Length of the Linking Domain of Human pro-Tumor Necrosis Factor Determines the Cleavage Processing

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ABSTRACT: Several studies have indicated that only one cleavage site (Ala−1/Val+1) is involved in the release of mature TNF from human pro-TNF, whereas others have suggested that the linking sequence (residues −20 to −1) may be important. We previously demonstrated that a pro-TNF deletion mutant, Δ −20- −1, was able to form a trimeric structure and mediate TNF cytotoxicity in a juxtacrine fashion without releasing mature TNF. We constructed seven mutants with smaller deletions within this region. Three 15-residue deletion mutants, Δ −20- −6, Δ −15- −1 and Δ −20- −16, −10- −1, were noncleavable, although able to form a trimer and to mediate cytotoxicity through cell-to-cell contact. Three five- or ten-residue deletion mutants, Δ −20- −16, Δ −10- −1, and Δ −5- −1, behaved like the wild-type TNF; all formed a trimer and released mature TNF. These results suggested that in pro-TNF (1) the number of residues between the base of the trimer and the plasma membrane determines accessibility of the cleavage site to the pro-TNF processing enzyme(s) since small deletions did not block cleavage whereas large ones did regardless of the presence of the native cleavage site (−1/+1), (2) the native cleavage site is not sufficient for releasing mature TNF because mutant Δ −20- −6, in which the native cleavage site was intact, was noncleavable, and (3) alternative cleavage site(s) may exist since mutants Δ −10- −1 and Δ −5- −1, which lack the native cleavage site, were cleavable.

TNF-α is a cytokine produced mainly by activated monocytes/macrophages (Old, 1985). There are two forms of TNF-α: pro-TNF, a 26-kDa type II transmembrane protein, and its 17-kDa mature secretory form, which is generated by proteolytic cleavage of the extracellular domain of pro-TNF between Ala(−1) and Val(+1) (Wang et al., 1985). Through two distinct TNF receptors, p55 and p75 (Loetscher et al., 1990; Schall et al., 1990), TNF-α can induce a wide range of physiologic and pathologic responses in the host, including such local effects as growth induction, differentiation, immunoregulation, antitumor activity, and antiviral activity, and such systemic effects as shock, cachexia, multiple sclerosis, and cerebral malaria (Jacob, 1992). It has been suggested that pro-TNF is mainly involved in mediating the local effects and mature TNF in mediating the systemic effects (Kriegler et al., 1988). A recent report has demonstrated that the pro-TNF is the main activating ligand for the p75/p80 TNF receptor (Grell et al., 1995). So understanding how pro-TNF is processed to release mature TNF will be valuable in designing ways to modulate TNF-induced pathophysiological effects.

The cleavage site between Ala(−1) and Val(+1) in pro-TNF was determined by N-terminal sequence analysis of mature TNF from PMA-stimulated HL-60 cells (Aggarwal et al., 1985). Recently, three reports based on protease inhibitor studies have suggested that a metalloproteinase is the sole cleavage enzyme involved in pro-TNF processing (Mohler et al., 1994; Gearing et al., 1994; McGeehan et al., 1994). Furthermore, they also indicated that the cleavage site between Ala(−1) and Val(+1) was the only one required for cleavage by the enzyme, based on N-terminal sequence analysis of the 17-kDa cleavage product (Gearing et al., 1994). However, other studies (Scuderi, 1989; Kim et al., 1993, Robache-Gallea et al., 1995) have suggested that a serine protease is involved in pro-TNF processing. Nevertheless, this enzyme activity is probably expressed in most cell types, since many studies of transfection of human TNF cDNA in different host cells have demonstrated proper maturation to biologically active, mature TNF (Muller et al., 1986; Perez et al., 1990; Utsumi et al., 1993; Tang et al., 1996).

Although the major species of mature TNF has an N-terminal Val(+1), there is also strong evidence that alternative cleavage site(s) may exist, possibly located in the linking region of pro-TNF (between residues −20 and −1). Muller and co-workers reported that in the supernatant of human TNF cDNA-transfected CHO cells and of *Xenopus laevis* oocytes injected with TNF mRNA, an 18.5-kDa doublet and a 20-kDa TNF-reactive protein could be detected in addition to the 17-kDa mature TNF, suggesting that intermediate precursor forms, which could be generated by a cleavage(s) within the linking region, may also exist. Also, pro-TNF apparently could be processed in other cell types besides its major natural hosts, monocytes/macrophages, suggesting that the enzyme activity is a common component in many cell types (Muller et al., 1986). In a mouse macrophage system, RAW 264.7, besides 17-kDa mature TNF, a secreted and biologically inactive 18.5-kDa form of mature TNF with 10 additional residues in its N-terminus could be detected. These results again pointed to the existence of alternative cleavage site(s) in the linking sequence (Cseh & Beutler, 1989).

