1/+1), such as Δ −3- −1, Δ+1- +5 and Δ −3- +5, could not block the release of the mature TNF, suggesting that a larger deletion may be needed to generate a noncleavable mutant. To study the relationship between the structure and function of pro-TNF, two deletion mutants were constructed. These included mutant Δ −20- −1, lacking the entire linking sequence between the transmembrane domain and mature domain, and mutant Δ −20- −11, lacking only the half of the linking sequence proximal to the transmembrane domain, preserving the structure around the native cleavage site.

The results are shown in Figure 6. Mutant −20- −1 expressed pro-TNF protein of expected size and comparable amount to that of wild-type TNF after transfection into COS-7 cells (Figure 6A). The neutral red TNF cytotoxicity assay showed that no detectable mature TNF activity was released into the supernatant from the mutant transfectants while wild-type transfectants expressed TNF activity at

~20 000 units/mL (data not shown). The sensitivity of this assay will allow detection of activity at as low as 15 unit/mL; thus, the mature TNF activity of the mutant Δ −20- −1 is less than 0.1% of the wild type. Furthermore, the 17-kDa mature TNF protein was not detected in the supernatant from mutant-transfected COS-7 cells by Western blotting following immunoprecipitation, whereas the wild-type TNF transfectant released high levels of 17-kDa TNF (Figure 6B). These results suggested that mutant Δ −20- −1 may be noncleavable due to the blockage of the cleavage processing rather than the release of an inactive mature TNF. The mutant pro-TNF protein could be cross-linked by EGS and BSOCOES in a trimeric structure on intact cells, as could wild-type pro-TNF (Figure 6C).

Since mutant Δ −20- −1 was apparently noncleavable and could be assembled into a trimeric structure, we further investigated the relationship between pro-TNF trimer formation and the cytotoxic activity mediated by trimeric pro-TNF using a ^{51}Cr release co-culture experiment. The results are shown in Figure 6D. As expected, the supernatant from the mutant Δ −20- −1 transfectant displayed minimal effects on the L929 cells (~2% ± 1% cytotoxicity); however, the transfectant itself could induce substantial levels of co-culture cytotoxicity (~30% cytotoxicity). These results indicated that although this mutant pro-TNF could not release significant detectable mature TNF, it could fold properly on the plasma membrane, interact as a trimer with TNF receptors on the target cell, and mediate its biologic activity through a juxtacrine interaction. These results suggest that the linking sequence of pro-TNF is not essential for proper pro-TNF folding, assembly, and targeting processes, but it is very important in the proteolytic process that releases mature TNF.

Mutant Δ −20- −11 also expressed substantial levels of pro-TNF protein in transfected COS-7 cells (Figure 6A). However, this protein was apparently noncleavable since it generated neither detectable mature TNF protein (Figure 6B) nor supernatant cytotoxicity against L929 cells (Figure 6D; supernatant), Also, this transfectant expressed no direct cytotoxicity against L929 cells (Figure 6D; co-culture), suggesting that this mutant had an inappropriate three-dimensional structure which rendered it biologically inactive. The fact that we could not detect any anti-TNF reactive protein, either pro-TNF monomer or pro-TNF trimer, after treatment of transfected COS-7 cells with cross-linkers and extraction with RIPA buffer (Figure 6C), suggested this mutant pro-TNF may be associated with some relatively insoluble cellular structure. Indeed, there are two lines of evidence supporting this suggestion. First, the extraction efficiency of mutant Δ −20- −11 pro-TNF with RIPA buffer containing 0.1% SDS was inferior to that of the wild-type, even in the absence of cross-linkers; that is, a large portion of it was still left in the pellet, which only could be solubilized with a sample buffer containing 3.3% SDS (data not shown). Secondly, following cross-linking, the mutant pro-TNF monomer could only be partially solublized in RIPA buffer containing 1% SDS (data not shown). Therefore, we propose that mutant Δ −20- −11 behaves anomalously compared to the other linking domain deletion mutants and is incapable of forming a trimeric structure which is important for its activity. However, we cannot rule out the possibility that the pro-TNF trimer is expressed at very low levels, below detection.

