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A Part of the Transmembrane Domain of pro-TNF Can Function as a Cleavable Signal Sequence That Generates a Biologically Active Secretory Form of TNF[1]

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To determine the minimum requirement in the 76-residue leader sequence of pro-tumor necrosis factor (TNF) for membrane translocation across the endoplasmic reticulum (ER) and for the maturation of pro-TNF, we constructed pro-TNF mutants in which a part of the transmembrane domain of pro-TNF was directly linked to the N-terminus of the mature domain, and evaluated their translocational behavior across the ER-membrane and their secretion from the transfected cells. The *in vitro* translation/translocation assay involving a canine pancreatic microsomal membrane system including a mutant, Δ−75-47, −32-1, revealed that the N-terminal half of the transmembrane domain of pro-TNF consisting of 14 residues functioned as a cleavable signal sequence; it generated a cleaved form of TNF having a molecular mass similar to that of mature TNF. Analysis of the cleavage site by site-directed mutagenesis indicated that the site was inside the leader sequence of this mutant. When the mutant, Δ−75-47, −32-1, was expressed in COS-1 cells, efficient secretion of a biologically active soluble TNF was observed. Further deletion of the hydrophobic domain from this mutant inhibited the translocation, indicating that some extent of hydrophobicity is indispensable for the membrane translocation of the mature domain of TNF. Thus, the N-terminal half of the transmembrane domain of pro-TNF could function as a cleavable signal sequence when linked to the mature domain of TNF, and secretion of a biologically active secretory form of TNF could be achieved with this 14-residue hydrophobic segment. In intact pro-TNF, however, this 14-residue sequence could not function as a cleavable signal sequence during intracellular processing, indicating that the remainder of the 76-residue leader sequence of pro-TNF inhibits the signal peptide cleavage and thus enables the leader sequence to function as a type II signal-anchor sequence that generates a transmembrane form of TNF.

Key words: membrane translocation, processing, secretion, signal sequence, tumor necrosis factor.

Tumor necrosis factor (TNF) is an extremely pleiotropic proinflammatory cytokine with a wide range of biological effects (*1*). It is initially synthesized and expressed as a functional transmembrane prohormone, linked to a membrane *via* a leader sequence. Synthesis of TNF begins with an unusually long 76 amino acid propeptide sequence. This leader sequence does not serve as a typical N-terminal signal sequence during processing and intracellular targeting (*2*). Rather, the leader sequence functions as a signal-anchor sequence, and synthesis and insertion into the ER membrane occur cotranslationally. Then, probably *via* Golgi-mediated transfer, TNF appears on the plasma membrane as a trimeric, type II transmembrane protein (*3*). This transmembrane form of TNF was found to be biologically active and can transduce signals to adjacent cells through cell-cell contact (*4*). Recent results suggest that it is superior to mature TNF in activating the human p80 TNF receptor for a number of cellular functions (*5*). In fact, it was recently reported that apoptosis of $CD8^+$T cells during human immunodeficiency virus (HIV) infection is mediated by interaction between the transmembrane form of TNF on macrophages and a p80 TNF receptor on $CD8^+$T cells (*6*). Finally, pro-TNF cleavage occurs on the outer surface of the plasma membrane, probably by a metallo-

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Abbreviations: TNF, tumor necrosis factor; ER, endoplasmic reticulum; PCR, polymerase chain reaction; ECL, enhanced chemiluminescence; DPBS, Dulbecco's phosphate-buffered saline; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; rhTNF, recombinant human tumor necrosis factor; FMLP, formyl-methionyl-leucyl-phenylalanine; KRP, Krebs-Ringer phosphate; $O_2^{\cdot-}$, superoxide anion; Cyt.c., cytochrome *c*; SRP, signal recognition particle.

proteinase (*7, 8*) and/or another enzyme, to release mature, soluble TNF. Thus, the pathophysiological effects of TNF seem to be mediated by both juxtacrine interaction of the transmembrane form of TNF and autocrine-paracrine interaction of the mature, secreted molecule.

The 76-residue leader sequence entails an extracellular linking domain from residues −20 to −1, a hydrophobic transmembrane domain from residues −46 to −21, as well as a cytoplasmic domain from residues −76 to −47. However, the role of these domains in the processing of pro-TNF is poorly understood.

To evaluate the role of the leader sequence of pro-TNF in the intracellular processing of pro-TNF, we previously analyzed the behavior, both in a microsomal translocational system and by transfection analysis, of a series of pro-TNF mutants with deletions in the linking, transmembrane, and cytoplasmic domains (*9, 10*). We established that the entire linking domain, and most of the cytoplasmic domain, could be deleted without the membrane translocation, sorting, or maturation of pro-TNF being affected. Part of the transmembrane domain, in contrast, was found to be essential for the pro-TNF processing. In this case, the hydrophobicity required for this domain was found to be comparable to that of a typical N-terminal signal sequence. Thus, the proper membrane translocation, type II topology formation, and maturation of human pro-TNF seemed to be accomplished with as few as 14 of the 26 residues of the transmembrane domain.

In the present study, in order to determine the minimum requirement in the 76-residue leader sequence of pro-TNF for the cellular processing and maturation of pro-TNF, we have constructed pro-TNF mutants in which a part of the transmembrane domain of pro-TNF is directly linked to the N-terminus of the mature domain, and evaluated their translocational behavior across the ER-membrane and their secretion from the transfected cells.

MATERIALS AND METHODS

Materials—Restriction endonucleases, DNA-modifying enzymes, RNase inhibitor, and Taq DNA polymerase were purchased from Takara Shuzo Kyoto. The mCAP RNA capping kit and proteinase K were from Stratagene. RNase was purchased from Boehringer-Mannheim, Germany. The canine pancreatic microsomal membrane and rabbit reticulocyte lysate were from Promega. [^{3}H]Leucine, [^{35}S]-methionine and Amplify were from Amersham (UK). The Dye Terminator Cycle Sequencing kit was from Applied Biosystems. Anti-human TNF polyclonal antibodies were purchased from R&D Systems. Protein G Sepharose was from Pharmacia Biotech. Other reagents purchased from Wako Pure Chemical, Tokyo, Daiichi Pure Chemicals, Tokyo, and Seikagaku Kogyo, Tokyo, were of analytical or DNA grade.