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Unlike the transmembrane precursors of EGF and TGF-α (Wong et al., 1989; Brachmann et al., 1989; Anklesaria et al., 1990), in which noncleavable mutants could be generated by eliminating the native −1/+1 cleavage site, deletions between residues −3 and +5 around the native cleavage site in pro-TNF did not block the release of mature human TNF in a transfected NIH3T3 cell system (Perez et al., 1990). These results also suggested that alternative site(s) for cleavage enzyme(s) may exist. In fact, the only noncleavable pro-TNF mutant so far reported was obtained by deletion of the first 12 residues in the N-terminus of the mature TNF (Perez et al., 1990). This blocked maturation may result from the loss of accessibility of the cleavage site by its positioning too close to the plasma membrane; it is unlikely that any alternative cleavage site(s) with physiological significance would be located in this region of mature TNF.

We have recently reported that deletion of the entire linking domain (residues −20 to −1) abolished the ability of pro-TNF to release mature TNF in COS-7 cells, although this pro-TNF mutant retained the ability to form a trimer and to mediate juxtacrine TNF cytotoxicity (Tang et al., 1996). Here we report further characterization of the mechanism underlying the behavior of this noncleavable mutant, determining whether it involves the loss of a specific cleavage site(s) or involves steric hindrance of the cleavage enzyme to its site of action.

MATERIALS AND METHODS

Cells and Medium. COS-7, an SV40-transformed African green monkey kidney cell line, and L929, a mouse fibrosarcoma cell line, were obtained from ATCC (Rockville, MD) and maintained in DMEM/F12 medium (GIBCO BRL, Gaithersburg, MD) supplemented with 10% (v/v) fetal bovine serum (FBS; GIBCO BRL).

Site-Directed mutagenesis. All oligonucleotide primers were synthesized either by the M. D. Anderson Cancer Center Macromolecular Synthesis Facility or by Dr. Hua Chen of the Department of Tumor Biology. 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, followed by inactivation of the enzyme at 65 °C for 10 min. The *Pst*I fragment of TNF cDNA was subcloned into puc19M (Clontech, Palo Alto, CA) to serve as the template, and trans oligo NdeI/NcoI (Clontech) was used as the selective primer. All mutants were made with a "Transformer site-directed mutagenesis kit" (Clontech) following instructions of the vendor. Briefly, 0.1 μg each of template, selective primer, and mutagenesis primer were mixed with annealing buffer in a total reaction volume of 20 μL. DNA double helix was denatured by boiling at 100 °C for 3 min, and annealing was allowed to occur by immediately chilling the reaction mixture in ice-cold water for 5 min. Synthesis of the mutated 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 the restriction enzyme NdeI (20 units/μL, New England BioLabs, Beverly, MA) was added for primary selection, and then this mixture was used to transform competent MutS cells. The miniprep DNA obtained from it was cut with *Nde*I again (secondary selection) 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 DNA Core Sequencing Facility. The mutated DNA was cloned back into pcDNAI (Invitrogen, San Diego, CA), which contains a human cytomegalovirus (CMV) promoter and the origins of SV40 and polyoma viruses for expression and analysis in mammalian cells.

Transfection and Cross-Linking of COS-7 Cells. COS-7 cells were plated into 100 mm dishes 1 day before transfection. Eight micrograms of pcDNAI containing either wild-type or mutated pro-TNF cDNA was used to transfect each plate of COS-7 cells with 24 μL of LipofectAmine (2 mg/mL, GIBCO BRL) in serum-free medium. After incubation for 5 h at 37 °C, the cells were fed with medium containing 10% fetal calf serum and incubated for another 24 h. The cells were then collected with cell scrapers (Costar, Cambridge, MA) and lysed with RIPA (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS, and several protease inhibitors), either directly or following cross-linking with 5 mg/mL each of ethylene glycol bis(succinimidylsuccinate) (EGS; arm length 1.61 nm) and bis[2-(succinimidyloxycarbonyoxy)-ethyl]sulfone (BSOCOES; arm length 1.3 nm) (Pierce Chemical, Rockford, IL) for 2 h at 4 °C. The supernatant of each group was collected at the same time for analysis of mature TNF by the neutral red cytotoxicity assay, immunoprecipitation, and ^{51}Cr release assay.

Neutral Red Assay and Immunoprecipitation. The neutral red assay was a 24-h TNF cytotoxicity assay using L929 target cells in the presence of the RNA synthesis inhibitor actinomycin D (Sigma, St. Louis, MO) and was performed as previously described (Tang et al., 1994).

Immunoprecipitation was performed as follows: One milliliter of supernatant from each transfection group was first immunoprecipitated with 10 μg of anti-human TNF goat IgG polyclonal antibody (R&D Systems, Minneapolis, MN) and 50 μL of protein G Sepharose 4 Fast Flow (Pharmacia Biotech, Piscataway, NJ) overnight. The precipitates were then separated on a 12.5% SDS−PAGE gel and analyzed by Western blotting. Prominent bands corresponding to the heavy and light chains of the primary goat IgG were observed in the Western blots.

