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Membrane interaction of TNF is not sufficient to trigger increase in membrane conductance in mammalian cells

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Abstract **Tumor necrosis factor (TNF) can trigger increases in membrane conductance of mammalian cells in a receptor-independent manner via its lectin-like domain. A lectin-deficient TNF mutant, lacking this activity, was able to bind to artificial liposomes in a pH-dependent manner, but not to insert into the bilayer, just like wild type TNF. A peptide mimicking the lectin-like domain, which can still trigger increases in membrane currents in cells, failed to interact with liposomes. Thus, the capacity of TNF to trigger increases in membrane conductance in mammalian cells does not correlate with its ability to interact with membranes, suggesting that the cytokine does not form channels itself, but rather interacts with endogenous ion channels or with plasma membrane proteins that are coupled to ion channels.**

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Key words: Tumor necrosis factor; Channel; Membrane; Unfolding

1. Introduction

Apart from mediating a plethora of effects by means of cross-linking its two types of receptors (reviewed in [1,2]), the proinflammatory cytokine tumor necrosis factor (TNF) also has receptor-independent activities. At acidic pH, the TNF trimer was shown to interact with artificial lipid membranes [4] possibly due to partial acidic unfolding [3,4] and exposure of hydrophobic residues. Moreover, lipid bilayer experiments indicated a channel-like activity of TNF. These observations led to the proposal that TNF might function as a sodium channel [4,5]. TNF was also shown to bind specific oligosaccharides, such as *N*,*N*′-diacetylchitobiose and branched trimannoses, that are present in the immunosuppressive glycoprotein uromodulin and in the variant specific glycoprotein (VSG) of African trypanosomes, via a lectin-like domain [6–8]. As a consequence of its interaction with *Trypanosoma brucei brucei*, TNF can lyse the trypanosomes, in a temperature- and pH-dependent manner, resulting in the disruption of lysosomal integrity [8,9]. The lectin-like and the trypanolytic activities of TNF appear to be mediated by the same domain, namely the tip region of the trimeric TNF molecule, which is spatially distinct from its receptor binding sites (Fig. 1) [9]. We recently reported that this domain of TNF is also able to increase the membrane conductance in endothelial cells and macrophages, with residues T104, E106 and E109 being critical for both the lectin-like and the membrane conductance increasing effect (correspond to residues T105, E107 and E110 in human TNF, Fig. 1) [10].

In this study, we analyzed the ability of an inactive triple TNF mutant (T104A-E106A-E109A) and of peptides derived from the TNF tip domain (Fig. 1) to interact with membranes. We found that the TNF mutant, like the wild type protein, was able to bind to membranes in a pH-dependent manner. In contrast, the tip peptide, which has retained its trypanolytic and membrane conductance increasing effect, did not interact with membranes. These results indicate that the ability of TNF to increase the membrane conductance in mammalian cells does not correlate with its ability to interact with membranes. We propose that at acidic pH an α-helix forms at the tip domain of TNF and mediates the interaction with an endogenous ion transporter in mammalian cells or alternatively with a plasma membrane protein that is coupled to ion channels.

2. Materials and methods

2.1. TNF and peptides

Escherichia coli-derived recombinant murine TNF (further referred to as TNF in the text) and an *E. coli*-derived recombinant (T104A-E106A-E109A) triple TNF mutant (mutTNF) were synthesized as described elsewhere [11]. TNF-derived peptides were synthesized with the use of Fmoc-α-amino group protection [12] and purified with a C18 reverse-phase high performance liquid chromatography column. The following TNF-derived peptides were synthesized:

Long tip peptide 99–115 (LTip)	GG-CGPKDTPEGAELKPWYC
Mutated tip peptide 99–115 (mutTip)	GG-CGPKD<u>A</u>P<u>A</u>GA<u>A</u>LKPWYC
Scrambled tip peptide (scramblTip)	GG-CGTKPWELGPDEKPAYC

To theoretically retain the original TNF conformation as much as possible, LTip, mutTip and scramblTip peptides were circularized. Ser^{99} of the TNF sequence was replaced by Cys, and Cys^{100} by Gly so that the disulfide bridge could be formed between Cys^{99} and Cys^{115} in the peptides. The scramblTip peptide could not be circularized.

