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Human Pro-Tumor Necrosis Factor: Molecular Determinants of Membrane Translocation, Sorting, and Maturation

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Human pro-tumor necrosis factor (pro-TNF) is a type II transmembrane protein with a highly conserved 76-residue leader sequence. We have analyzed the behavior, both in a microsomal translocational system and by transfection, of a series of mutants with deletions from the cytoplasmic, transmembrane, and linking domains. Cytoplasmic deletions included the Arg doublet at −49 and −48 and/or the Lys doublet at −58 and −57; additional mutants included deletion of residues −73 to −55 and −73 to −55, −49, and −48. The transmembrane and linking domain mutants included deletions in the −42 to −35 region, combined with the deletion of residues −32 to −1. Two hybrid mutants combined the cytoplasmic deletions with the deletion of residues −32 to −1. All of the cytoplasmic deletion mutants were properly translocated, as were the transmembrane deletion mutants with deletions up to residues −36, −35, −32 to −1, although the last one exhibited reduced efficiency; further incremental deletions, including deletions of residues −38 to −35 and −32 to −1, completely blocked translocation. Both hybrid mutants were effectively translocated; furthermore, transfection analysis revealed competent expression and maturation of both the cytoplasmic and hybrid mutants. Thus, proper expression and maturation of human pro-TNF can be accomplished with as few as ~12 of the 26 residues of the native transmembrane domain and with a net negative charge in the cytoplasmic domain flanking the transmembrane region.

Tumor necrosis factor (TNF) is an extremely pleiotropic proinflammatory cytokine. It is initially synthesized and expressed as a functional transmembrane prohormone with a type II orientation (12, 16). The pathophysiological effects of TNF may be mediated by juxtacrine interactions of the prohormone and/or autocrine-paracrine interactions of the mature, secreted molecule. The pro-TNF leader sequence does not serve as a classical signal sequence during processing and intracellular targeting. Indeed, the role of this leader sequence is poorly understood.

In human pro-TNF, the 76-residue leader sequence entails a linking domain from Gly-20 to Ala-1, a hydrophobic transmembrane domain from Leu-46 to Ile-21, as well as a cytoplasmic domain from Met-76 to Cys-47. We have previously shown that the entire linking domain and the periexoplasmic half of the transmembrane domain could be deleted and that, under these circumstances, membrane targeting and orientation and maturation mechanisms were still intact (3). However, deletion of residues −55 to −34 resulted in blocked translocation and secretory mechanisms. This result was rationalized on the basis of inadequate hydrophobicity of the remaining transmembrane domain (16, 26). However, since the latter mutant also lacked the Arg-49–Arg-48 doublet, a role for cytoplasmic positive charge in effective translocation of human pro-TNF might also exist.

In eukaryotic cells, the orientation of transmembrane proteins is determined by topogenic sequences (3, 22, 27, 32). Frequently, an internal uncleaved signal sequence-membrane anchor sequence directs the cotranslational transmembrane insertion and initiates this via binding to the endoplasmic reticulum membrane (reviewed in reference 21). The topogenic sequences include a hydrophobic domain of ~19 to 22 residues (1, 5, 6, 25, 28), which spans the membrane, and the flanking regions, ~15 residues in length, on either side of the transmembrane domain (8). For naturally occurring transmembrane proteins surveyed with a cytoplasmic amino terminus and exoplasmic carboxy terminus (N_{cyt} C_{exo} orientation; type II, as for human pro-TNF), ~90% have a net positive cytoplasmic charge in the 15-residue transmembrane-flanking region of the nontranslocated amino terminus (8). The least frequently observed transmembrane protein is the type III protein, which also has an uncleaved signal sequence and is in the N_{exo} C_{cyt} orientation, typified by the cytochrome P-450 proteins (17, 23).

Two hypotheses have been proposed to explain this orientation of type II and III proteins. The "charge difference" rule (8) states that when the net differences in the sums of positive and negative charges in the flanking regions are determined, the cytoplasmic side is the more positive. The "positive inside" rule (30, 31) states that the determinants are the positive charges alone and that the cytoplasmic side will have the greater number. In human pro-TNF, a net charge of +4, as well as four positive residues, exist in the 15-residue cytoplasmic flanking region, whereas two positive charges and a net charge of −1 exist in the 15-residue exoplasmic flanking region, consistent with both rules (26).

However, a systematic mutational analysis of two other type II proteins, the asialoglycoprotein receptor H1 and the paramyxovirus HN polypeptide, have failed to support either the charge difference or the positive inside rule. Beltzer and coworkers evaluated the influence of charged residues in the signal-anchor sequence of the asialoglycoprotein receptor H1 (2). They determined that both the cytoplasmic charged residues and the amino-terminal domain influenced orientation;