Western Blot Analysis and Scanning of the X-ray Films. The protein concentration of each total cell lysate was determined by Bio-Rad protein assay kit (Bio-Rad Laboratories, Richmond, CA) with MR5000 Deluxe (Dynatech Laboratories, Chantilly, VA). From each group 75 μg of total cellular protein was separated on a 12.5% SDS−PAGE gel, transferred to Immobilon-P Transfer membrane (Millipore, Burlington, MA), preblotted with 4% nonfat milk (Carnation, Glendale, CA) in DPBS containing 0.1% Tween 20 for 1 h, and blotted with 0.5 μg/mL TNF antibody overnight. After being washed 3−5 times with DPBS containing 0.1% Tween 20, the membrane was blotted with rabbit anti-goat secondary antibody (1:2000 dilution, 1.5 mg/mL, Zymed, South San Francisco, CA) for 1 h at room temperature, washed again, and developed with ECL Western blotting reagent (Amersham, Arlington Heights, IL). The bands of pro-TNF proteins (both 26 and 24 kDa bands) on X-ray film were scanned with AMBIS (Image Acquisition

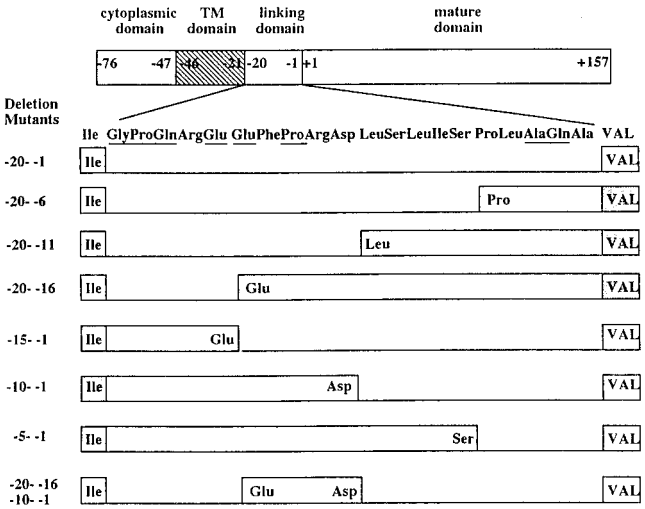

FIGURE 1: Primary structure of wild-type pro-TNF and mutants with deletions in the linking domain. The amino acid sequences of the linking domain are shown with the conserved residues among species underlined.

& Analysis, San Diego, CA), and the mean and standard deviation were calculated.

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

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

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

RESULTS

Previously, we reported that a 20-residue deletion (mutant Δ −20- −1) of the linking domain abolished the ability of pro-TNF to release mature TNF (Tang et al., 1996), suggesting that this region may play an important role in pro-TNF processing. To further investigate the direct role of this region in processing, the 20-residue linking domain was incrementally deleted from either the transmembrane domain side or the mature domain side. This generated three pairs of complementary mutants with preserved junction with either the transmembrane domain or the mature domain (Figure 1). Thus, systematically evaluating these mutants would provide information about the effects not only of the size of the linking domain but also of the particular region of the linking domain on pro-TNF processing.

Protein Expression of pro-TNF. Both wild-type and mutant TNF cDNAs in vector pcDNAI were transfected into

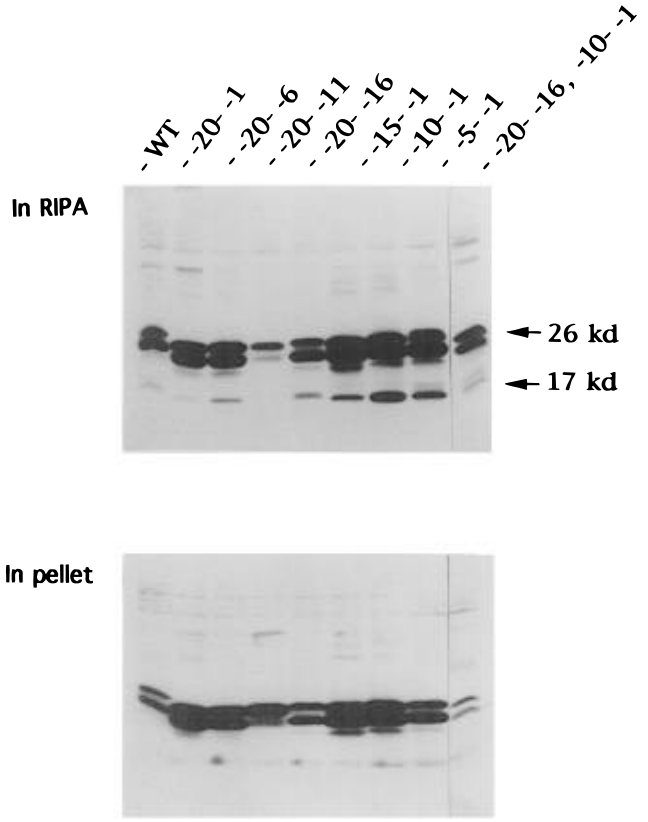

FIGURE 2: Expression of pro-TNF in COS-7 transfectants. The expression level of wild-type and of mutant pro-TNFs from 2.5×10^5 transfected COS-7 cells in RIPA extraction and pellet fraction after transfection was analyzed by Western blotting.