Plasmid Construction—Plasmid pBluescript II SK(+) lacking *Apa*I and *Hin*dIII sites was constructed as previously described (*10*). This plasmid was designated as pB.

Plasmid pBpro-TNF, which contains the full-length human pro-TNF cDNA, plasmid pBΔpro-TNF, which contains the cDNA coding for the mature domain of TNF, and plasmids pBΔ−32-1 and pBΔ−20-1 were constructed as described (*10, 11*). Plasmid pBΔ−75-47, −32-1 was constructed by means of PCR. For this procedure, pBΔ−32-1 served as a template and two oligonucleotides (A-1, 5′-GCGCGGATCCATGTTGTTCCTCAGCCTCTTC-3′, A-2, 5′-GCCGGGATCCTAGGGCGAATTGGGTACC-3′) as primers. After digestion with *Bam*HI and *Pst*I, the amplified product was subcloned into pB at the *Bam*HI and *Pst*I sites.

Plasmids pBΔ−75-47, −32-1-V1M and pBΔ−75-47, −32-1-S5M were constructed by means of PCR. In this case, pBΔ−75-47, −32-1 served as a template and two mutagenic primers (B-1, 5′-GCGCGGCGCCATGAGATC-ATCTTCTCGA-3′; B-2, 5′-GCGCGGCGCCGTCAGATC-ATCTATGCGAACCCCGAGTGAC-3′, respectively) and A-2 as primers.

Plasmids pBΔ−75-47, −36, −35, −32-1, pBΔ−75-47, −38-35, −32-1, and pBΔ−75-47, −40-35, −32-1 were constructed from plasmids pBΔ−36, −35, −32-1, pBΔ−38-35, −32-1, and pBΔ−40-35, −32-1, respectively, by means of PCR. Plasmids pBΔ−36, −35, −32-1, pBΔ−38-35, −32-1, and pBΔ−40-35, −32-1 were constructed as previously described (*10*). For these procedures, plasmids pBΔ−36, −35, −32-1, pBΔ−38-35, −32-1, and pBΔ−40-35, −32-1 served as templates, and two oligonucleotides (A-1, A-2) as primers. The amplified products were subcloned into pB at the *Bam*HI and *Pst*I sites as described above.

The DNA sequences of these recombinant cDNAs were confirmed by the dideoxy-nucleotide chain termination method (*12*).

In Vitro Transcription and Translation—Methods essentially identical to those described previously were employed (*10*). T3 polymerase was used to obtain transcripts of these cDNAs subcloned into pB. They were purified by phenol-chloroform extraction and ethanol precipitation before use in the translation reaction.

A canine pancreatic microsomal membrane system including a rabbit reticulocyte lysate deficient in a selected amino acid (Promega) was used to characterize the endoplasmic processing of these transcripts. As previously reported (*2, 9*), a metabolically-labeled ([^{3}H]Leu) parental pro-TNF translate could be detected with or without microsomes. In the former case, it was transported across the microsomal membrane, with the mature domain in the lumen. In this orientation, the amino-terminal cytoplasmic domain was lost upon proteinase K treatment. The remainder was degraded if the membrane was permeabilized with Triton X-100. In the present study, the proper translocation of a mutant pro-TNF across the microsomal membrane was established if a proteinase K-resistant protein band could be detected in the absence of a detergent; a diminished signal was interpreted as indicating reduced translocation or translocation with the reversed orientation.

Transfection of COS-1 Cells—The simian virus 40-transformed African Green monkey kidney cell line, COS-1, was maintained in Dulbecco modified Eagle's medium (GIBCO BRL) supplemented with 10% fetal calf serum (GIBCO BRL). Cells (2×10^5) were plated on 35-mm-diameter dishes 1 day before transfection. pcDNA3 (2 μg; Invitrogen, San Diego, CA) containing either wild-type or mutant pro-TNF cDNA was used to transfect each plate of COS-1 cells along with 4 μl of LipofectAmine (2 mg/ml; GIBCO BRL) in 1 ml of serum-free medium. After incubation for 5 h at 37°C, the cells were refed with serum-containing medium and then incubated again for an appropri-

ate period. The supernatants were frozen, and the cells were washed three times with Dulbecco's phosphate-buffered saline (DPBS) and collected with cell scrapers, and then lysed with 200 μl of RIPA buffer (50 mM Tris-HCl [pH 7.5], 150 mM NaCl, 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS, protease inhibitors) on ice for 20 min. The cell lysates were centrifuged for 15 min at 15,000 rpm at 4°C in a microcentrifuge (HITACHI-CF15D2), and the resulting supernatants were analyzed by SDS-PAGE and fluorography.

Western Blotting—Total cell lysates from each group of transfected cells were resolved by 12.5% SDS-PAGE and then transferred to an Immobilon-P transfer membrane. After blocking with nonfat milk, the membrane was probed with anti-hTNF antibodies as described (*9*). Immunoreactive proteins were specifically detected by incubation with horseradish peroxidase-conjugated protein A (Bio-Rad). The membrane was developed with ECL Western blotting reagent (Amersham) and exposed as needed to X-ray film. Quantitative analysis of immunoreactive proteins on the membrane was carried out using a storage phosphor imaging screen and a GS-250 Molecular Imager (Bio-Rad).

Immunoprecipitation—[^{3}H]-labeled samples were immunoprecipitated with anti-hTNF polyclonal antibodies (R&D Systems) as described (*10*).

SDS-PAGE and Fluorography—Samples were denatured by boiling for 3 min in SDS-sample buffer and then analyzed by SDS-PAGE on a 12.5% gel according to Laemmli (*13*). Thereafter, the gel was fixed and soaked in Amplify™ (Amersham) for 30 min. The gel was dried under vacuum and then exposed to XAR-5 X-ray film (Kodak) for an appropriate period.

TNF Cytotoxicity Assay—The mouse L929 fibrosarcoma cell line, sensitized with actinomycin D, was used to assay TNF secreted into transfected cell supernatants as previously described (*10*).