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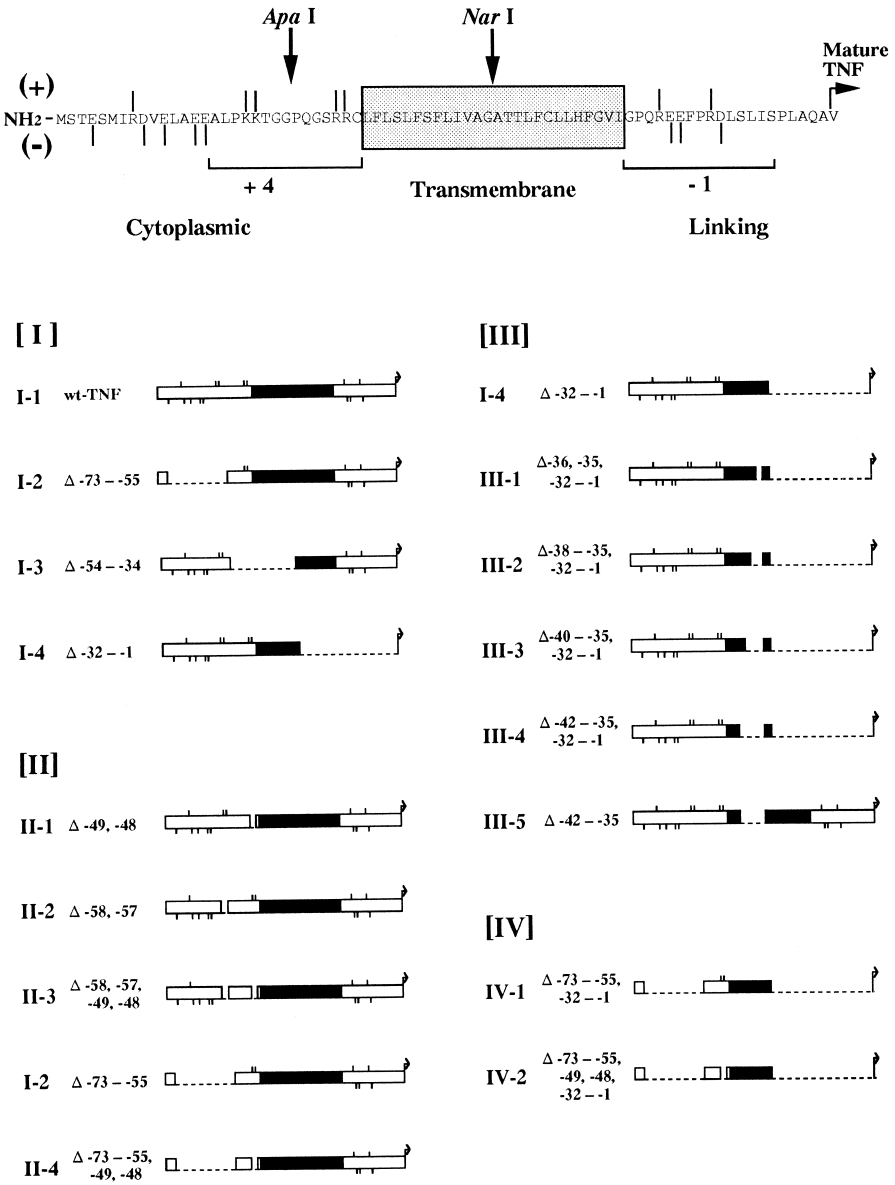

FIG. 1. Structures of parental and mutant pro-TNFs. (Top) Pro-hormone sequence of parental pro-TNF shown in one-letter code, with positively charged (vertical lines above sequence) and negatively charged (vertical lines below sequence) residues indicated. Stippled area indicates hydrophobic transmembrane domain from Leu-46 to Ile-21. A linking domain of 20 residues connects the transmembrane and mature domains. The net charges of the 15-residue sequences flanking the transmembrane domain are shown. *Apa*I and *Nar*I sites used for mutant construction are indicated by arrows. (Bottom) Schematic structures of parental (wt-TNF) and mutant pro-TNFs, with deleted regions shown as dashed lines and the transmembrane domain shown as a solid bar. I, mutants to roughly localize the region indispensable for translocation of pro-TNF; II, mutants to define the role of cytoplasmic charged residues; III, mutants to define the essential hydropathy of the transmembrane domain; IV, hybrid mutants.

when reversed-charge gradient mutants were constructed, only when the amino-terminal domain was also truncated did efficient reversal of membrane orientation occur in COS-7 cells. Furthermore, Parks and Lamb (18) have shown that the addition of positive charges to the C_{exo} region flanking the transmembrane domain of the paramyxovirus HN polypeptide did not significantly affect topology.

Other studies have provided insight into the mechanism of signal sequence-dependent translocation of proteins across membranes. Hikita and Mizushima (9, 10) and Sasaki et al. (24) have developed and characterized model bacterial presecretory proteins to evaluate the role of amino-terminal positive charge and hydrophobicity in their signal sequences. They observed that the functions of these two determinants might be coexpressed, as in mammalian systems (23). Positive charges were strongly required when the length of the hydrophobic domain was minimal, whereas they were dispensable when this domain was optimal. In their studies, the hydrophobic domain was composed of poly(Leu) or poly(AlaLeu) sequences. Translocation rates were optimal with sequences 8 to 10 residues in length, far shorter than the 19 to 22 residues usually found in natural presecretory proteins; however, as few as 6 highly hydrophobic residues in a eukaryotic protein have been reported (20) and as few as 7 to 10 Leu residues have been reported for a natural signal peptide (23). Whether such coordinated influences exist for native signal-anchor sequences remains to be

TABLE 1. Charge distribution of transmembrane domain flanking regions of pro-TNF deletion mutants

Mutant no.	Deleted region	N-terminal flanking charge	C-terminal flanking charge	N−C[a]
I-1	None (wt-TNF)[b]	+4	−1	+5
I-2	−73 to −55	+2	−1	+3
I-3	−54 to −34	−1	−1	0
I-4	−32 to −1	+4	+2	+2
II-1	−49,−48	0	−1	+1
II-2	−58,−57	0	−1	+1
II-3	−58,−57,−49,−48	−2	−1	−1
II-4	−73 to −55,−49,−48	0	−1	+1
III-1	−36,−35,−32 to −1	+4	+2	+2
III-2	−38 to −35,−32 to −1	+4	+2	+2
III-3	−40 to −35,−32 to −1	+4	+2	+2
III-4	−42 to −35,−32 to −1	+4	+2	+2
III-5	−42 to −35	+4	−1	+5
IV-1	−73 to −55,−32 to −1	+2	+2	0
IV-2	−73 to −55,−49,−48,−32 to −1	0	+2	−2

[a] N and C, charges of the N-terminal and C-terminal flanking regions, respectively.
[b] wt-TNF, pro-TNF.