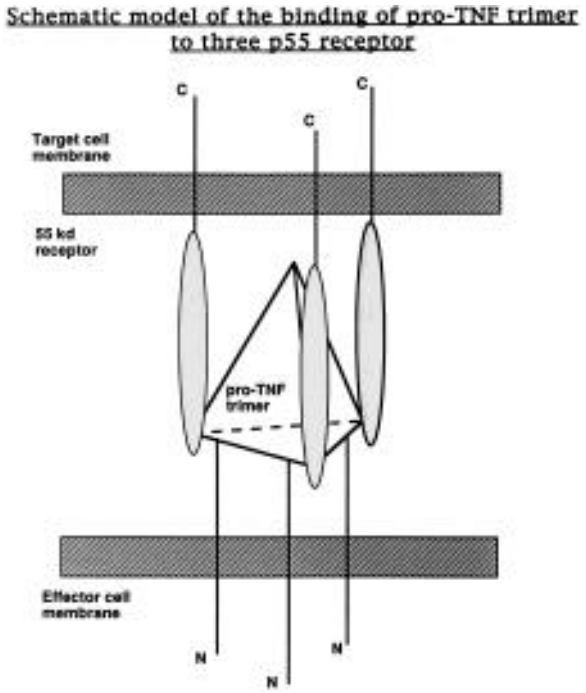

FIGURE 7: Schematic model for the interaction between pro-TNF trimer and TNF receptors.

DISCUSSION

Great understanding of the structure of mature TNF has been achieved following the cloning of its cDNA (Jones et al., 1992). Crystallography of the complex of TNF-β and the extracellular domain of the p55 TNF receptor showed that TNF is a bell-shaped trimer that can interact with three TNF receptor molecules (Banner et al., 1993). Here, for the first time, we have shown that the transmembrane form of pro-TNF, which ultimately displays the mature TNF domain on the outside of the plasma membrane, is also a trimer and that this trimeric structure is required for its biological activity.

Structure−Function Model. On the basis of the results of the present studies and many previous ones (Van Ostade et al., 1991; Zhang et al., 1992; Banner et al., 1993), we propose a model (Figure 7) to interpret the interaction between the pro-TNF trimer on the effector cells and the receptors on the target cells. In this model, the mature TNF domains of pro-TNF are arranged as a compact bell-shaped trimer, very similarly to their arrangement in the mature TNF crystal (Jones et al., 1989; Eck & Sprang, 1989). In this structure, the N-terminal 6−8 residues were found to be flexible, since their coordinates in the crystal structure could not be assigned. Near the base of this trimer, the mobile N-terminus from each subunit is linked to the 20-residue linking sequence, which is in turn connected to the transmembrane domain and ultimately the intracellular domain of pro-TNF. Thus, when effector cells are juxtaposed to the target cells, pro-TNF on the effector cells can interact with TNF receptors on the target cells and thereby induce their TNF responses via receptor aggregation. At this point of the interaction, the base of the trimer is proximal to the effector cells, the linking domain is connected to the effector plasma membrane, and the top of the trimer is proximal to the target cells. The extracellular domains of TNF receptors on target cells may interact with the pro-TNF trimer much as mature TNF does.

Our cross-linking data have strongly suggested that pro-TNF exists as a trimeric structure. Although we observed less trimer than dimer of pro-TNF following cross-linking, the detection of the dimeric species alone strongly supports the concept of the oligomerization of pro-TNF. The distribution among the trimer, dimer, and monomer of pro-TNF, we believe, reflects a balance between inefficient cross-linking and disruption of oligomeric structure in the denaturing SDS−PAGE system. Our kinetic data strongly suggest that the trimer of pro-TNF is formed intracellularly. This is similar to observations on the hemagglutinin (HA) of influenza virus, which shares the same "jelly roll" structure with TNF-α (Jones & Stuart, 1990) and forms a trimer within a few minutes of its synthesis in the ER (Gething et al., 1986). The folding and assembly of HA monomers into a trimeric structure takes about 7−10 min and is completed before the protein leaves the ER, based on the pulse−chase experiments and analysis of the resistance to endoglycosidase H of monomeric and trimeric HA molecules (Gething et al., 1986).