Priming and Stimulation of Neutrophils—Human peripheral neutrophils were isolated from venous blood of healthy humans by Ficoll-Hypaque density gradient centrifugation using Mono-Poly Resolving medium as described (*14*). FMLP (1.25×10^{-8} M) was used for receptor-mediated activation of neutrophils. To prime neutrophils, 10^6 cells/ml were preincubated with TNF for 10 min at 37°C.

Determination of $O_2^{\cdot-}$ Generation—$O_2^{\cdot-}$ generation was assayed as the reduction of Cyt.c as previously described using a dual beam spectrophotometer (Shimadzu UV-3000) equipped with a water-jacketed cell holder and a magnetic stirrer (*14*). Briefly, the reaction mixture contained, in a final volume of 2 ml KRP, 1 mM $CaCl_2$, 20 μM Cyt.c, 10 mM glucose, and $0.2\text{-}1\times10^6$ cells/ml. The reaction was started by adding a stimulant at 37°C and then the change in absorbance at 550-540 nm ($A_{550-540}$) was monitored. $O_2^{\cdot-}$ generation was calculated from the superoxide-dependent reduction of Cyt.c, using an extinction coefficient of 21.0 mM^{-1}.

RESULTS

The N-Terminal Half of the Transmembrane Domain of pro-TNF Can Function as a Cleavable Signal Sequence—To determine whether or not the N-terminal half of the transmembrane domain alone can direct the translocation of the mature domain of pro-TNF, we generated a mutant in which the entire cytoplasmic region of the $\Delta-32$-1 mutant was deleted. Figure 1 compares the translation/translocation patterns of the wild-type pro-TNF (Pro-TNF) with those of the $\Delta-32$-1 and $\Delta-75$-47, -32-1 mutants. The translocational behavior of the $\Delta-32$-1 mutant was

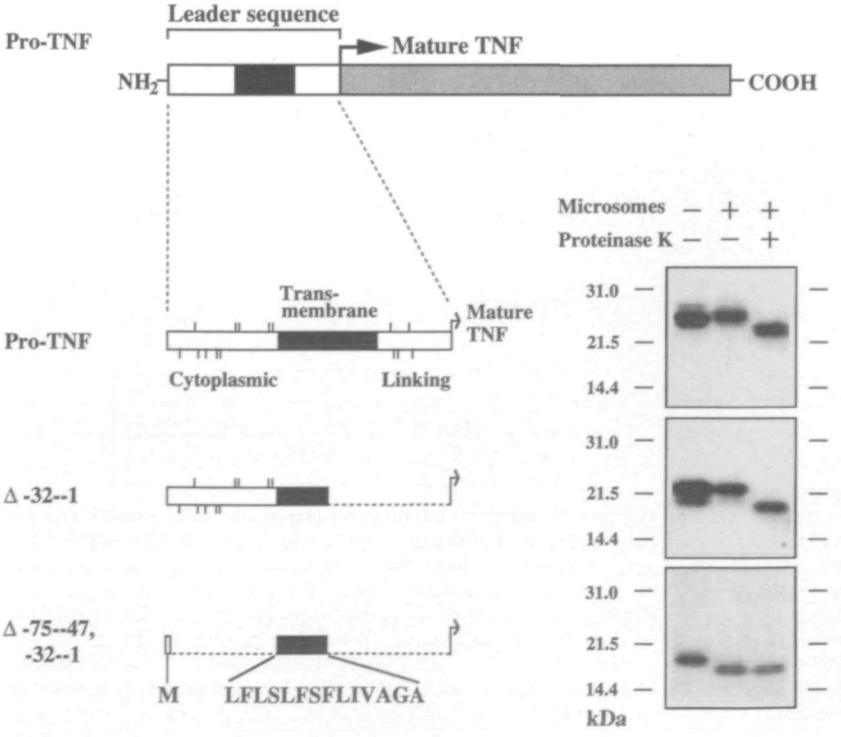

Fig. 1. ***In vitro* translocation of the parental pro-TNF, $\Delta-32$-1 mutant and $\Delta-75$-47, -32-1 mutant across the microsomal membrane.** The structures of the leader sequences of these mutants are schematically shown. The deleted regions are shown as dashed lines and the transmembrane domain is shown as a solid bar. Positively charged (vertical lines above boxes) and negatively charged (vertical lines below boxes) residues are also shown. The [^{3}H]Leu-labeled proteins, detected by fluorography after SDS-polyacrylamide gel electrophoresis, among the *in vitro* translation products of mutant pro-TNF mRNAs in the presence (+) or absence (–) of microsomes are shown. The effects of proteinase K digestion of the microsome-dependent translates are also shown.

indistinguishable from that of the wild-type pro-TNF, as previously observed (*10*), indicating that this mutant was localized on the ER-membrane with the correct type II orientation. In contrast, the translation/translocation pattern of the Δ−75-47, −32-1 mutant was different from those of the wild-type pro-TNF and the Δ−32-1 mutant. In the presence of microsomes, a protein band corresponding to a slightly reduced molecular weight was observed, and the material in this band was not degraded by treatment with proteinase K. Therefore, deletion of the cytoplasmic region (residues −75 to −47) from the Δ−32-1 mutant unexpectedly caused the remaining 14-residue hydrophobic region (residues −46 to −33) to function as a typical cleavable signal sequence.

As shown in Fig. 2, when microsomes were added after completion of the translation reaction, the proteinase K-resistant band was undetectable in the translocation assay with either the wild-type pro-TNF or the Δ−75-47, −32-1 mutant. These results indicated that the membrane translocation of these proteins across the ER-membrane was achieved only cotranslationally, as is the case with a typical cleavable signal sequence or signal-anchor sequence.

In order to determine whether or not the cleavage site was localized inside of the leader sequence or in the mature domain of this mutant, we constructed two additional mutants (Δ−75-47, −32-1 V1M- and Δ−75-47, −32-1 S5M-TNF) in which Val located at the N-terminus (+1 site) or Ser at the +5 site of the mature domain of the Δ−75-47, −32-1 mutant was replaced with Met, and evaluated their translocational behavior. In this experiment, we utilized the fact that the mature domain of TNF does not contain a Met residue. If the Met residue at the +1 site is not removed upon signal peptide cleavage indicates that the cleavage site is localized inside of the leader sequence of the Δ−75-47, −32-1 mutant.