TABLE 2. Hydropathy of transmembrane domains of pro-TNF deletion mutants

Mutant no.	Deleted region	Sum of hydropathy	No. of amino acid residues	Average hydropathy
I-1	None (wt-TNF)[a]	57.1	26	2.2
I-2	−73 to −55	57.1	26	2.2
I-3	−54 to −34	25.0	13	1.92
I-4	−32 to −1	38.1	15	2.54
II-1	−49,−48	57.1	26	2.2
II-2	−58,−57	57.1	26	2.2
II-3	−58,−57,−49,−48	57.1	26	2.2
II-4	−73 to −55,−49,−48	57.1	26	2.2
III-1	−36,−35,−32-−1	32.1	13	2.47
III-2	−38 to −35,−32 to −1	23.8	11	2.16
III-3	−40 to −35,−32 to −1	21.8	9	2.42
III-4	−42 to −35,−32 to −1	15.2	7	2.17
III-5	−42 to −35	34.2	18	1.9
IV-1	−73 to −55,−32 to −1	38.1	15	2.54
IV-2	−73 to −55,−49,−48,−32 to −1	38.1	15	2.54

[a] wt-TNF, pro-TNF.

established; human pro-TNF presents an opportunity to examine this question.

In our previous study of transmembrane deletion mutants of human pro-TNF, we also deleted proximal aspects of either flanking region (26). Because of the incomplete and, in some cases, apparently conflicting results from studies of either model proteins, native proteins, or their mutants with respect to the relative roles of the transmembrane domain and its flanking regions, we have herein evaluated the effects of cytoplasmic, transmembrane, and linking domain deletions on translocational behavior and transfection of human pro-TNF.

MATERIALS AND METHODS

The domain structure, the charge distributions of the domains flanking the transmembrane domains, and the net hydropathy (13) of the transmembrane domains of the pro-TNFs encoded by the parental and mutant TNF plasmids are shown in Fig. 1 and Tables 1 and 2, respectively.

Plasmid construction. Since unique *Apa*I and *Nar*I sites are located in the cytoplasmic and transmembrane domains of the pro-TNF cDNA, respectively, we utilized PCR to construct several sets of deletion mutants. A pBluescript

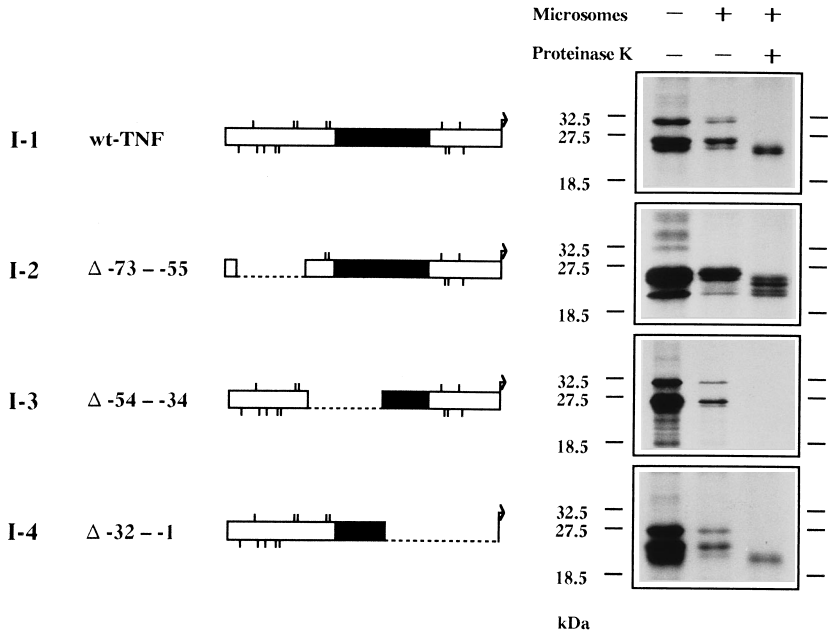

FIG. 2. In vitro translocation of parental pro-TNF (wt-TNF) and deletion mutants (group I mutants). [^{35}S]Cys-labeled proteins, detected by fluorography after SDS-polyacrylamide gel electrophoresis, from in vitro translation of pro-TNF mRNAs in the presence (+) or in the absence (−) of microsomes are shown. The effects of proteinase K digestion of the microsome-dependent translates are shown. Molecular mass markers (in kilodaltons) are indicated. The symbols in the schematic structures are as described in the legend to Fig. 1.