COS-7 cells, and 24 h later about 2.5×10^5 cells of each group were lysed with 50 μL of RIPA buffer (sample 1). The resulting pellet was then solublized in 30 μL of sample buffer containing 3.3% SDS (sample 2). Both samples from each group were subjected to Western blotting analysis. The purpose of checking the levels of pro-TNF in both RIPA extracts and remaining pellets from each group was to study the RIPA extraction efficiency for each mutant because we have previously observed that the major portion of pro-TNF from mutant Δ −20- −11 remains in the pellet (Tang et al., 1996). The results are shown in Figure 2. All mutants expressed the expected size and reasonable amounts of pro-TNF compared to that of the wild-type. The smaller band (~24 kDa) of TNF-specific protein has been consistently observed from transfectants with this particular vector. The source of this 24 kDa TNF is not known, but it likely arises from an alternative initiation site as opposed to premature termination.

The relative expression levels of pro-TNF from each group is shown in Table 1. The level of pro-TNF from each mutant was fairly constant in the combined RIPA buffer extracts and the pellet fractions. However, when we compared the efficiency of RIPA buffer extraction among the pro-TNF mutants, we found that most of them showed a lower extraction efficiency than that of the wild-type, especially the mutant Δ −20- −11. This lower efficiency may have resulted from a stronger association between these mutant pro-TNFs and certain detergent-insoluble cellular structure-(s).

The Size of the Deletion in the Linking Region Affects the Efficiency of the Release of the Biologically Active Mature

Table 1: Relative pro-TNF Expression Levels[a]

	RIPA	pellet	total
wild-type	1.00	1.00	1.00
−20- −1	1.24	1.83	1.48
−20- −6	1.39	1.72	1.53
−20- −11	0.70	1.42	1.01
−20- −16	1.06	1.21	1.12
−15- −1	1.63	2.02	1.79
−10- −1	1.52	1.93	1.69
−5- −1	1.37	1.34	1.36
−20- −16, −10- −1	0.79	0.53	0.69

[a] Wild-type and mutant TNF cDNA were transfected into COS-7 cells (2.5×10^5 cells/well of six-well plate). The cells were collected 24 h after transfection and lysed with 50 μL of RIPA buffer (RIPA), and the resulting pellets were solubilized in 30 μL of sample buffer containing 3.3% SDS (Pellet). Both samples were separated on SDS−PAGE followed by Western blotting analysis. The intensity of each pro-TNF band was scanned and normalized to that of the wild-type, and the mean from both samples was calculated as total.

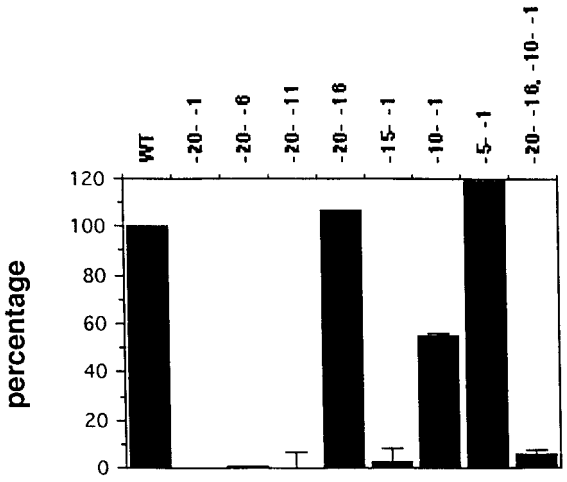

FIGURE 3: Supernatants of each group were assayed by neutral red assay, and the units/mL of TNF activity was normalized to the total pro-TNF expression level.

TNF. The abilities of both wild-type and mutant TNFs to generate biologically active mature TNF varied greatly as determined after normalizing the values from the individual neutral red assays of the supernatants to the corresponding pro-TNF expression levels (Figure 3). Using this analysis, two five-residue deletion mutants (Δ −20- −16 and Δ −5- −1) were found to express supernatant TNF cytotoxicity similar to that of the wild type. This suggests that cleavage operated properly in these mutants, even though mutant Δ −5- −1 lacked the native cleavage site (−1/+1), becoming Ser−6/Val+1. Mutant Δ −10- −1, also lacking the native cleavage site (Asp−11/Val+1) expressed about 50% of wild-type TNF activity, and three 15-residue deletion mutants (Δ −20- −6, Δ −15- −1, and Δ −20- −16, −10- −1) and one 20-residue deletion mutant (Δ −20- −1) showed minimal supernatant TNF activity, regardless of the presence or absence of the native cleavage site. Thus, there was an inverse correlation between the size of the deletion and the efficiency of the release of the mature TNF; that is, large deletions in the linking domain blocked processing of pro-TNF, while small deletions did not. Thus, these results suggest that the efficiency of the maturation of pro-TNF depends not only on the actual amino acid sequence but also on the size of the linking domain.