As shown in Fig. 3, the apparent molecular sizes of the *in vitro* translation products (−Microsomes) and cleaved products (+Microsomes) of Δ−75-47, −32-1-, Δ−75-47, −32-1 V1M- and Δ−75-47, −32-1 S5M-TNF were similar to each other, indicating that the substitution of the amino acid in these two mutants did not affect the processing by microsomes. The molecular sizes of these three mutants translated in the presence of microsomal membranes (lanes 5, 6, and 7) were approximately the same as that of the Δpro-TNF mutant, which lacks the entire leader sequence of pro-TNF, translated in the absence of microsomal membranes (lane 1), indicating that the cleavage site was located at, or at least close to, the C-terminus of the 14-residue-leader sequence of this mutant.

Figure 4 compares the translation/translocation pattern of the Δ−75-47, −32-1 mutant with that of the Δ−75-47, −32-1 V1M- and Δ−75-47, −32-1 S5M-TNF mutants. The translocational behavior of the Δ−75-47, −32-1 V1M- and Δ−75-47, −32-1 S5M-TNF, analyzed by [^{3}H]leucine labeling was indistinguishable from that of the Δ−75-47, −32-1-TNF, indicating that the replacement of Val 1 or Ser 5 with Met did not affect the translocational behavior of this mutant. When the incorporation of [^{35}S]-methionine into these two substitution mutants was analyzed by the same assay, the pattern of the labeled proteins was exactly the same as that obtained on [^{3}H]leucine labeling, and [^{35}S]methionine labeling was detected for the processed form of TNF even after treatment with protein-

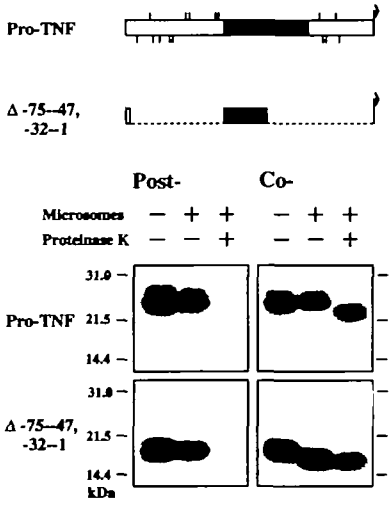

Fig. 2. **Cotranslational translocation of the Δ−75-47, −32-1 mutant across the endoplasmic reticulum membrane.** The mRNAs coding for pro-TNF and the Δ−75-47, −32-1 mutant were translated at 30°C for 90 min with a rabbit reticulocyte lysate in the absence of microsomal membrane and then translation was terminated with cycloheximide (final concentration, 100 μg/ml). Microsomes were then added to the reaction mixture, followed by further incubation at 30°C for 30 min. The membrane translocation was then analyzed as described under "MATERIALS AND METHODS." The symbols for the schematic structures are as described in the legend to Fig. 1. Post-, posttranslational, Co-, cotranslational.

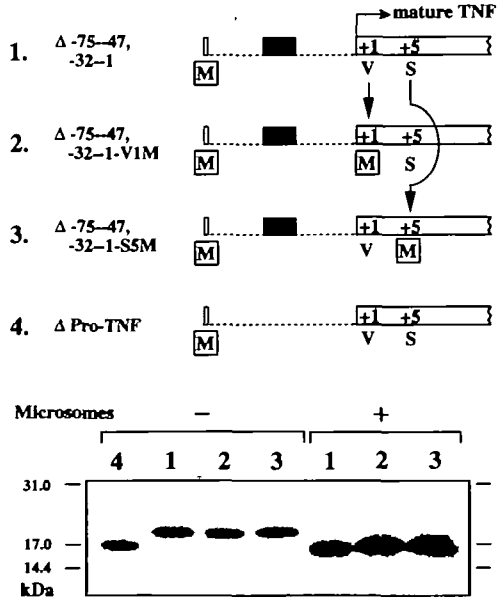

Fig. 3. **Molecular size determination of the microsomal processing product of the Δ−75-47, −32-1, Δ−75-47, −32-1-V1M and Δ−75-47, −32-1-S5M mutanst.** The structures of these mutants are schematically shown. The positions of the methionine residues (M) are shown. The molecular sizes of the *in vitro* translation product and the microsomal processing product of the Δ−75-47, −32-1, Δ−75-47, −32-1-V1M and Δ−75-47, −32-1-S5M mutants are compared with that of the *in vitro* translation product of Δpro-TNF.

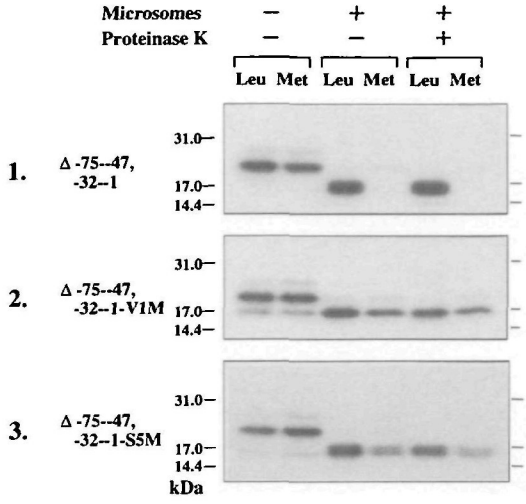

Fig. 4. **Analysis of the cleavage site of the Δ−75-47, −32-1 mutant by metabolic labeling of the microsomal processing products of the Δ−75-47, −32-1, Δ−75-47, −32-1-V1M and Δ−75-47, −32-1-S5M mutants.** The [^{3}H]Leu- or [^{35}S]Met labeled proteins, detected by fluorography after SDS-polyacrylamide gel electrophoresis, among the *in vitro* translation products of mutant pro-TNF mRNAs in the presence (+) or absence (−) of microsomes are shown. The effects of proteinase K digestion of the microsome-dependent translates are also shown.