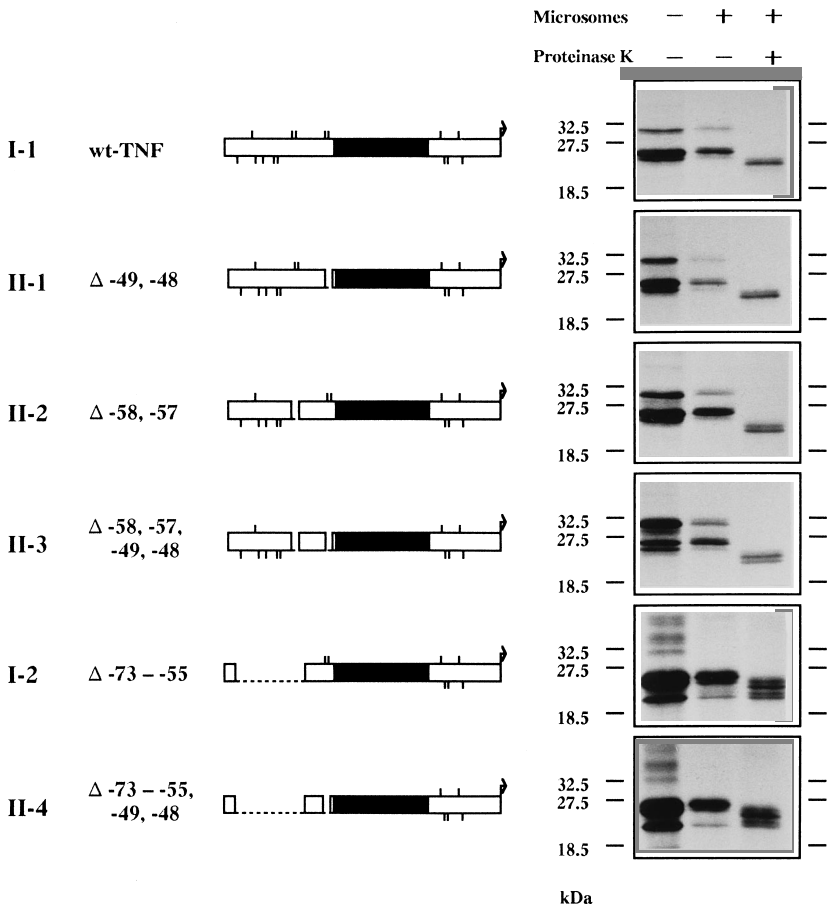

FIG. 3. In vitro translocation of parental pro-TNF (wt-TNF) and cytoplasmic charge deletion mutants (group II mutants). The conditions used are described in the legend to Fig. 2. The symbols in the schematic structures are as described in the legend to Fig. 1.

SK(+) vector lacking *Apa*I and *Hin*dIII sites [pB(-AH)] was first constructed by digesting pBluescript SK(+) (Stratagene) with *Apa*I and *Hin*dIII, blunt-ending with mung bean nuclease, and ligating with T4 ligase. A 1.1-kb *Pst*I fragment of cDNA encoding full-length human pro-TNF or a 1.0-kb *Pst*I fragment of TNF OL-1 cDNA (26) was subcloned into the *Pst*I site of pB(-AH). In these procedures, pro-TNF cDNA or TNF OL-1 cDNA (only for mutant Δ−32-−1 [12]) subcloned into pB(-AH) served as a template for PCR in which the primer bearing the mutated sequence was incorporated into the amplified product. The presence of the same restriction site in the mutagenic primer and in the template DNA permitted direct replacement of a wild-type DNA segment with the mutated segment. The double and triple deletion mutants II-3, II-4, III-1, III-2, III-3, III-4, IV-1, and IV-2 were constructed by replacement of the mutated region of one mutant with the corresponding region of the other mutant.

DNA sequencing was used to confirm the structures of all the mutants.

In vitro transcription and translation. Methods essentially identical to those described previously were employed (26). T3 polymerase was used to obtain transcripts of these cDNAs subcloned into pB(-AH). These were purified by phenol-chloroform extraction and ethanol precipitation before use in the translation reaction.

A canine pancreatic microsomal system using rabbit reticulocyte lysate kits deficient in a selected amino acid (Promega) was used to characterize endoplasmic processing of these transcripts. As previously reported (12, 26), a metabolically labeled (with [^{35}S]Cys) 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 also permeabilized with Triton X-100. In the present study, 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 detergent; a diminished signal was interpreted as indicating reduced translocation or translocation with mixed orientation.

Transfection of COS-7 cells. The simian virus 40-transformed African green monkey cell line, COS-7, was maintained in Dulbecco modified Eagle medium-F12 (GIBCO BRL, Gaithersburg, Md.) supplemented with 10% fetal calf serum (GIBCO BRL). Cells (2×10^6) were plated into 100-mm-diameter dishes 1 day before transfection. pcDNAI (8 μg; Invitrogen, San Diego, Calif.) containing either wild-type or mutant TNF cDNA was used to transfect each plate of COS-7 cells along with 24 μl of LipofectAmine (2 mg/ml; GIBCO BRL) in 4 ml of serum-free medium. After incubation for 5 h at 37°C, the cells were refed with serum-containing medium and incubated for another 24 h. The supernatants were frozen, and the cells were collected with cell scrapers, lysed with radioimmunoprecipitation assay buffer (50 mM Tris-HCl [pH 7.5], 150 mM NaCl, 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulfate [SDS], protease inhibitors), and stored at −70°C. The 24-h time point was selected on the basis of preliminary experiments which indicated a high flux of pro-TNF synthesis and that maturation was underway (data not shown).

Western blotting (immunoblotting). Seventy-five micrograms of total protein of cell lysates from each group of transfected cells was resolved on an SDS–12.5% polyacrylamide gel and then transferred to an Immobilon-P Transfer membrane (Millipore, Bedford, Mass.). The membrane was pretreated for 1 h with 4% nonfat milk in Dulbecco's phosphate-buffered saline (DPBS) containing 0.1% Tween 20, blotted with 0.5 μg of purified goat anti-recombinant human TNF immunoglobulin G (R & D Systems, Minneapolis, Minn.) per ml overnight at 4°C, washed three times with DPBS containing 0.1% Tween 20, and then blotted with secondary antibody (purified rabbit horseradish peroxidase-coupled immunoglobulin G raised against goat immunoglobulin G, 1.5 μg/ml, 1:2,000 dilution; Zymed, South San Francisco, Calif.). The membrane was developed with ECL Western blotting reagent (Amersham, Arlington Heights, Ill.) and exposed as needed to X-ray film. Discrimination between nonspecific and TNF-specific bands was achieved by incubation of the first antibody in the absence or in the presence of 8 μg of free recombinant human TNF (Genentech, South San Francisco, Calif.) per ml.

TNF cytotoxicity assay. The mouse L929 fibrosarcoma cell line, sensitized with actinomycin D, was used to assay for TNF secreted into transfected cell supernatants collected 24 h after transfection, as previously described (26).