2.2. Tryptophan fluorescence

Fluorescence measurements were made with a PTI spectrofluorimeter. The excitation wavelength was 295 nm and slit widths were 5 nm and 2.5 nm for excitation and emission respectively. For each recorded spectrum, the Raman scatter contribution was removed by subtraction of a buffer blank. All buffers contained 150 mM NaCl,

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and 20 mM of N-(2-morpholino)ethanesulfonic acid (MES) buffer at the desired pH. The samples were allowed to incubate for 1.5 h at the desired pH before measuring the emission spectrum. The wild type and mutant TNF concentrations were 6 μg/ml.

2.3. Preparation of liposomes

Large unilamellar liposomes were prepared by reverse-phase evaporation as previously described [13]. Liposomes were prepared of either 100% egg phosphatidylglycerol (EPG) or a mixture of egg phosphatidylcholine and EPG (1:1 w/w) in a buffer containing 100 mM KCl, 20 mM N-(2-hydroxyethyl)piperazine-N'-(2-ethanesulfonic acid) (HEPES), pH 7.4 and 1.5 mg/ml of 6-methoxy-N-(3-sulfopropyl)quinolinium (SPQ).

2.4. Chloride efflux measurements

All fluorescence experiments were carried out using a PTI spectrofluorimeter equipped with a thermostated cell holder (37°C). The dye was excited at 350 nm and emission was recorded at 422 nm, both excitation and emission bandwidths were set to 5 nm. Liposomes were diluted to a final concentration of 50 μg/ml in a solution containing 100 mM KNO_3 and 20 mM MES pH 6.1 or 20 mM HEPES, pH 7.4. Wild type and mutant TNF were added to a final concentration of 3 μg/ml.

2.5. Circular dichroism (CD)

CD experiments were carried out at room temperature on a Jasco 700 spectrometer. Quartz cells with a 0.01 cm path length were used. The peptides were diluted to a concentration of 0.5 mg/ml in a buffer containing 145 mM NaCl, 3 mM KCl, 2 mM $CaCl_2$, 2 mM $MgCl_2$, 10 mM D-glucose, 10 mM HEPES, pH 7.3. The buffer spectrum was subtracted for each sample.

3. Results

3.1. MutTNF undergoes partial unfolding at acidic pH

It has been previously shown that TNF interacts with lipids in a pH-dependent manner and that this membrane interaction correlates with partial unfolding of the protein. We therefore investigated whether the lack of effect of mutTNF on the membrane conductance in lung MVEC at acidic pH [10] could be due to its inability to undergo partial unfolding and therefore to interact with membranes. The conformation of mutTNF at various pH values was followed by measuring the intrinsic fluorescence of the two tryptophan residues found in the molecule (shown space-filled in Fig. 1). As shown in Fig. 2, the fluorescence intensity dropped upon acidification of the medium, and the maximum emission wavelength underwent a red shift from 318 nm at pH 6 to 339 nm at pH 4.6. These observations indicate that the initially buried tryptophan residues became exposed to the solvent. However, the protein was not fully unfolded since the spectrum at pH 4.6 was not as red shifted as that of mutTNF in 6 M GuHCl. These results show that mutTNF was able to undergo acidic unfolding. Acidic unfolding of mutTNF was in fact more rapid and slightly more extended than that of wild type TNF (not shown).

3.2. MutTNF interacts with membranes at acidic pH

We next investigated whether mutTNF was in fact unable to interact with membranes at acidic pH, by following its ability to induce chloride leakage from liposomes containing the chloride-sensitive dye SPQ. These experiments were per-