Deletion Mutants. The studies with our pro-TNF deletion mutants have shown a strong correlation between trimer formation and biological activity, suggesting the trimeric structure of pro-TNF is very important for its biological activity. The noncleavable mutant, Δ −20- −1, was able to form a trimeric structure and was also able to mediate cytotoxicity in a juxtacrine fashion. In contrast, the noncleavable mutant, Δ −20- −11, behaved anomalously; although it was expressed at a high level (Figure 6A), we could detect neither its trimeric structure nor cytotoxic capacity in the co-culture assay.

The lower cytotoxicity expressed by mutant Δ −20- −1 compared to the wild-type in the co-culture assay may be attributable to the following. First, there was no mature TNF to mediate paracrine cytotoxicity with the mutant pro-TNF transfectant because it was noncleavable, whereas cytotoxicity expressed by the wild-type would include both juxtacrine and paracrine pathways. Secondly, the 20-residue deletion of the linking domain that normally extends between the plasma membrane and the base of trimeric structure may have decreased the flexibility of the pro-TNF molecule and thereby its ability to bind, cross-link, and activate its receptors.

The lack of pro-TNF activity in the co-culture assay and the absence of any TNF-specific protein after treatment with chemical cross-linking reagents suggested that mutant Δ −20- −11 existed in an anomalous three-dimensional structure, which was biologically inactive and strongly associated with certain detergent-insoluble cellular membrane components. The lack of cytotoxic activity in coculture of this mutant pro-TNF could be due to its inverted orientation in the plasma membrane, but we think this is unlikely in light of our other studies (Utsumi et al., 1993, 1995) which indicate that even larger deletions in this part of the leader sequence did not affect targeting and orientation. Why the larger deletion in mutant Δ −20- −1 would still allow formation of a biologically active pro-TNF trimer, but the smaller deletion in mutant Δ −20- −11 would not, is unclear.

The most important region affecting TNF trimer assembly appeared to be located in the mature domain. Site-directed mutagenesis studies (Van Ostade et al., 1991; Zhang et al., 1992) have shown that substitution of certain residues in the mature domain can abolish the TNF trimer formation and its biological activity. X-ray crystallography has shown that most amino acids in mature TNF are involved in the formation of the compact TNF trimer, except for the first 10 residues, which are proximal to the linking domain. We propose that the region including the entire linking domain and the first 10 residues of the mature domain may mainly serve to connect the mature domain to the cell surface. As such, this region would give the mature domain reasonable flexibility to interact with TNF receptors on adjacent cells. The high number of proline residues in this region is consistent with this suggestion. It is unlikely that this region could significantly affect the assembly process of pro-TNF, since deletion mutants Δ −20- −1 and Δ +1- +12 (Perez et al., 1990) still retain biological activity which requires the intact trimeric structure.

Chemical Cross-Linking. Although there are limitations of chemical cross-linking due to its low efficiency, it is the best approach available at present to study the oligomerization of transmembrane proteins. Although we observed that dimers in particular are the major species in cross-linked groups of pro-TNF producing cells, we believe that the trimer is the major species of pro-TNF existing in intact cells. Data from X-ray crystallography have clearly shown that TNF monomeric subunits in the mature trimer are extremely tightly associated with each other, with hydrophobic residues clustering in the core and charged residues decorating the surface (Jones & Stuart, 1990). This tight interaction between the facades of the subunits would be compromised and the binding interaction would be lost in a dimeric model. Since we propose that assembly of the mature domains of pro-TNF is essential for pro-TNF oligomerization, the trimeric structure also seems the most energetically favorable configuration. The fact that we could detect the trimeric structure of pro-TNF shortly after its synthesis also suggests that the trimer may be the most stable structure for pro-TNF.