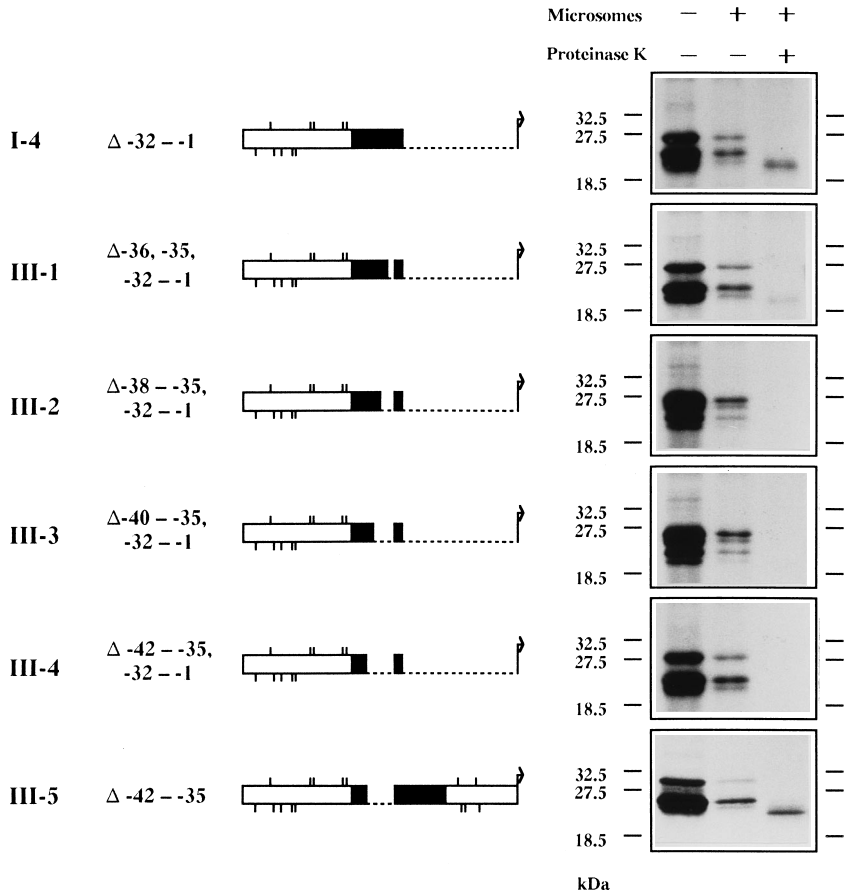

FIG. 4. In vitro translocation of transmembrane and linking domain deletion mutants (group III mutants). The conditions used are described in the legend to Fig. 2. The symbols in the schematic structures are as described in the legend to Fig. 1.

RESULTS

The results shown in Fig. 2 compare the translation-translocation pattern of the parental pro-TNF with those of the Δ−73-−55, Δ−54-−34, and Δ−32-−1 mutants. Consistent with our previous results (26), retention of the pericytoplasmic half of the transmembrane domain (residues −46 to −33), even with deletion of the entire linking domain (residues −20 to −1), resulted in proper targeting and orientation of the

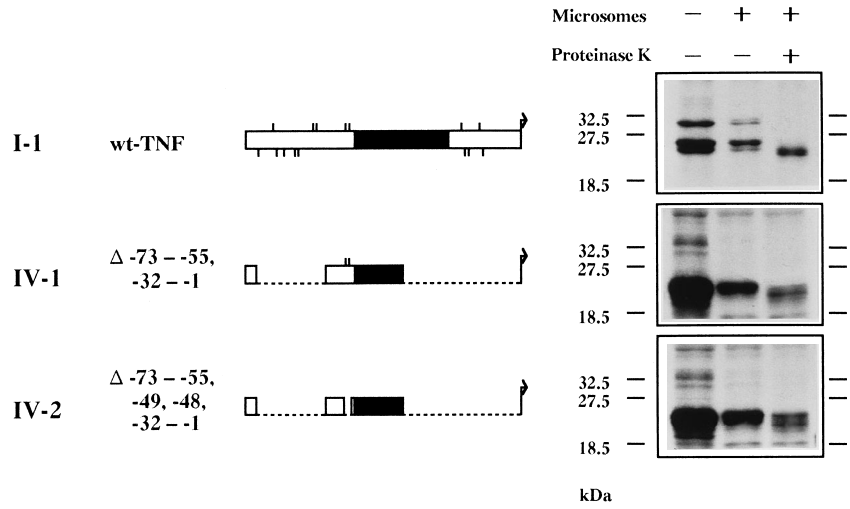

FIG. 5. In vitro translocation of parental pro-TNF (wt-TNF) and hybrid mutants (group IV mutants). The conditions used are described in the legend to Fig. 2. The symbols in the schematic structures are as described in the legend to Fig. 1.

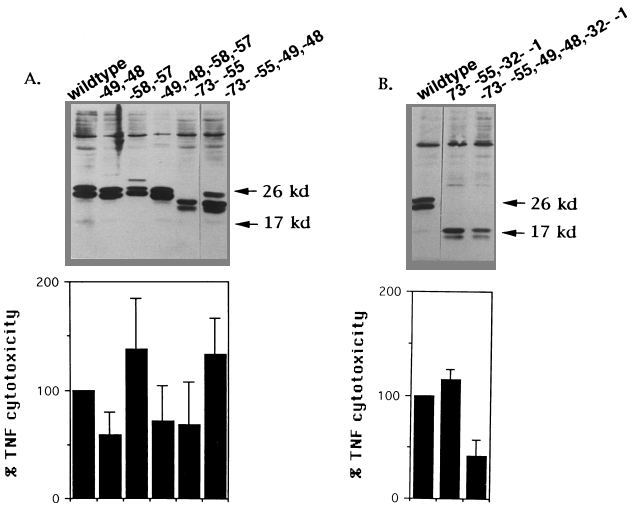

FIG. 6. Western analysis of cell lysates and bioassay of cell supernatants derived from COS-7 cells transfected with mutant pro-TNF cDNAs. (A) Cytoplasmic deletion mutants; (B) hybrid deletion mutants.

pro-TNF mutant Δ−32-−1. Elimination of this half of the transmembrane domain along with 8 flanking residues (mutant Δ−54-−34) blocked proper translocation. Deletion of a similar length of the cytoplasmic domain had no effect (mutant Δ−73-−55).