Lack of Mature TNF Activity Is Not due to the Release of Inactive Mature TNF. In a mouse macrophage system, it has been reported that a macrophage cell line could release a biologically inactive mature TNF with 10 additional residues in its N-terminus (Cseh & Beutler, 1989). In the human pro-TNF system, a biologically inactive 17 kDa TNF was also observed from two two-residue deletion mutants, Δ +1, +12 and Δ +1, +13 in the N-terminal portion of mature TNF (Perez et al., 1990). To rule out the possibility that the low TNF cytotoxicity observed with our large deletions in pro-TNF was due to the release of biologically inactive mature TNF, an immunoprecipitation assay of the supernatant from each transfected group was performed followed by Western blotting analysis (Figure 4). We were able to detect a very strong signal of 17 kDa mature TNF from the wild-type transfectant as well as from transfectants expressing the three five- to ten-residue deletion mutants, Δ −20- −16, Δ −10- −1, and Δ −5- −1. With longer exposures, a small amount of 17-kDa TNF from the supernatants of three 15-residue deletion mutants, Δ −20- −6, Δ −20- −16, −10- −1, and Δ −15- −1, was revealed, although at much lower levels than in the wild-type and the mutants with smaller deletions (data not shown). However, we could not detect *any* 17-kDa TNF from mutants Δ −20- −1 and Δ −20- −11 by this assay. The levels of 17-kDa protein of each group agreed well with the biological assay of the supernatants, suggesting that the lack of mature TNF cytotoxicity was not due to the release of an inactive TNF, but rather to the blocking of the cleavage process itself. Again, the results indicate that the extent of the inhibition may have been related to the size of the deletion in the linking domain. Prominent bands corresponding to the heavy and light chains of the primary goat IgG were observed in the Western blots.

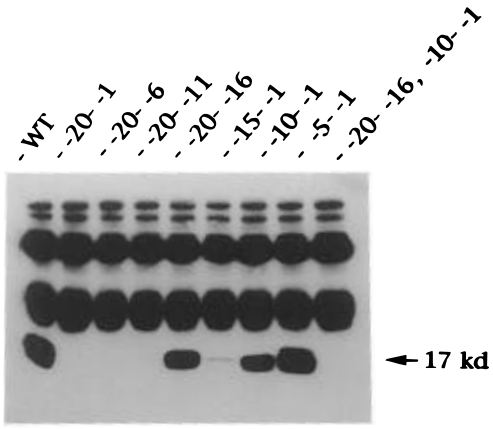

FIGURE 4: One milliliter of supernatant of each group was collected 24 h after transfection, immunoprecipitated with anti-TNF antibody and protein G Sepharose, and analyzed by Western blotting.

Trimer Formation by the Mutant pro-TNFs. Previously, we have reported that pro-TNF is a trimer on the plasma membrane of both LPS-induced THP-1 cells and human TNF cDNA transfected COS-7 cells (Tang et al., 1996). Here, we investigated the influence on trimer formation of these linking domain deletions. The mutant pro-TNFs were expressed in the intact transfected COS-7 cells, which were then cross-linked with EGS and BSOCOES. The cell lysates were analyzed by Western blotting and representative results from several experiments are shown in Figure 5. We found that pro-TNF trimers were detected in all mutant groups, regardless of their ability to release mature TNF, except for mutant Δ −20- −11 as we observed previously (Tang et al., 1996). The pro-TNF expression level as well as the

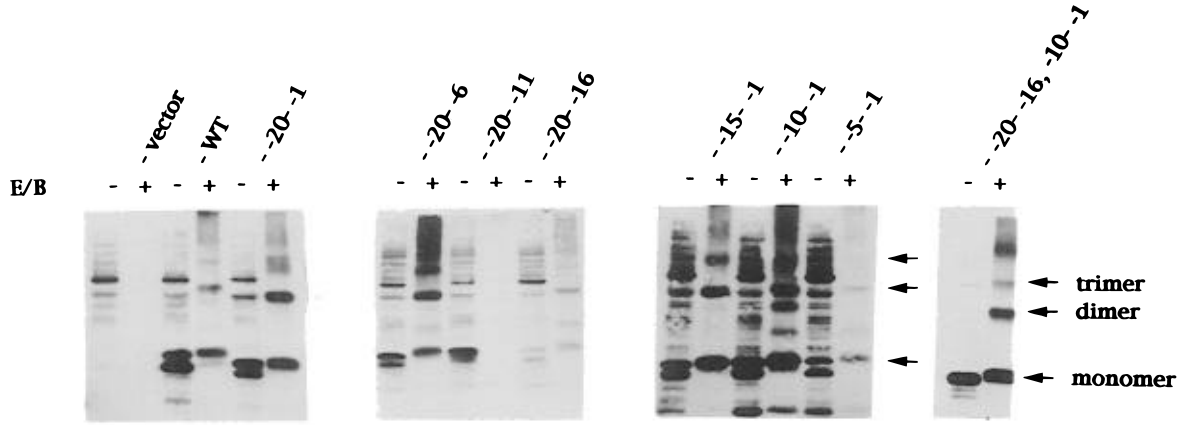

FIGURE 5: $(1-2) \times 10^7$ COS-7 cells were collected from each group 24 h after transfection, cross-linked with EGS and BSOCOES in DPBS for 2 h at 4 °C, and lysed with RIPA buffer. A 75 μg aliquot of total protein from each group was analyzed by Western blotting.