The driving force for trimer formation is unclear. Based on gel filtration data, both native and recombinant mature TNF readily form a trimer in the absence of the pro-TNF leader sequence, suggesting that the leader sequence may not be required for trimer formation, at least for mature TNF (Smith & Baglioni, 1987). However, Kriegler et al. (1988) showed that pro-TNF from *in vitro* translation in the absence of a microsomal membrane did not display cytotoxicity. In contrast, in the presence of microsomes, pro-TNF became cytotoxic only after the mature TNF domain was exposed by sonication, suggesting that proper anchoring of the leader sequence in the ER may be required for proper folding and assembly of pro-TNF trimer, which is required for mediating its biological activity. This is supported by the studies of Tanabe et al. (1991), who showed that recombinant pro-TNF purified from *Escherichia coli* following dialysis in PBS lacked biological activity. These studies suggested that although the leader sequence is not important in the trimerization of mature TNF, it may be involved in facilitating trimer formation of pro-TNF: it may properly anchor pro-TNF in the ER membrane so that the pro-TNF monomer could be folded properly and the pro-TNF trimer could be assembled from it under physiological conditions.

There are six potential NHS ester-reactive lysyl residues in mature TNF, at positions 11, 66, 90, 98, 112, and 128. Five (all except residue 98) are reactive with the NHS ester of caprylic acid based on the results from HPLC/mass spectrophotometric analysis of TNF after tryptic digestion

(Akimaru et al., 1995). The level of contribution of two additional lysyl residues located in the cytoplasmic domain of pro-TNF in our cross-linking system is unknown because no data on either their reactivity with NHS esters or the existence of any association between the cytoplasmic tails are available at this time.

Of the cross-linkers we examined, only EGS and BSOCOES could cross-link the pro-TNF trimer, which agreed with our data and previous studies on mature TNF (Figure 1; Smith & Baglioni, 1987). It is unclear why the sulfo forms of EGS and BSOCOES were not able to cross-link the pro-TNF trimer; perhaps the sulfo group is so close to the functional group that it decreases its accessibility to the lysyl residues on pro-TNF by simple steric hindrance. Which of these lysyl residues reacts with the cross-linkers in our experiments is unknown, but we have consistently observed that these two cross-linkers work better than either one alone. These results may reflect the preferences of each cross-linker for particular lysyl residues. Thus, either cross-linker alone would yield mainly dimers, whereas together they would yield more dimers and trimers. Since both EGS and BSOCOES are water-insoluble and membrane-permeable cross-linkers, they can cross-link proteins both outside and inside of the cells. Thus, the cross-linked pro-TNF from intact cells reflects the total cellular 26-kDa pro-TNF located in the ER, Golgi apparatus, and the plasma membrane.

Conclusion. TNF-α belongs to the TNF superfamily. An increasing number of members have been identified recently, including TNF-α, TNF-β (or LT-α) (Gray et al., 1984), LT-β (Browning et al., 1993), and the ligand for Fas antigen (Suda et al., 1993), CD 27 (Goodwin et al., 1993), CD 30 (Smith et al., 1993), and CD 40 (Armitage et al., 1993). Like TNF-α, all of them are type II transmembrane proteins except TNF-β, a typical secretory protein. However, TNF-β can also associate with the plasma membrane by forming a heteromeric complex with membrane-anchored LT-β (Androlewicz et al., 1992; Ware et al., 1992). The fact that the homology among TNF family members mainly localizes in the β-sheet regions of the extracellular domains suggests that these proteins may share a similar three-dimensional structure with TNF-α. A recent report has indicated that the transmembrane pro-TNF is the primary activating ligand for the p75/80 as opposed to the p55/60 TNF receptor (Grell et al., 1995). This suggests that the signaling hierarchy ascribed to these two receptors by many investigators needs to be reexamined in light of the differential receptor selectivity of the mature vs pro-TNF ligands. Thus, our study provides important information on the structure of pro-TNF and the possible mechanism of interaction between membrane-bound pro-TNF and its receptor. It may also provide insight into structural features of other TNF family members.

ACKNOWLEDGMENT

We thank Ms. Pam O'Sullivan in the M. D. Anderson Cancer Center Macromolecular Synthesis Facility (supported by NCI Core Grant CA-16671) for synthesis of our primers and Ms. Rashmi Pershad in the M. D. Anderson Cancer Center DNA Sequencing Facility (supported by NCI Core Grant CA 16672-20) for sequencing TNF mutants. We also thank Mr. Walter J. Pagel in the M. D. Anderson Cancer Center Department of Scientific Publications for helpful editoral suggestions.