Cytoplasmic charged residues were incrementally deleted from the parental pro-TNF, beginning with the Arg-49–Arg-48 doublet, because of its proximity to the transmembrane domain and the reported greater influence of Arg residues compared with Lys residues in determining orientation (31). Neither elimination of this doublet, elimination of the Lys-58–Lys-57 doublet, nor elimination of both doublets influenced translocation (Fig. 3). As noted above, the mutant Δ−73-−55 was properly targeted and oriented, and elimination of all of the charged residues and ~70% of the cytoplasmic domain (mutant Δ−73-−55,−49,−48) still allowed proper translocation.

The role of the pericytoplasmic half of the transmembrane domain was further evaluated by an additional deletion in this region. Whereas, as noted before, the translocational behavior of the Δ−32-−1 mutant was essentially indistinguishable from that of the parental pro-TNF, the mutant Δ−36,−35,−32-−1 exhibited diminished translocation, at least in the proper orientation, as determined by the reduced intensity of the proteinase K-resistant band (Fig. 4). The corresponding bands were undetectable in the translocation assays with the Δ−38-−35,−32-−1; Δ−40-−35,−32-−1; and Δ−42−35,−32-−1 mutants. However, the Δ−42-−35 mutant was properly translocated, indicating that structural determinants critical to translocation were not located in this region.

To evaluate possible coordinate roles of the cytoplasmic charges and the pericytoplasmic half of the transmembrane domain in translocation, the behavior of the mutants Δ−73-−55,−32-−1 and Δ−73-−55,−49,−48,−32-−1 was evaluated. As shown in Fig. 5, both of these mutants exhibited proper targeting and orientation in the endoplasmic reticulum membrane. Therefore, residues −46 to −33 of the transmembrane domain still constituted an adequate determinant for translocation despite elimination of all cytoplasmic charge, thereby nullifying or even inverting the charge gradient (Table 1), elimination of the entire linking domain (residues −20 to −1), and elimination of ~70% of the cytoplasmic domain.

The cytoplasmic domain deletion mutants were also analyzed by transfection in COS-7 cells. Figure 6A shows both the results of a Western blotting analysis of the lysates of COS-7 cells transfected with the series of mutants as well as a bioassay for secreted TNF found in the cell supernatants 24 h after transfection. The Western analysis and the bioassay data indicated that, compared with the case for the wild type, competent expression and maturation occurred for all of these cytoplasmic domain deletion mutants, including mutant II-3, which has a negative charge gradient, and mutant II-4, which retained only ~30% of the cytoplasmic domain.

The two hybrid deletion mutants were also subjected to transfection analysis. Figure 6B shows the Western blot of the transfected cell lysates, which reveals that these mutants were competently expressed in COS-7 cells compared with the parental pro-TNF. Similarly, sorting and proteolytic events resulting in the secretion of mature TNF were largely unaffected by these extensive deletions in the cytoplasmic, transmembrane, and linking domains, since the bioassays revealed minimal differences in the levels of cell-lytic activity in the supernatants compared with those for the parental prohormone (Fig. 6B).

DISCUSSION

The orientation of naturally occurring transmembrane proteins in the plasma membrane has been empirically observed to be an orientation with a positively charged cytoplasmic domain, with rare exceptions (8); this has led to the formulation of the so-called charge difference and positive inside rules (8, 30, 31). Because of the high level of interspecies sequence homology of the pro-TNF leader sequence (4), we directly assessed the role of flanking charge distribution in the orientation of the transmembrane domain.

We found that the positively charged residues in the flanking cytoplasmic domain 15 residues upstream from the transmembrane domain of pro-TNF were not critical to proper membrane translocation. This was apparent with the successfully translocated mutant Δ−58,−57,−49,−48 (II-3 [Fig. 3]) which has a net negative charge gradient from cytoplasm to exoplasm (Table 1). Furthermore, inversion of this charge gradient influenced neither sorting to the plasma membrane nor the subsequent proteolytic processing in intact cells; the supernatant levels of mature TNF detected for this mutant (II-3) as well as for the others in this series were comparable to those of the parental pro-TNF (Fig. 6). These results also obviate a preferential effect of charge proximity to the transmembrane domain, such as the Lys residues at positions −58 and −57 (mutant II-2) compared with the Arg residues at positions −49 and −48 (mutant II-1), on influencing the orientation and processing in these systems. This is in direct contrast to the effects of the cytoplasmic Arg residues on the orientation of another type II transmembrane protein, paramyxovirus HN protein (19). In the latter system, the Arg residues were systematically converted to Gln or Glu residues. These investigators determined that substitution of any of the Arg residues, and in particular the one closest to the signal-anchor domain, caused inversion of orientation to that of type III; replacement with Glu had a greater effect on inversion than did replacement with Gln.

In fact, translocation and ultimately maturation of human pro-TNF proceeded in the absence of any cytoplasmic charged residues and without ~70% of the cytoplasmic domain (Fig. 3 and 6; mutant Δ−73-−55,−49,−48 [II-4]). Thus, for pro-TNF, electrostatic interaction between membrane components on the cytoplasmic surface of the endoplasmic reticulum mem-

brane and N-terminal positive charges is not an obligatory prelude to translocation of the C-terminal portion, including the linking domain and the entire mature domain, as has been proposed for other systems (reviewed in reference 21).

This mutant (II-4) has a 19-residue deletion near its amino terminus; it also lacks charged residues. Although our approach and that of Beltzer and coworkers in their studies of the asialoglycoprotein receptor H1 (2) are not identical, we did not observe a coordinate influence of the amino terminus and the charge gradient on orientation. Moreover, a subsequent proteolytic event(s), which was not operant in the receptor model, was unperturbed here. It remains possible that inversion of orientation would occur if we truncated even more of the amino terminus and introduced positively charged residues to the exoplasmic flank of the transmembrane domain. However, even the present results focus future attention on the as-yet-unknown bases for both the existence of the amino-terminal positive charges and the structural conservation among species of the cytoplasmic domain of the pro-TNF leader sequence (4).