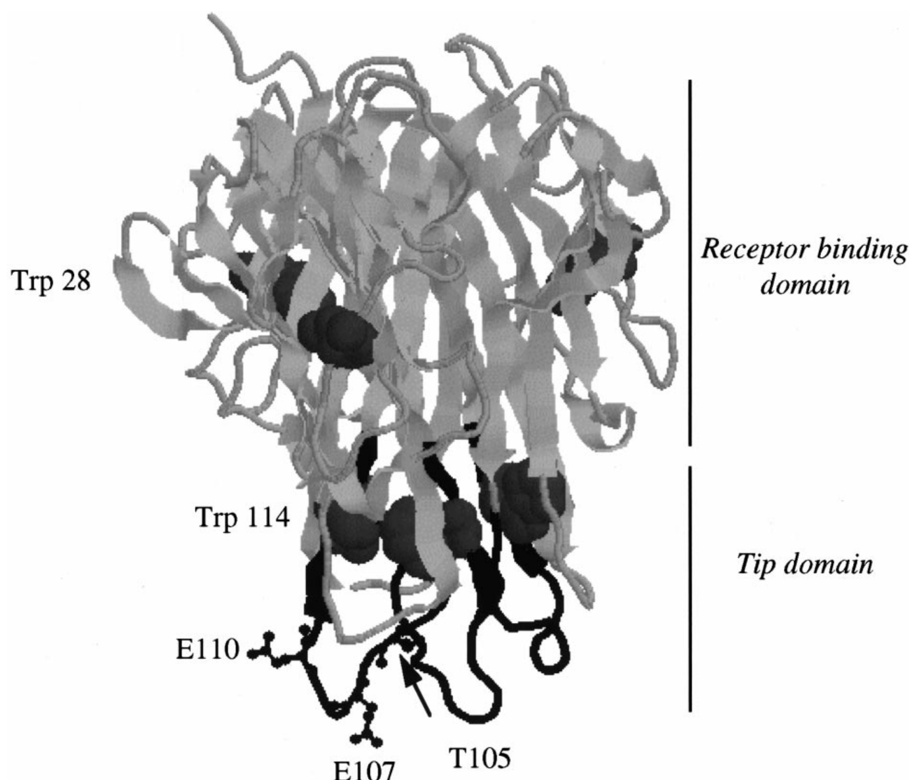

Fig. 1. Ribbon diagram of the human TNF trimer according to the crystal structure [22,23]. The sequence corresponding to that of the LTip peptide (LTip, see Section 2) is shown in black. The two tryptophan residues are shown in black space-filled representation. The three critical amino acids that were mutated in the TNF mutant (mutTNF) as well as in the peptide (mutTip) are shown in black ball and stick representation in one of the monomers in the trimer.

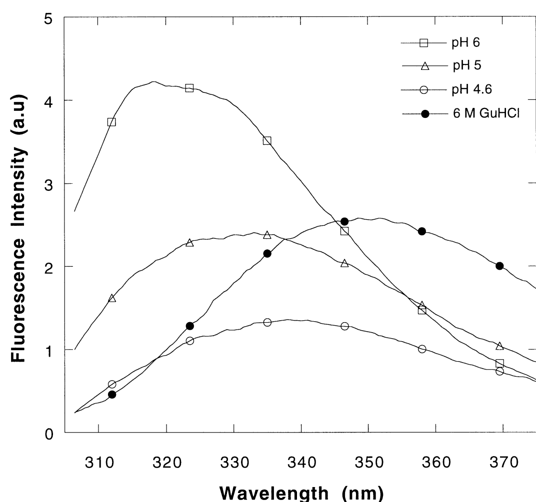

Fig. 2. Acidic unfolding of the triple TNF mutant. The tryptophan fluorescence spectrum of mutTNF (6 μg/ml) was measured in 150 mM NaCl, and 20 mM MES buffered at various pH values, as described in Section 2 (25°C). The fluorescence spectrum of the fully unfolded mutant in 6 M GuHCl (in 150 mM NaCl and 20 mM HEPES, pH 7.4) was also measured. The excitation wavelength was 295 nm.

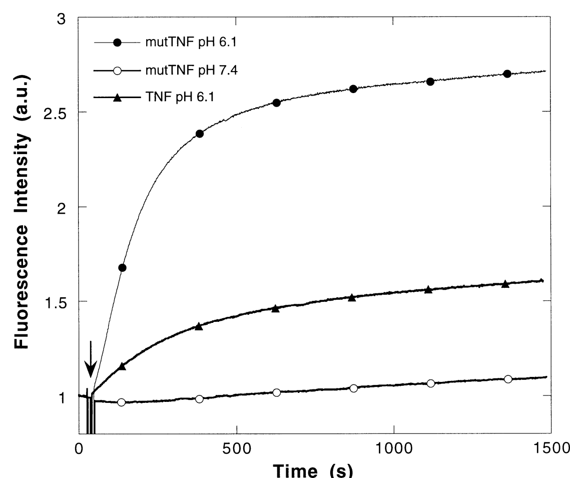

Fig. 3. Both wild type and mutTNF trigger chloride efflux from negatively charged phospholipid vesicles in a pH-dependent manner. Chloride efflux, followed by measuring SPQ fluorescence, from liposomes containing 100% EPG was measured for native (TNF) and triple mutant TNF (mutTNF) in 100 mM KNO_3, 20 mM MES, pH 6.1 or 100 mM KNO_3, 20 mM HEPES, pH 7.4. The time at which the proteins were added (3 μg/ml) is indicated by an arrow.

formed using liposomes containing 100% EPG. As can be seen in Fig. 3, mutTNF induced rapid chloride efflux at pH 6.1, but not at neutral pH, as previously observed for wild type TNF [4]. The effect of mutTNF on SPQ fluorescence was even more pronounced than that of wild type TNF (Fig. 3), most likely because its acidic unfolding was more rapid than that of wild type TNF. As can be seen in Fig. 2, mutTNF was still folded at pH 6. However, we have previously shown that the pH at the surface of 100% EPG vesicles is far lower than that of the bulk pH. More specifically, for a bulk pH of 6, the pH at the membrane surface of 100% EPG vesicles is 4.35 [13]. Therefore, mutTNF is likely to have undergone partial unfolding at the surface of the EPG vesicles.