REFERENCES

Akimaru, K., Akimaru, Y., Hayman, A. C., Auzenne, E., Leroux, M. E., Utsumi, T., Soma, G.-I., & Klostergaard, J. (1995) *Cytokine Mol. Ther. 1*, 197−210.

Androlewicz, M. J., Browning, J. L., & Ware, C. F. (1992) *J. Biol. Chem. 267*, 2542−2547.

Armitage, R. J., Fanslow, W. C., Strockbine, L., Sato, T. A., Clifford, Ky, N., Macduff, B. M., Anderson, D. M., Gimpel, S. D., Davis-Smith, T., Maliszewski, C. R., Clark, E. A., Smith, C. A., Grabstein, K. H., Cosman, D., & Spriggs, M. K. (1993) *Nature 357*, 80−82.

Banner, D. W., D'Arcy, A., Janes, W., Gentz, R., Schoenfeld, H. J., Broger, C., Lostscher, H., & Lesslauer, W. (1993) *Cell 73*, 431−445.

Browning, J. L., Ngam-ek, A., Lawton, P., DeMarinis, J., Tizard, R., Chow, E. P., Hession, C., O'Brine-Greco, B., Foley, S. F., & Ware, C. F. (1993) *Cell 72*, 847−856.

Creasey, A. A., Doyle, L. V., Reynolds, M. T., Jung, T., Lin, L. S., & Vitt, C. R. (1987) *Cancer Res. 47*, 145−149.

Davies, G. E., & Start, G. R. (1970) *Proc. Natl. Acad. Sci. U.S.A. 66*, 651−656.

Decker, T., Lohmann-Matthes, M.-L., & Gifford, G. E. (1987) *J. Immunol. 138*, 957−962.

Eck, M. J., & Sprang, S. R. (1989) *J. Biol. Chem. 264*, 17595−17605.

Eck, M. J., Ultsch, M., Rinderknecht, E., de Vos, A. M., & Sprang, S. R. (1992) *J. Biol. Chem. 267*, 2119−2122.

Engelmann, H., Holtmann, H., Brakebusch, C., Anvi, Y. S., Sarov, I., Nophar, Y., Hadas, E., Leitner, O., & Wallach, D. (1990) *J. Biol. Chem. 265*, 14497−14504.

Espevik, T., & Nissen-Meyer, J. (1987) *Immunology 61*, 443−448.

Fiers, W. (1992) in *Tumor Necrosis Factors: Structure, Function, and Mechanism of Action* (Aggarwal, B. B., & Vilcek, J., Eds.) pp 79−82, New York.

Gearing, A. J. H., Beckett, P., Christodoulou, M., Churchill, M., Clements, J., Davidson, A. H., Drummond, A. H., Galloway, W. A., Gilbert, R., Gordon, J. L., Leber, T. M., Mangan, M., Miller, K., Nayee, P., Owen, K., Patel, S., Thomas, W., Wells., G., Wood, L. M., & Woolley, K. (1994) *Nature 370*, 555−557.

Gething, M-J., McCammon, K., & Sambrook, J. (1986) *Cell 46*, 939−950.

Goodwin, R. G., Alderson, M. R., Smith, C. A., Armitage, R. J., VandenBos, T., Jerzy, R., Tough, T. W., Schoenborn, M. A., Davis-Smith, T., Hennen, K., Falk, B., Cosman, D., Baker, E., Sutherland, G. R., Grabstein, K. H., Farrah, T., Giri, J. G., & Beckmann, M. P. (1993) *Cell 73*, 447−456.

Gray, P. W., Aggarwal, B. B., Benton, C. V., Bringman, T. S., Henzel, W. J., Jarrett, J. A., Leung, D. W., Moffat, B., Ng, P., Svedersky, L. P., Palladino, M. A., & Nedwin, G. E. (1984) *Nature 312*, 721−724.

Grell, M., Douni, E., Wajant, H., Lohden, M., Claus, M., Maxeiner, B., Georgopoulos, S., Lesslauer, W., Kollias, G., Pfizenmaier, K., & Scheurich, P. (1995) *Cell 83*, 793−802.