Translocation of human pro-TNF does require a minimum hydrophobic character for the transmembrane domain, which is nominally expressed in mutant $\Delta-36,-35,-32--1$; this mutant translocates somewhat less efficiently than the $\Delta-32--1$ mutant, which in turn translocates as efficiently as the native pro-TNF (Fig. 4). Its total hydropathy of 32.1 (III-1 [Table 2]) is similar to that of the hydrophobic domains of model signal sequences of the OmpF-LPP mutants which demonstrate optimal translocation rates (8, 9, 21). The length of the transmembrane domain of mutant III-1 (~12 residues of original sequence) is slightly longer than that determined to be optimal (8 to 10) in the presecretory models from the laboratory of Mizushima and coworkers (9, 10, 24) or in the cytochrome P-450-type signal-anchor sequences (23); perhaps this is attributable to the highly hydrophobic nature of the poly(Leu) or poly(AlaLeu) oligomers. However, it is substantially shorter than the ~19 or greater residues typically found for naturally occurring transmembrane proteins (1, 5, 6, 25, 28), which may reflect either the high average hydropathic value per residue (2.47 [Table 2]) or a favorable conformation(s) of this region which serves to maximize interaction energy with the lipid bilayer. On the other hand, there was no evidence for a critical transmembrane determinant required for translocation. This is perhaps not surprising, since there exists little homology among peptide sequences which can function in targeting and translocation of membrane proteins (29).

Perhaps most surprisingly, deletion of all cytoplasmic charged residues apparently did not affect the translocation and cellular processing of pro-TNF with a minimal functional transmembrane domain (Fig. 5 and 6; mutant $\Delta-73--55,-49,-48,-32--1$ [IV-2]), suggesting a lack of coordinate influences of cytoplasmic charge and transmembrane hydrophobicity in regulating these mechanisms. The positive supernatant bioassay results with this mutant established that maturation did occur to yield a bioactive TNF, although precise definition of the scissile bond would require sequencing, as with the other mutants. In fact, this mutant has an inverted charge gradient (Table 1), and its net hydropathy of 38.1 (Table 2) is only marginally greater than the value of 34.2 for the $\Delta-42--35$ mutant (III-5 [Table 2]), which exhibits completely effective translocation. Our results do not rule out the possibility that other constructs could demonstrate such coordinate influences. However, our present findings are striking, not only because they are contrary to the observations obtained with the presecretory models from the laboratory of Mizushima and coworkers (9, 10, 24) as well as those obtained with the amino-terminal domain-transmembrane domain chimeric models of Sakaguchi and coworkers (23), but also because they indicate that most of the leader sequence of pro-TNF (53 of 76 residues) is dispensable for its proper translocation and maturation.

Since our studies did not reveal an anticipated role for much of the pro-TNF leader sequence in the mechanisms leading to the secretion of mature TNF, one may speculate about other possible functions for this highly conserved (4) region. Kolias and coworkers (11) have demonstrated normal development in transgenic mice expressing the entire human TNF gene, as well as 0.6 to 0.8 kb of the 5′- and 3′-flanking sequences. TNF mRNA was detected in tissues, although secreted TNF was not detected in plasma. It would be of interest to determine if the deletions from the leader sequence characterized in our studies affect possible juxtracrine mechanisms expressed by pro-TNF during ontogeny.

Our approach has focused entirely on the construction of deletion mutants rather than substitution mutants. We believed that this would be the most direct way to ascertain the roles of the domains of the native leader sequence of pro-TNF. Future studies using amino acid substitutions could be directed to ascertain the roles of particular residues within the minimized domains we have herein defined. As noted above, our evidence obtained from experiments with the hybrid mutants is that ~70% of the entire leader sequence could be deleted without affecting mechanisms leading to maturation; only a role for a minimum transmembrane domain in translocation could be established. In light of several recent reports indicating a role for a ubiquitous metalloproteinase in the maturation of pro-TNF via the necessary and sufficient cleavage of the Ala(−1)–Val(+1) bond (7, 14, 15), it is of interest that our deletion mutants were apparently properly processed. For steric reasons alone, it is difficult to envision that a protease could cleave at the Ala(−33)–Val(+1) bond, as in the hybrid mutants, given its proximity to the plasma membrane. Indeed, in other studies, deletion of the linking domain has been shown to block maturation (25a). It remains to be established exactly where cleavage occurs with the hybrid mutants, as well as whether the same metalloproteinase is operant.

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REFERENCES

1. **Adams, G. A., and J. K. Rose.** 1985. Structural requirements of a membrane spanning domain for protein anchoring and cell transport. Cell **41:**1007–1015.
2. **Beltzer, J. P., K. Fiedler, C. Fuhrer, I. Geffen, C. Handschin, H. P. Wessels, and M. Spiess.** 1991. Charged residues are major determinants of the transmembrane orientation of a signal-anchor sequence. J. Biol. Chem. **266:**973–978.
3. **Blobel, G.** 1980. Intracellular protein topogenesis. Proc. Natl. Acad. Sci. USA **77:**1496–1500.
4. **Caput, D., B. Beutler, K. Hartog, S. Brown-Shimer, and A. Cerami.** 1986. Identification of a common nucleotide sequence in 3′-untranslated region of mRNA molecules specifying inflammatory mediators. Proc. Natl. Acad. Sci. USA **83:**1670–1674.
5. **Davis, N. G., and P. Model.** 1985. An artificial anchor domain: hydrophobicity suffices to stop transfer. Cell **41:**607–614.
6. **Garoff, H.** 1985. Using recombinant DNA techniques to study protein targeting in the eucaryotic cell. Annu. Rev. Cell Biol. **1:**403–445.
7. **Gearing, A. J. H., M. C. Beckett, M. Churchill, J. Clements, A. H. Davidson, A. H. Drummond, W. A. Galloway, R. Gilbert, J. L. Gordon, T. M. Leber, M. Mangan, K. Miller, P. Nayee, K. Owen, S. Patel, W. Thomas, G. Wells, L. M. Wood, and K. Woolley.** 1994. Processing of tumour necrosis factor-α precursor by metalloproteinases. Nature (London) **370:**555–557.
8. **Hartmann, E., T. A. Rapoport, and H. F. Lodish.** 1989. Predicting the

orientation of eukaryotic membrane-spanning proteins. Proc. Natl. Acad. Sci. USA **86:**5786–5790.