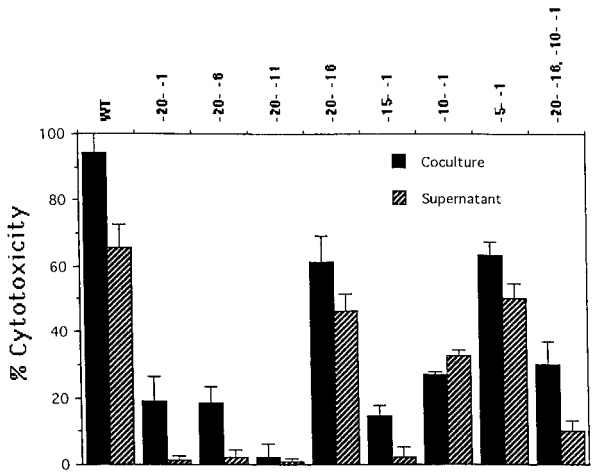

FIGURE 6: COS-7 cells transfected with wild-type and mutant TNF cDNA were immediately plated into a 96-well plate (2.5×10^4 cells per well) and incubated for 24 h. ^{51}Cr-labeled L929 cells (2.5×10^4 cells per well) were then added to these cultures and to wells containing a 1:1 dilution of supernatant from each group 24 h posttransfection. The plates were incubated for another 24 h, and a fraction of the supernatant of each well was collected and counted in a gamma counter. The percent cytotoxicity normalized to the total pro-TNF expression level was shown.

intensity of the cross-linked trimer band for the mutant $\Delta -20- -16$ were consistently lower than those of the wild-type; these bands were quite evident with longer exposure times (data not shown). These results suggest that the defect in pro-TNF processing of the noncleavable mutants ($\Delta -20- -6$, $\Delta -15- -1$ and $\Delta -20- -16$, $-10- -1$) occurred late, possibly after their reaching the plasma membrane.

Mutant pro-TNF Mediates Cytotoxicity through Cell-to-Cell Contact. Pro-TNF can mediate cytotoxicity in a juxtacrine fashion (Perez et al., 1990; Tang et al., 1996). To further analyze the folding and orientation of these mutants on the plasma membrane, the cytotoxicity of these mutants expressed in transfected COS-7 cells was evaluated in a co-culture ^{51}Cr release assay using L929 target cells; the results from individual ^{51}Cr release assays were normalized to the corresponding transfectant pro-TNF expression levels (Figure 6). Pro-TNF from all noncleavable mutants that could form detectable trimers (mutants $\Delta -20- -6$, $\Delta -20- -11$, $-5- -1$, and $\Delta -15- -1$) mediated TNF cytotoxicity through cell-to-cell contact in the co-culture assay; the cytotoxicity level was inversely related to the size of the deletion in the linking domain, with the exception of mutant $\Delta -20- -11$, which could not form a detectable pro-TNF trimer and had no pro-TNF cytotoxic activity (Tang et al., 1996). This suggested that these mutants were displayed on the cell surface with proper folding and conformation and that the trimeric structure was the prerequisite for pro-TNF-mediated activity. The supernatant of each group was also evaluated in the ^{51}Cr release assay. Mutants with smaller deletions had higher cytotoxicity levels than those with the larger deletions, regardless of the presence of the native ($-1/+1$) cleavage site, which is consistent with our data from the neutral red assay and immunoprecipitation analysis.

DISCUSSION

The Length of the Linking Domain Determines pro-TNF Processing. Perez's studies have shown that pro-TNF mutants with deletions around the native cleavage site, from -3 to $+5$, could not block the release of mature TNF, and deletions of 11 or fewer residues downstream from the known cleavage site could also not block the cleavage. In fact, only after the deletion of the first 12 residues of the mature TNF domain did the pro-TNF mutant become noncleavable (Perez et al., 1990). However, this region of the N-terminus of mature TNF is unlikely to have any regulatory role in pro-TNF processing under physiological conditions.

Many studies have suggested that the linking domain of pro-TNF plays a role in pro-TNF processing (Muller et al., 1986; Cseh & Beutler, 1989). We have previously reported that for pro-TNF mutant $\Delta -20- -1$, which is devoid of the entire linking sequence, the release of the mature TNF was completely blocked; furthermore, the mutant pro-TNF was assembled into a trimeric structure with proper orientation on the plasma membrane and displayed biological activity on the target cells through a juxtacrine interaction (Tang et al., 1996). This result suggested that the linking domain, without affecting the protein assembly, is very important in the maturation of pro-TNF. Several mechanisms may account for why mutant $\Delta -20- -1$ is noncleavable. First, the cleavage site(s) may be inaccessible to the protease because the base of the trimeric structure is too close to the plasma membrane. Secondly, perhaps the cleavage site(s) located in this region was lost.

To dissect these possibilities, we constructed seven mutants with smaller deletions in this region with incremental size differences from either the side of the transmembrane domain or the side of the mature domain. We observed a strong