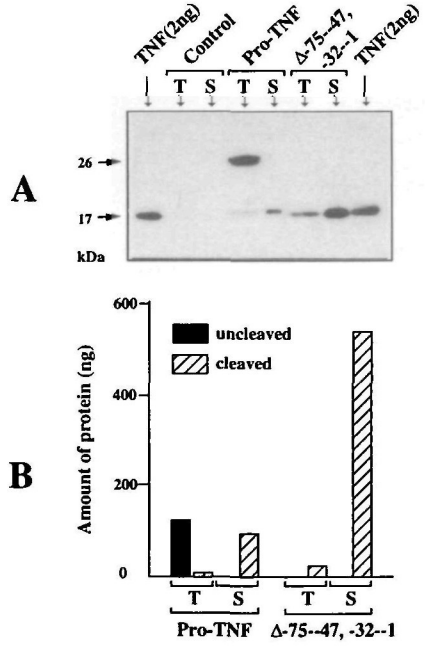

Fig. 5. **Secretion of the secretory form of TNF from COS-1 cells transfected with cDNA coding for the Δ−75-47, −32-1 mutant.** The cDNAs encoding pro-TNF and the Δ−75-47, −32-1 mutant were transfected into COS-1 cells, and then their expression in total cell lysates and their secretion into cell culture supernatants were evaluated by Western blotting analysis (A). T, total cell lysates; S, cell culture supernatants. The amounts of protein present in total cell lysates and in cell culture supernatants of transfected cells 48 h after transfection were determined by Western blotting analysis (B). T, total cell lysates; S, cell culture supernatants.

ase-K. These results strongly indicated that the signal cleavage site was located at, or at least upstream of, the −1/+1 site.

Secretion of a Biologically Active Secretory Form of TNF from COS-1 Cells Transfected with cDNA Coding for the Δ−75-47, −32-1 Mutant—To determine whether or not the N-terminal 14-residues (−46 to −33) of the transmembrane domain of pro-TNF can function as a cleavable signal sequence in intact cells, cDNAs encoding pro-TNF and Δ−75-47, −32-1 mutant were transfected into COS-1 cells, and then their expression and the secretion of TNF were evaluated by Western blotting analysis.

In control COS-1 cells, no protein was detected in total cell lysates or in culture supernatants as shown in Fig. 5A, lanes 2 and 3. When pro-TNF cDNA was transfected into COS-1 cells, a remarkable amount of 26 kDa pro-TNF and a trace amount of a processed form of TNF (17 kDa) were detected in the total cell lysates, as shown in lane 4. In the cell supernatants, only a 17 kDa mature TNF was detected (lane 5). In contrast, total cell lysates and cell supernatants

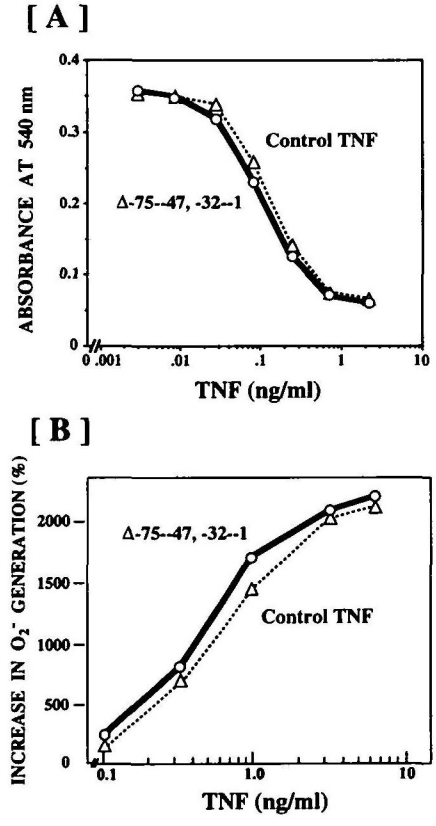

Fig. 6. **Dose-response curves for the cytotoxic activity and neutrophil-priming activity of a cell culture supernatant derived from COS-1 cells transfected with the Δ−75-7, −32-1 mutant cDNA.** A cell culture supernatant of transfected cells was subjected to cytotoxicity assay using actinomycin D-treated L-929 cells. The absorbance of the incorporated neutral red dye was measured at 540 nm and was plotted (A). The neutrophil-priming activity of a cell culture supernatant of transfected cells was measured as described under "MATERIALS AND METHODS." To prime neutrophils, 10^6 cells/ml were preincubated with various concentrations of TNF secreted into the culture supernatant. The dose-response curves for the stimulus-dependent O_2^- generation are expressed as the means of three separate experiments and presented as percentages of the control (B). △, rhTNF; ○, Δ−75-47, −32-1 mutant.

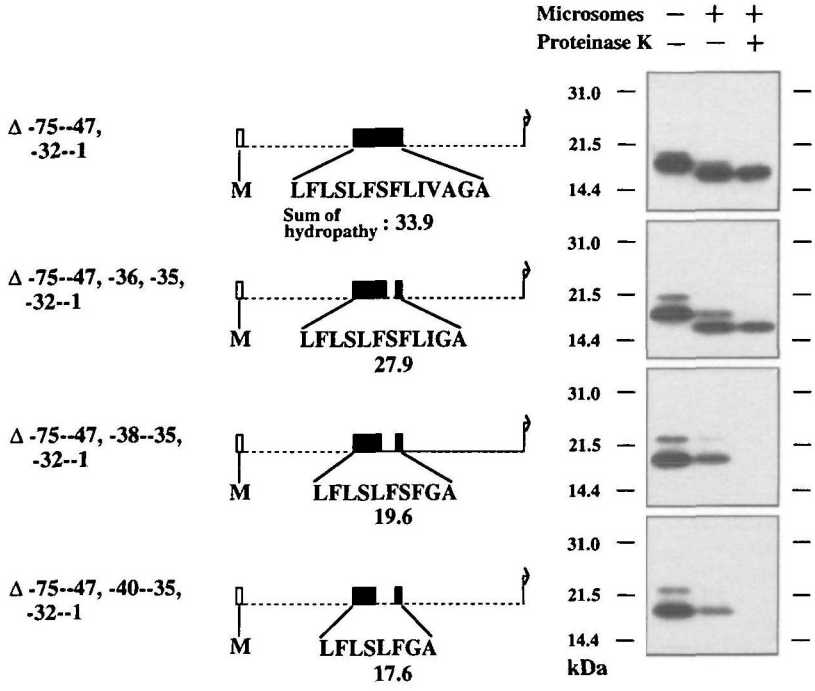

Fig. 7. **The minimum requirement for the hydrophobicity of the N-terminal half of the transmembrane domain for membrane translocation.** The structures of the deletion mutants are schematically shown. The sum of the hydropathy of the hydrophobic domain of each mutant is also shown. The translation/translocation patterns of the Δ−75-47, −36, −35, −32-1, Δ−75-47, −38-35, −32-1, and Δ−75-47, −40-35, −32-1 mutants are compared with that of the Δ−75-47, −32-1 mutant.