In order to investigate whether chloride efflux was due to membrane binding or membrane insertion of TNF, we analyzed whether brominated lipids were able to quench the intrinsic fluorescence of TNF and mutTNF upon membrane interaction. Brominated lipids have been useful in determining the topology of membrane proteins [14,15] as well as in studying the membrane interaction of pore-forming toxins [16–18]. TNF contains two tryptophan residues, one at the top of the receptor binding domain (Trp-28 in human TNF, Fig. 1) and one at the top of the tip domain (Trp-114 in human TNF, Fig. 1). If the tip of the TNF trimer were to insert into the lipid bilayer, the fluorescence of the latter tryptophan should be quenched upon insertion into liposomes composed of dioleoylphosphatidylglycerol, which has bromines attached at positions 9 and 10 of the acyl chains. We have indeed previously observed that even tryptophans located at the boundary between the lipid head groups and the acyl chains were susceptible to bromide quenching [18]. We were, however, unable to see any fluorescence quenching when adding either TNF or mutTNF at acidic pH to vesicles formed of brominated lipids (not shown).

The above observations show that mutTNF undergoes partial unfolding at acidic pH and is then able to interact with membranes. However, the lack of quenching by brominated lipids indicates that chloride release is due to binding of the partially unfolded TNF molecules and thereby perturbation of the lipid bilayer rather than to membrane insertion of the molecule.

3.3. Structural analysis of the TNF tip peptides

Previously, we have shown that the peptide corresponding to the tip domain of TNF (LTip) was sufficient, albeit at higher concentrations than full-length TNF, to increase membrane currents in microvascular endothelial cells and macrophages [10]. Mutations of key residues in this peptide (in ball and stick in Fig. 1) (mutTip) or scrambling of the amino acids in the peptide (scramblTip) inactivated the peptide [10]. Here we analyzed the secondary structure of these various peptides by far-UV CD. As shown in Fig. 4, all peptides largely adopt a random structure as indicated by the minimum ellipticity at 203 nm. However, the long tip peptide (LTip) also has a clear

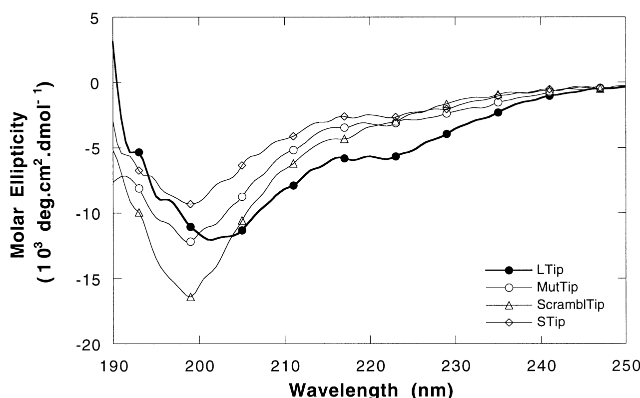

Fig. 4. Far UV CD spectra of TNF tip peptides. CD spectra were measured in the far UV for the long, the short, the mutant and the scrambled peptides. Measurements were performed at room temperature at a concentration of 0.5 mg/ml in 150 mM NaCl, 20 mM HEPES pH 7.4. The buffer scan was subtracted from each scan. Each spectrum represents the mean of 13 scans.

minimum at 222 nm, which is not observed in the other peptides. This minimum is characteristic of helical structure. The percentage of α-helices was estimated to be approximately 10%. The CD spectra of the various peptides were not affected by changes in buffer pH down to pH 4.5 (not shown).

We next tested whether the TNF tip peptides were able to induce chloride efflux from SPQ containing vesicles and whether tryptophan quenching could be observed upon interaction with brominated lipids at various pH values. Liposomes containing either 100% neutral lipids, 100% acidic lipids or a 1:1 mixture of both were tested. For none of the lipid compositions and for peptide concentrations up to 300 μg/ml could we observe any change in SPQ fluorescence or any quenching by brominated lipids with all four peptides (not shown). These experiments strongly suggest that the long tip as well as the modified tip peptides are unable to interact with membranes.