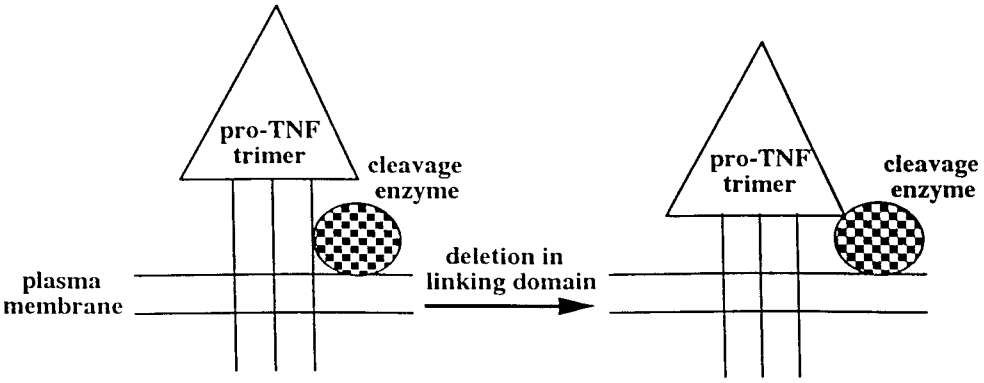

FIGURE 7: Schematic model of the influence of the length of the linking domain on the efficiency of the cleavage process of pro-TNF.

correlation between the size of the deletion in the linking region and the efficiency of the pro-TNF processing to release mature TNF; that is, small deletions could not block this process, while larger ones could. The results from these mutants lead us to propose that the space between the plasma membrane and the base of the trimer (including the entire linking domain and the N-terminal approximately six to eight residues of the mature domain) determines the fate of the cleavage processing, while the actual amino acid sequence is less important (Figure 7).

The mutants with small deletions, Δ −20- −16 and Δ −5- −1, with or without the intact native cleavage site, respectively, showed similar TNF activity compared to the wild-type in both supernatant cytotoxicity assays and in co-culture cytotoxicity assays. Mutant Δ −10- −1, without the native cleavage site, showed an intermediate level of TNF activity from both co-culture and supernatant assays. The three mutants with 15-residue deletions from either side (Δ −20- −6, Δ -15- −1, and Δ −10- −1, −20- −16) were unable to release mature TNF, nor could mutant Δ −20 - −1, although they all could be detected as trimers and could cause pro-TNF-mediated juxtacrine cytotoxicity in the coculture assay.

These results suggest that the maturation of pro-TNF is closely related to the accessibility of the protease to the cleavage site. Thus, while short deletions in this region decreased the efficiency of cleavage, the large deletion could completely block the cleavage process. Our results have shown that at least 10 residues of the linking domain are required to achieve a reasonable level of pro-TNF maturation. The data of Perez et al. (1990) are also consistent with this proposal because the only noncleavable mutant required deletion of the first 12 residues of the mature TNF; other deletions of 11 residues or fewer could not completely block the secretion of mature TNF, although they did decrease the efficiency of maturation, as we observed with our deletion mutants.

The studies from Utsumi et al. (1995) have shown that the pro-TNF deletion mutant, Δ −32- −1, could be properly targeted and oriented on the plasma membrane. Thus, the current mutants with deletions in the linking region (residues −20 to −1) would also most likely have an intact targeting process. Our previous studies (Tang et al., 1996) have shown that mutant Δ −20- −1 can assemble into a trimeric structure and mediate cell-to-cell cytotoxicity, suggesting that the smaller mutants in this region may also be able to form a trimer, be displayed on the plasma membrane, and be available for the pro-TNF processing enzyme. However, we observed that mutant Δ −20- −11 behaved anomalously. It was able to express a comparable amount of pro-TNF but was unable to form any detectable pro-TNF trimer.

The truncated 24-kDa form of pro-TNF protein may result from an alternative initiation. Gase et al. (1990) have shown that a two-residue deletion of the C-terminus of TNF resulted in a drastic reduction of TNF cytotoxicity. This suggests that the C-terminal amino acids of human TNF are critical for its biological activity due to their role in trimer formation (Van Ostade et al., 1991; Zhang et al., 1992; Banner et al., 1993). So, since this 24-kDa TNF seems to be able to form a trimer (Tang et al., unpublished data), it likely has an intact mature TNF domain.

Note also the 17-kDa mature TNF observed in some groups following RIPA extractions (Figure 2); its source is also unclear. It is not likely due to the secreted 17-kDa TNF bound to the cell surface TNF receptors since the intensity of the band in the extract from each group (Figure 2) did not correlate with the secreted TNF activity in the supernatant (Figure 3) or with the mature TNF protein level in the supernatant (Figure 4).

A Specific Amino Acid Sequence in the Linking Domain Is Not Essential for the pro-TNF Processing. Our data also suggest, surprisingly, that the actual amino acid sequence does not play an overriding role in pro-TNF processing: regardless of where the deletion was placed in the linking domain, the cleavage efficiency seemed similar as long as the deletion size was the same. By comparing the amino acid sequence homology of pro-TNF among six different species (Fiers, 1992), we observed clusters of homology in each domain. Two regions had the highest homology (∼75%): the region between residues −76 and −20 (cytoplasmic domain plus transmembrane domain) and the region between residues +10 and +157 (the trimeric portion of the mature TNF). However, the linking domain plus the first 10 residues of the mature domain only share about 40−50% homology. This may reflect a broad recognition and existence of multiple scissile bonds for the cleavage enzyme(s) located in the linking region. However, it is also possible that the specificity determinants for the cleavage may be located C-terminal to the −1/+1 processing site.