derived from COS-1 cells transfected with cDNA coding for the Δ−75-47, −32-1 mutant were found to contain only the 17 kDa form of TNF (lanes 6 and 7). The fact that the 18.5 kDa unprocessed form of the Δ−75-47, −32-1 mutant was not detected in the total cell lysates suggested that this mutant was cotranslationally processed to generate 17 kDa TNF on the ER-membrane. The amount of protein present in the total cell lysates (T) or in the cell supernatants (S) 48 h after transfection was determined by quantitative analysis of immunoreactive proteins using a storage phosphor imaging screen (Bio-Rad), and the results are summarized in Fig. 5B. When compared with pro-TNF-producing cells, Δ−75-47, −32-1 mutant-producing cells yielded approximately 6-fold more secretory TNF per 48 h (Fig. 5B). These results clearly indicated that the N-terminal 14-residues of the transmembrane domain of pro-TNF functioned as a cleavable signal sequence in COS-1 cells and efficiently generated the 17 kDa mature form of TNF.

In order to determine the biological activity of the TNF molecule secreted from COS-1 cells transfected with cDNA coding for the Δ−75-47, −32-1 mutant, cytotoxic activity toward L929 cells and priming activity toward human peripheral neutrophils of the secreted TNF were evaluated. As shown in Fig. 6, A and B, the dose response curves for these two biological activities of native TNF and the TNF molecule found in the supernatants of Δ−75-47, −32-1 mutant-producing cells were comparable.

Thus, secretion of biologically active TNF could be achieved with as few as 14 of the 26 residues of the native transmembrane domain of the 76-residue pro-TNF leader sequence.

Minimum Requirement in the 76-Residue Leader Sequence of pro-TNF for Membrane Translocation across the Endoplasmic Reticulum and Maturation of pro-TNF—To determine the minimum requirement for the hydrophobicity of the N-terminal half of the transmembrane domain

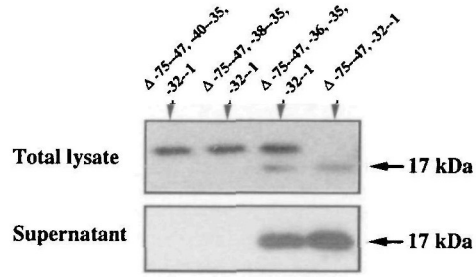

Fig. 8. **Western blotting analysis of total cell lysates and cell culture supernatants derived from COS-1 cells transfected with cDNAs coding for the Δ−75-47, −32-1, Δ−75-47, −36, −35, −32-1, Δ−75-47, −38-35, −32-1, and Δ−75-47, −40-35, −32-1 mutants.** The cDNAs encoding the Δ−75-47, −32-1, Δ−75-47, −36, −35, −32-1 , Δ−75-47, −38-35, −32-1, and Δ−75-47, −40-35, −32-1 mutants were transfected into COS-1 cells, and then their expression in the total cell lysates and their secretion into the cell culture supernatants were evaluated by Western blotting analysis.

for membrane translocation of the Δ−75-47, −32-1 mutant, we constructed a series of deletion mutants with increasing deletions in the −46 to −33 region. The structure and the sum of hydropathy of the leader sequence of these mutants are shown in Fig. 7. Figure 7 compares the translation/translocation patterns of the Δ−75-47, −32-1 mutant with those of the Δ−75-47, −36, −35, −32-1, Δ−75-47, −38-35, −32-1, and Δ−75-47, −40-35, −32-1 mutants. Efficient translocation and effective signal cleavage was observed with the Δ−75-47, −32-1 and Δ−75-47, −36, −35, −32-1 mutants, as judged from the existence of an intense proteinase K-resistant band. In contrast, this proteinase K-resistant band was undetectable in the translocation assay with the Δ−75-47, −38-35, −32-1, and Δ−75-47, −40-35, −32-1 mutants, indicating their inability to be translocated across the

ER-membrane. In accordance with these results, only the $\Delta-75$-47, -32-1 and $\Delta-75$-47, -36, -35, -32-1 mutants, which showed efficient translocation across the ER-membrane, could produce the secreted TNF when transfected into the COS-1 cells, as shown in Fig. 8. These results clearly demonstrated that some extent of hydrophobicity within this region is indispensable for the ER-translocation and maturation of pro-TNF.

DISCUSSION

In this study, in order to determine the minimum requirement in the 76-residue leader sequence of pro-TNF for the membrane translocation across the endoplasmic reticulum (ER) and for the maturation of pro-TNF, we constructed pro-TNF mutants in which a part of the transmembrane domain of pro-TNF was directly linked to the N-terminus of the mature domain, and then evaluated their translocational behavior across the ER-membrane and their secretion from the transfected cells.

As few as 14 of the 26 residues of the native transmembrane domain in the 76-residue pro-TNF leader sequence were found to function as a cleavable signal sequence in both *in vitro* and *in vivo* expression systems, and efficient secretion of biologically active soluble TNF could be achieved with this 14 residue-hydrophobic region.