4. Discussion

We have previously shown that TNF can trigger an increase in the membrane conductance of lung microvascular endothelial cells and peritoneal macrophages [10]. This effect of TNF occurred independently of the TNF receptors, since we observed a similar activity in cells isolated from mice deficient in both TNF receptors, and is mediated by the tip domain of the molecule, which is spatially and functionally distinct from its receptor binding sites (Fig. 1) [9,11]. Indeed, a TNF mutant in which the three residues critical for its trypanolytic activity were replaced by alanine residues [11] no longer induced this effect in the primary murine cells tested. A circularized 17 amino acid peptide mimicking the tip region of TNF was also able to exert the TNF ion channel activity, a property that was lost upon mutation of residues T104, E106 and E109. Here we show that the ability to trigger an increase in membrane conductance does not correlate with the protein's ability to interact with membranes, thereby strongly suggesting that the effect on mammalian cells is due to binding of TNF to endogenous ion channels, or alternatively to plasma membrane proteins that are coupled to ion channels, rather than to channel formation by TNF itself.

Since acidic unfolding is a prerequisite for the interaction of TNF with artificial lipid bilayers, we first compared the capacity of the wild type molecule and the triple TNF mutant to undergo acidic unfolding. Interestingly, even though it was unable to exert any ion channel activating effect in cells, the mutTNF molecule underwent acidic unfolding similarly to wild type TNF, and also had the ability to interact with artificial liposomes in a pH-dependent manner. However, we were unable to observe membrane penetration of either wild type or mutant TNF. It therefore appears that partial unfolding, which generally leads to the exposure of hydrophobic patches at the protein surface, allowed TNF to bind to membranes, but not to insert. This membrane binding ability could well explain the channel-like activity [19] that was observed for TNF in lipid bilayers [5]. Altogether, these observations indicate that acidic unfolding, although it may be necessary [10], is not sufficient to trigger channel activity.

It is not clear whether there is any relationship between the ability of TNF to interact with membranes and its ability to trigger an increase in membrane conductance in cells. The observation that the tip peptide, which is able to increase membrane currents in mammalian cells, did not show any interaction with liposomes suggests that these two properties of TNF are not related. In fact, several features of the LTip peptide suggest that it is unlikely to penetrate or cross a lipid bilayer. Since the peptide is circularized, it can only penetrate the membrane as an α-helical or β-sheet hairpin. The CD data suggest that the peptide has a certain propensity to form α-helices. However, a transmembrane helical conformation is unlikely since the peptide (i) would be too short to span even half the membrane, (ii) contains three proline residues which are known to be helix breakers and (iii) contains only three hydrophobic residues but five charged residues. A β-sheet transmembrane conformation is also unlikely because again the peptide is slightly too short and does not contain a sufficient number of hydrophobic residues that would allow at least one face of the β-hairpin to interact with the lipid acyl chains.

The present observations thus raise the possibility that the ability of TNF to trigger changes in membrane conductance might not be due to the direct interaction of this molecule with the membrane, but to the interaction of TNF with endogenous ion channels, or alternatively with plasma membrane proteins that are coupled to ion channels. This hypothesis is supported by the observation that the TNF-triggered increase in membrane conductance is amiloride-sensitive [10]. Based on our observations and data in the literature, we propose the following model. At acidic pH, at the surface of the target membrane, the TNF trimer would undergo partial unfolding, presumably due to protonation of histidine residues (there are three histidine residues). This conformational change, which does not lead to trimer dissociation [3], would lead to a greater exposure of the three tip domains in the trimer. Using CD, Hlodan and Pain observed a 16% increase in α-helical structure in TNF upon acidic unfolding at the expense of aperiodic structure [3]. It is therefore tempting to speculate that at acidic pH, the very tip domains, including residues T104, E106 and E109, which do not have any structure in native TNF (Fig. 1), would adopt a helical fold in agreement with secondary structure predictions. The tip would thus be in the proper conformation to interact with proteins on the target cell surface. Binding of TNF to these surface molecules would then lead to opening of ion channels. TNF receptors appear not to be involved since increases in membrane conductance were also observed in cells lacking both known TNF receptors [9,11]. However, it remains to be established whether TNF binds directly to ion channels, which could have similarities to identified amiloride-sensitive neuropeptide-gated sodium channels [20,21], or to proteins coupled to ion channels.

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