It is interesting to observe that, in our experimental system, the mutants which contain the −15 to −11 region, with one of the three proline residues and two of the three anionic side chains in the linking domain, are all cleavable. The significance of this result is unknown. Further study with a −20- −16 and −5- −1 combination deletion mutant or by moving the −15- −11 sequence around the linker could

verify the role of this sequence. Alternatively, replacing the entire linker sequence with a Ser/Gly linker may further define the role of the actual amino acid sequence in pro-TNF processing.

The Native Cleavage Site Is Not Sufficient for pro-TNF Cleavage. Ala(−1)/Val(+1) has been determined to be the native cleavage site based on N-terminal sequence analysis of mature TNF (Aggarwal et al., 1985; Gearing, et al., 1994). However, the deletion mutant Δ −20- −6, which has the intact native cleavage site, was unable to release mature TNF into the supernatant. Thus, for the first time we have shown that the native cleavage site is not sufficient to release mature TNF. This data again suggest that accessibility of the cleavage enzyme is very important in the process.

Alternative Cleavage Site(s) Exist. Several studies have suggested the existence of an alternative cleavage site(s) (Muller et al., 1986; Perez et al., 1990; Utsumi et al., 1993; Cseh & Beutler, 1989). Perez et al. (1990) reported that deletions of the region around Ala−1/Val+1 (including residues +1 to +5, −3 to −1, or −3 to +5) could not block the release of the mature TNF. The levels of mature TNF released from these mutant pro-TNFs were roughly equivalent to that of the wild-type, suggesting that there is an alternative cleavage site(s) located outside this region. Our results here with mutants Δ −10- −1 and Δ −5- −1, which lacked the intact native cleavage site yet were able to release mature TNF, also suggest that alternative cleavage site(s) exist, possibly located in the linking domain. However, the physiological significance of alternative sites is unclear.

Cleavage Enzyme in pro-TNF Processing. TNF-α shares certain properties with TGF-α (Massague, 1990); the precursor molecules for both exist as transmembrane prohormones, and their mature secretory components are generated through proteolytic cleavage of the extracellular domain of the prohormone, interestingly, both at an Ala/Val cleavage site. However, deletion of this site could completely block the release of the secretory component of TGF-α (Wong et al., 1989; Brachmann et al., 1989; Anklesaria et al., 1990), but not of TNF-α. This suggests that either the cleavage enzyme in pro-TNF processing does not need to recognize a specific cleavage site or that more than one cleavage enzyme activity with a different specificity may be involved.

The cleavage enzyme activity seems to exist in many cell types, including CHO cells (hamster) and *X. laevis* oocytes (Muller et al., 1986; Perez et al., 1990), NIH3T3 cells (mouse; Perez et al., 1990; Utsumi et al., 1993), and COS-7 cells (monkey; Tang et al., 1996). This is similar to interleukin-1β-converting enyzme (ICE), which also exists in many cell types besides macrophage/monocyte cells (Cerretti et al., 1992).

The native Ala −1/Val+1 cleavage site has been proposed to be the target for an elastase-like enzyme (Carrell & Boswell, 1986). Scuderi (1989) reported that a serine protease inhibitor *p*-toluenesulfonyl-L-arginine methyl ester (TAME) could block mature TNF secretion in LPS-induced human leukocytes without detectable effects on the expression of either TNF mRNA or surface TNF protein. We have observed similar results with LPS-induced THP-1 cells and human TNF cDNA-transfected COS-7 cells using a similar dose level of TAME (unpublished data). Kim et al. (1993) reported the effect of treatment of the activated mouse macrophage cell line RAW 264.7 with various protease inhibitors. They found that specific serine protease inhibitors had some effect in this model, while the cleavage activity was not sensitive to metal ion chelators such as EDTA or 1,10-phenanthroline. More recently, Robecha-Gallea et al. (1995) reported that a serine protease activity (possibly proteinase-3), from human monocytes or monocytic cell lines, can cleave pro-TNF between +1 Val and +2 Arg in an *in vitro* system. However, three groups independently reported (Mohler et al., 1994; Gearing et al., 1994; McGeehan et al., 1994) that a metalloprotease is responsible for pro-TNF cleavage based on the effects of a hydroxamic acid-based specific metalloprotease inhibitor. This inhibitor could block the release of mature TNF both *in vitro* and *in vitro*, and the same pattern was seen in human, rat, and mouse, despite the fact that the native cleavage sites are different: Ala/Val in human and Thr/Leu in mouse and rat pro-TNFs. These results also suggest that the cleavage enzyme need not recognize a specific site. These apparent contradictions as to the nature of the pro-TNF processing enzyme can be clarified only after this enzyme(s) is purified or cloned.

Conclusion. The data presented here let us conclude that the length of the linking domain of human pro-TNF plays a critical role in the release of mature TNF. By comparing the sequence of the remaining residues within the possible cleavage region in pro-TNF located between residues −21 and +10 of the two cleavable mutants, Δ−5- −1 and Δ−10- −1, we were not able to find any candidate matrix metalloproteinase cleavage site(s) according to the homology provided by Gearing et al. (1995), suggesting that the actual amino acid sequence play a less important role in pro-TNF processing. N-terminal sequence analysis of the mature TNF from these cleavable mutants and substitutive mutation in this region may provide us more detailed information on the pro-TNF processing.

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