Signal sequences, whether of prokaryotic or eukaryotic origin, share common structural features in the absence of an apparent amino acid consensus sequence (*15, 16*). The N-terminal end of a signal sequence (N-region) is characterized by its hydrophilicity due to the presence of basic residues. This region is followed by a hydrophobic core (H-region) of some 7-15 amino acids that most often is predicted to exist as an α-helix. The carboxyl terminus (C-region) is generally more polar than the H-region and contains the site for signal peptide cleavage after membrane translocation has been successfully initiated. To facilitate translocation across the ER membrane, it is believed that a signal sequence, and in particular its H-region, interacts with a number of soluble and membrane-bound components of the cellular export machinery (*17, 18*). Extensive mutational analyses in both prokaryotic and eukaryotic H-regions have shown that the H-region is the dominant structure in determining the signal sequence function and that the H-region must exhibit a minimal degree of hydrophobicity to facilitate efficient protein translocation (*19, 20*). Concerning the N-region, mutational analyses have shown that removing these basic amino acid residues has relatively little effect on translocation efficiency. Recent studies indicated that there are at least two steps in the recognition of signal peptides: recognition by signal recognition particle (SRP) in the cytosol and subsequent recognition by the translocational machinery in the ER membrane (*21, 22*). A recent study also revealed that the total hydrophobicity of the H-region is a determinant for recognition by both SRP and the membrane-embedded translocation machinery (*23*). The present finding that a part of the intact transmembrane domain functions as a signal sequence without having any charged residues at the N-terminus is totally consistent with the above mentioned observations. Since it was indicated that signal/signal-anchor sequences with short hydrophobic segments were treated differently by the translocation machinery compared to long ones (*24-26*), the present observation may not simply imply that the 14-residue segment is directly involved in the translocation mechanism of pro-TNF. However, this result still clearly indicated that the N-terminal half of the transmembrane region of pro-TNF by itself can be a sufficient determinant for the membrane translocation of the following portion of the polypeptide without having any positive charge at the N-terminus.

The carboxyl terminal end of the general signal sequence is usually hydrophilic and contains the site for signal peptide cleavage, whereas the leader sequence present in the $\Delta-75$-47, -32-1 mutant is a part of the hydrophobic transmembrane segment having no such hydrophilic residues. It is unclear why the signal peptide cleavage occurred in the $\Delta-75$-47, -32-1 mutant. Analysis of the cleavage site by site-directed mutagenesis indicated the signal cleavage site was located at, or at least upstream of, the $-1/+1$ site. The amino acid sequence of the C-terminus of the leader sequence is Ala^{-3}-Gly^{-2}-Ala^{-1} and the Ala-X-Ala sequence is frequently found upstream of the cleavage site in many secretory proteins. A recent report on the crystal structure of the active site of a bacterial signal peptidase clearly explained the requirement for small residues such as alanine at positions -1 and -3, upstream of the cleavage site (*27*). Therefore, it is reasonable to speculate that the cleavage occurs at the C-terminus of this motif. To determine the exact cleavage site, we are currently determining the N-terminal sequence of the secreted TNF.

The present experimental results clearly demonstrated that the leader sequence of pro-TNF contains a cryptic signal peptide within its transmembrane domain. In intact pro-TNF, however, this sequence could not function as a cleavable signal sequence during intracellular processing. These results indicate that the remainder of the 76-residue leader sequence inhibits the signal peptide cleavage and enables the leader sequence to function as a type II signal-anchor sequence that generates a transmembrane form of TNF. As the signal peptide cleavage was not observed with a mutant $\Delta-32$-1 which contained the cytoplasmic domain of the leader sequence in addition to the N-terminal half of the transmembrane domain, the inhibition of the signal peptide cleavage seems to be mediated by the cytoplasmic domain of the leader sequence. Since selective activation of the p80 TNF receptor by the transmembrane form of TNF through juxtacrine interactions plays an important role in many immunological responses (*4-6*), retention of TNF on the cellular plasma membrane must be a critical step in these responses. Therefore, inhibition of the signal peptide cleavage might be one of the physiological roles of the relatively long (30 residues) cytoplasmic domain of the leader sequence of pro-TNF.

The active form of mature TNF has been determined to be a compact, bell-shaped trimer (*28-30*). Recently, it was revealed that pro-TNF, like the secreted mature TNF, has a trimer structure which is assembled intracellularly on the ER-membrane before transport to the cell surface (*3*). Therefore, it was speculated that the membrane localization mediated by the cotranslational integration into the ER-membrane might facilitate the trimerization of pro-TNF. In fact, the hemagglutinin (HA) of influenza virus, a single membrane-spanning protein with the same "jelly roll" structure as TNF, forms a trimer on the ER-membrane within a few minutes of its synthesis before the

protein leaves the ER (*31, 32*). The trimer is stabilized through interactions between the exoplasmic domain of the constituent polypeptides as well as through interactions between the three cytoplasmic and membrane-spanning domains. However, the present results revealed that the biologically active (probably trimeric) TNF could be secreted without membrane localization on the ER-membrane. These results clearly indicated that the membrane localization mediated by the 76-residue leader sequence of pro-TNF is not a prerequisite for the formation of the biologically active trimeric form of TNF.