Hakoshima, T., & Tomito, K.-I. (1988) *J. Mol. Biol. 201*, 455−457.

Jones, E. Y., & Stuart, D. I. (1990) *J. Cell. Sci. Suppl. 13*, 11−18.

Jones, E. Y., Stuart, D. I., & Walker, N. P. C. (1989) *Nature 338*, 225−228.

Jones, E. Y., Stuart, D. I., & Walker, N. P. C. (1992) in *Tumor Necrosis Factors: Structure, Function, and Mechanism of Action* (Aggarwal, B. B., & Vilcek, J., Eds.) pp 93−127, New York.

Kim, K.-U., Kwon, O.-J., & Jue, D.-M. (1993) *Immunology 80*, 134−139.

Kriegler, M., Perez, C., DeFay, K., Albert, I ., & Lu, D. (1988) *Cell 53*, 45−53.

McGeehan, G. M., Becherer, J. D., Bast, R. C., Jr., Boyer, C. M., Champion, B., Connolly, K. M., Conway, J. G., Furdon, P., Karp, S., Kidao, S., McElroy, A. B., Nichols, J., Pryzwansky, K. M., Schoenen, F., Sekut, L., Truesdale, A., Verghese, M., Warner, J., & Ways, J. P. (1994) *Nature 370*, 558−561.

Mohler, K. M., Sleath, P. R., Fitzner, J. N., Cerrettl, D. P., Alderson, M., Kerwar, S. S., Torrance, D. S., Otten-Evans, C., Greenstreet, T., Weerawarna, K., Kronhelm, S. R., Petersen, M., Gerhart, M., Kozlosky, C. J., March, C. J., & Black, R. A. (1994) *Nature 370*, 218−220.

Muller, R., Marmenout, A., & Fiers, W. (1986) *FEBS Lett. 197*, 99−104.
Perez, C., Albert, I., DeFay, K., Zachariades, N., Gooding, L., & Kriegler, M. (1990) *Cell 63*, 251−258.
Robache-Gallea, S., Morand, V., Bruneau, J. M., Schoot., B., Tagat, E., Realo, E., Chouaib, S., & Roman-Roman, S. (1995) *J. Biol. Chem. 270*, 23688−23692.
Smith, C. A., Gruss, H.-J., Davis, T., Anderson, D., Farrah, T., Baker, E., Sutherland, G. R., Brannan, C. I., Copland, N. G., Jemnkins, N. A., Grabstein, K. H., Gliniak, B., McAlister, I. B., Fanslow, W., Alderson, M., Falk, B., Gimpel, S., Gillis, S., Din, W. S., Goodwin, R. G., & Armitage, R. J. (1993) *Cell 73*, 1349−1360.
Smith, R. A., & Baglioni, C. (1987). *J. Biol. Chem. 262*, 6951−6954.
Suda, T., Takahashi, T., Golstein, P., & Nagata, S. (1993) *Cell 75*, 1169−1178.
Tanabe, Y., Noguchi, K., Morikawa, A., Mizuno, D.-I., & Soma, G.-I. (1991) *Biochem. Biophys. Res. Commun. 69*, 683−688.
Utsumi, T., Levitan, A., Hung, M.-C., & Klostergaard, J. (1993) *J. Biol. Chem. 268*, 9511−9516.
Utsumi, T., Akimaru, K., Kawabata, Z., Levitan, A., Tokunaga, T., Tang, P., Ide, A., Hung, M.-C., & Klostergaard, J. (1995) *Mol. Cell. Biol. 15*, 6398−6405.
Van Ostade, X., Travernier, J., Prange, T., & Fier, W. (1991) *EMBO J. 10*, 827−836.
Ware, C. F., Crowe, P. D., Grayson, M. H., Androlewicz, M. J., & Browning, J. L. (1992) *J. Immunol. 149*, 3881−3888.
Wingfield, P., Pain, R. H., & Craig, S. (1987) *FEBS Lett. 221*, 179−184.
Zhang, X. M., Weber, I., & Chen, M. J. (1992) *J. Biol. Chem. 267*, 24069−24075.

BI952182T