9. **Hikita, C., and S. Mizushima.** 1992. Effects of total hydrophobicity and length of the hydrophobic domain of a signal peptide on in vitro translocation efficiency. J. Biol. Chem. **267:**4882–4888.
10. **Hikita, C., and S. Mizushima.** 1992. The requirement of a positive charge at the amino terminus can be compensated for by a longer central hydrophobic stretch in the functioning of signal peptides. J. Biol. Chem. **267:**12375–12379.
11. **Kolias, G., H. Cazlaris, S. Georgopolos, E. Kaslaris, J. Keffer, D. Kioussis, and L. Probert.** 1992. Analysis of tumour necrosis factor gene expression and biological function in transgenic mice, p. 159–167. *In* T. Osawa and B. Bonavida (ed.), Tumor necrosis factor: structure-function relationship and clinical application. S. Karger. Basel.
12. **Kriegler, M., C. Perez, K. DeFay, I. Albert, and S. D. Lu.** 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.
13. **Kyle, J., and R. F. Doolittle.** 1982. A simple method for displaying the hydropathic character of a protein. J. Mol. Biol. **157:**105–132.
14. **McGeehan, G. M., J. D. Becherer, R. C. Bast, Jr., C. M. Boyer, B. Champion, K. M. Connolly, J. G. Conway, P. Furdon, S. Karp, S. Kidao, A. B. McElroy, J. Nichos, K. M. Pryzwansky, F. Schoene, L. Sekut, A. Truesdale, M. Verghese, J. Warner, and J. P. Ways.** 1994. Regulation of tumour necrosis factor-α processing by a metalloproteinase inhibitor. Nature (London) **370:** 558–561.
15. **Mohler, K. M., P. R. Sleath, J. N. Fitzner, D. P. Cerretti, M. Alderson, S. S. Kerwar, D. S. Torrance, C. Otten-Evans, T. Greenstreet, K. Weerawarna, S. R. Kronheim, M. Petersen, M. Gerhart, C. J. Kozlosky, C. J. March, and R. A. Black.** 1994. Protection against a lethal dose of endotoxin by an inhibitor of tumour necrosis factor processing. Nature (London) **370:**218–220.
16. **Muller, R., A. Marmenout, and W. Fiers.** 1986. Synthesis and maturation of recombinant human tumor necrosis factor in eukaryotic systems. FEBS Lett. **197:**99–104.
17. **Nelson, D. R., and H. W. Strobel.** 1988. On the membrane topology of vertebrate cytochrome P-450 proteins. J. Biol. Chem. **263:**6038–6050.
18. **Parks, G. D., and R. A. Lamb.** 1991. Topology of eukaryotic type II membrane proteins: importance of N-terminal positive charged residues flanking the hydrophobic domain. Cell **64:**777–787.
19. **Parks, G. D., and R. A. Lamb.** 1993. Role of NH_2-terminal positively charged residues in establishing membrane protein topology. J. Biol. Chem. **268:** 19101–19109.
20. **Paul, C., and J. P. Rosenbusch.** 1985. Folding patterns of porin and bacteriorhodopsin. EMBO J. **4:**1593–1597.
21. **Rapoport, T. A.** 1992. Transport of proteins across the endoplasmic reticulum membrane. Science **252:**931–936.
22. **Sabatini, D. D., G. Kreibich, T. Morimoto, and M. Adesnick.** 1982. Mechanisms for the incorporation of proteins in membranes and organelles. J. Cell Biol. **92:**1–22.
23. **Sakaguchi, M., R. Tomiyoshi, T. Kuroiwa, K. Mihara, and T. Omura.** 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.
24. **Sasaki, S., S. Matsuyama, and S. Mizushima.** 1990. In vitro kinetic analysis of the role of the positive charge at the amino-terminal region of signal peptides in translocation of secretory protein across the cytoplasmic membrane in Escherichia coli. J. Biol. Chem. **265:**4358–4363.
25. **Spiess, M., and C. Handschin.** 1987. Deletion analysis of the internal signal-anchor domain of the human asialoglycoprotein receptor H1. EMBO J. **6:**2683–2691.

25a. **Tang, P., M.-C. Hung, and J. Klostergaard.** Human pro-tumor necrosis factor is a homotrimer. Submitted for publication.

26. **Utsumi, T., A. Levitan, M.-C. Hung, and J. Klostergaard.** 1993. Effects of truncation of human pro-tumor necrosis factor transmembrane domain on cellular targeting. J. Biol. Chem. **268:**9511–9516.
27. **Verner, K., and G. Schatz.** 1988. Protein translocation across membranes. Science **241:**1307–1313.
28. **von Heijne, G.** 1985. Structural and thermodynamic aspects of the transfer of proteins into and across membranes. Curr. Top. Membr. Transp. **24:**151–179.
29. **von Heijne, G.** 1985. Signal sequence: the limits of variation. J. Mol. Biol. **184:**99–105.
30. **von Heijne, G.** 1986. Mitochondrial targeting sequences may form amphiphilic helices. EMBO J. **5:**1335–1342.
31. **von Heijne, G., and Y. Gavel.** 1988. Topogenic signals on integral membrane proteins. Eur. J. Biochem. **174:**671–678.
32. **Wickner, W. T., and H. F. Lodish.** 1985. Multiple mechanisms of protein insertion into and across membrane. Science **230:**400–407.