REFERENCES

1. Vilcek, J. and Lee, T.H. (1991) Tumor necrosis factor. New insights into the molecular mechanisms of its multiple actions. *J. Biol. Chem.* **266**, 7313-7316
2. Kriegler, M., Perez, C., DeFay, K., Albert, I., and Lu, S.D. (1988) A novel form of TNF/Cachectin is a cell surface cytotoxic transmembrane protein: Ramifications for the complex physiology of TNF. *Cell* **53**, 45-53
3. Tang, P., Hung, M-C., and Klostergaard, J. (1996) Human pro-tumor necrosis factor is a homotrimer. *Biochemistry* **35**, 8216-8225
4. Perez, C., Albert, I., DeFay, K., Zachariades, N., Gooding, L., and Kriegler, M. (1990) A nonsecretable cell surface mutant of tumor necrosis factor (TNF) kills by cell-to-cell contact. *Cell* **63**, 251-258
5. Grell, M., Douni, E., Wajant, H., Löhden, M., Clauss, M., Maxeiner, B., Georgopoulos, S., Lesslauer, W., Kollias, G., Pfizenmaier, K., and Scheurich, P. (1995) The transmembrane form of tumor necrosis factor is the prime activating ligand of the 80 kDa tumor necrosis factor receptor. *Cell* **83**, 793-802
6. Herbein, G., Mahlknecht, U., Batliwalla, F., Gregersen, P., Pappas, T., Butler, J., O'Brien, W.A., and Verdin, E. (1998) Apoptosis of $CD8^+$ T cells is mediated by macrophages through interaction of HIV gp 120 with chemokine receptor CXCR4. *Nature* **395**, 189-194
7. Black, R.A., Rauch, C.T., Kozlosky, C.J., Peschon, J.J., Slack, J.L., Wolson, M.F., Castner, B.J., Stocking, K.L., Reddy, P., Srinivasan, S., Nelson, N., Boiani, N., Schooley, K.A., Gerhart, M., Davis, R., Fitzner, J.N., Johnson, R.S., Paxton, R.J., March, C.J., and Cerretti, D.P. (1997) A metalloproteinase disintegrin that releases tumor necrosis factor-α from cells. *Nature* **385**, 729-733
8. Moss, M.L., Jin, S.-L.C., Milla, M.E., Burkhart, W., Carter, H.L., Chen, W.J., Clay, W.C., Didsburry, J.R., Hassler, D., Hoffman, C.R., Kost, T.A., Lambert, M.H., Leesnitzer, M.A., McCauley, P., McGeehan, G., Mitchell, J., Moyer, M., Pahel, G., Rocque, W., Overton, L.K., Schoenen, F., Seaton, T., Su, J.-L., Warner, J., Willard, D., and Becherer, J.D. (1997) Cloning of a disintegrin metalloproteinase that processes precursor tumor-necrosis factor-α. *Nature* **385**, 733-736
9. Utsumi, T., Levitan, A., Hung, M-C., and Klostergaard, J. (1993) Effect of truncation of human pro-tumor necrosis factor transmembrane domain on cellular targeting. *J. Biol. Chem.* **268**, 9511-9516
10. Utsumi, T., Akimaru, K., Kawabata, Z., Levitan, A., Tokunaga, T., Tang, P., Ide, A., Hung, M-C., and Klostergaard, J. (1995) Human pro-tumor necrosis factor: Molecular determinants of membrane translocation, sorting, and maturation. *Mol. Cell. Biol.* **15**, 6398-6405
11. Utsumi, T., Kuranami, J., Tou, E., Ide, A., Akimaru, K., Hung, M-C., and Klostergaard, J. (1996) *In vitro* synthesis of an N-myristoylated fusion protein that binds to the liposomal surface. *Arch. Biochem. Biophys.* **326**, 179-184
12. Sanger, F., Nicklen, S., and Coulson, A.R. (1977) DNA sequencing with chain-terminating inhibitors. *Proc. Natl. Acad. Sci. USA* **74**, 5463-5467
13. Laemmli, U.K. (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **22**, 680-685
14. Utsumi, T., Klostergaard, J., Akimaru, K., Edashige, K., Sato, E.F., and Utsumi, K. (1992) Modulation of TNF-α-priming and stimulation-dependent superoxide generation in human neutrophils by protein kinase inhibitors. *Arch. Biochem. Biophys.* **294**, 271-278
15. von Heijne, G. (1985) Signal sequences. The limits of variation. *J. Mol. Biol.* **184**, 99-105
16. von Heijne, G. (1990) The signal peptide. *J. Membr. Biol.* **115**, 195-201
17. Bieker, K.L., Phillips, G.J., and Silhavy, T.J. (1990) The *sec* and *prl* genes of *Escherichia coli*. *J. Bioenerg. Biomembr.* **22**, 291-310
18. Rapoport, T.A. (1992) Transport of protein across the endoplasmic reticulum membrane. *Science* **258**, 931-936
19. Gennity, J., Goldstein, J., and Inouye, M. (1990) Signal peptide mutants of *Escherichia coli*. *J. Bioenerg. Biomembr.* **22**, 233-269
20. Bird, P., Gething, M.-J., and Sambrook, J. (1990) The functional efficiency of a mammalian signal peptide is directly related to its hydrophobicity. *J. Biol. Chem.* **265**, 8420-8425
21. Jungnickel, B. and Rapoport, T.A. (1995) A posttargeting signal sequence recognition event in the endoplasmic reticulum membrane. *Cell* **82**, 261-270
22. Belin, D., Bost, S., Vassalli, J.-D., and Strub, K. (1996) A two-step recognition of signal sequences determines the translocation efficiency of proteins. *EMBO J.* **15**, 468-478
23. Hatsuzawa, K., Tagaya, M., and Mizushima, S. (1997) The hydrophobic region of signal peptide is a determinant for SRP recognition and protein translocation across the ER membrane. *J. Biochem.* **121**, 270-277
24. Sato, T., Sakaguchi, M., Mihara, K., and Omura, T. (1990) The amino-terminal structures that determine topological orientation of cytochrome P-450 in microsomal membrane. *EMBO J.* **9**, 2391-2397
25. Sakaguchi, M., Tomiyoshi, R., Kuroiwa, T., Mihara, K., and Omura, T. (1992) Functions of signal and signal-anchor sequences are determined by the balance between the hydrophobic segment and the N-terminal charge. *Proc. Natl. Acad. Sci. USA* **89**, 16-19
26. Nilsson, I., Whitley, P., and von Heijne, G. (1994) The COOH-terminal ends of internal signal and signal-anchor sequences are positioned differently in the ER translocase. *J. Cell Biol.* **126**, 1127-1132
27. Paetzel, M., Dalbey, R.E., and Strynadka, N.C.J. (1998) Crystal structure of a bacterial signal peptidase in complex with a β-lactam inhibitor. *Nature* **396**, 186-190
28. Wingfield, P., Pain, R.H., and Craig, S. (1987) Tumor necrosis factor is a compact trimer. *FEBS Lett.* **221**, 179-184
29. Jones, E.Y., Stuart, D.I., and Walker, N.P.C. (1989) Structure of tumor necrosis factor. *Nature* **338**, 225-228
30. Eck, M.J., Ultsch, M., Rinderknecht, E., de Vos, A.M., and Sprang, S.R. (1992) The structure of human lymphotoxin (tumor necrosis factor-β) at 1.9-Å resolution. *J. Biol. Chem.* **267**, 2119-2122
31. Gething, M.J., McCammon, K., and Sambrook, J. (1986) Expression of wild-type and mutant forms of influenza hemagglutinin: The role of folding in intracellular transport. *Cell* **46**, 939-950
32. Braakman, I., Hoover-Litty, H., Wagner, K.R., and Helenius, A. (1991) Folding of influenza hemagglutinin in the endoplasmic reticulum. *J. Cell Biol.* **114**, 401-411