nylon wool passage of total splenocytes from C57BL/6 mice ($NOS2^{+/+}$ or $NOS2^{-/-}$), analyzed by flow cytometry ($CD3^+$ >93%, $NK1.1^+$ <3%, $CD19^+$ <1%, and $F4/80^+$ <1%), and stimulated with rmIL-12 (0.1 to 1 ng/ml) or mouse IFN-α/β (100 to 500 U/ml).
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27. After stimulation, KY-1 cells or IL-2–expanded splenic NK cells were washed twice with ice-cold PBS (plus 100 μM sodium orthovanadate) and lysed in 20 mM tris buffer (pH 8.0) containing 150 mM NaCl; 1% Triton X-100; 0.5% NP-40; 1 mM each of EDTA, EGTA, sodium orthovanadate, sodium pyrophosphate, sodium fluoride, and PMSF; 0.1 mM sodium molybdate; and pepstatin A, aprotinin, chymostatin, and leupeptin (5 μg/ml each). Protein lysates were immunoprecipitated with 1 μg of affinity-purified rabbit anti-mouse Stat4 IgG (C-20, Santa Cruz Biotechnology, Santa Cruz, CA) in the absence or presence of the respective Stat4 blocking peptide (5 μg/ml) using protein A/G-Plus-agarose (Santa Cruz Biotechnology); separated by 7.5% SDS–polyacrylamide gel electrophoresis (SDS-PAGE); and transferred to nitrocellulose. Immunoblotting (IB) of tyrosine phosphorylated Stat4 was performed with anti-phosphotyrosine mouse IgG [PY99, Santa Cruz Biotechnology; 1 μg/ml in dilution buffer (tris-buffered saline with 5% nonfat dry milk and 0.05% Tween 20)], followed by incubation with peroxidase-conjugated goat anti-mouse IgG (Dianova; 160 ng/ml in dilution buffer) and detection with ECL Plus (Amersham Pharmacia Biotech). Blots were reprobed with anti-Stat4 IgG (200 ng/ml in dilution buffer).
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32. KY-1 cells, primary NK cells, or T cells were lysed (*27*) and immunoprecipitated with rabbit anti-Jak1 IgG (HR-785, Santa Cruz Biotechnology; 2 μg/ml), rabbit anti-Jak2 IgG (HR-758, Santa Cruz Biotechnology; 2 μg/ml), or rabbit anti-Tyk2 {C-15, specific for amino acids 1173 through 1187 [O. Colamonici *et al.*, *Mol. Cell. Biol.* **14**, 8133 (1994)]; 1:200} in the absence or presence of the respective blocking peptides (10 μg/ml) using protein A–Sepharose. They were then separated by 7.5% SDS-PAGE and transferred to nitrocellulose. Immunoblotting of tyrosine-phosphorylated Jak1, Jak2, or Tyk2 was performed with anti-phosphotyrosine mouse IgG (PY99, 1 μg/ml; Santa Cruz Biotechnology) as described (*27*). After stripping, blots were reprobed with antibody to Jak1 (800 ng/ml), Jak2 (600 ng/ml), or Tyk2 [C-15 combined with C-20 (Santa Cruz Biotechnology), 600 ng/ml].
33. Jak1, Jak2, or Tyk2 immunoprecipitates were washed with lysis buffer and with kinase buffer [20 mM Hepes (pH 7.4), 50 mM NaCl, 2 mM EGTA, 1 mM dithiothreitol, 1 mM sodium orthovanadate, 5 mM $MnCl_2$, and 5 mM $MgCl_2$]. The protein bead complexes were resuspended in 50 μl kinase buffer containing 10 μCi [γ-^{32}P]ATP (>3000 Ci/mmol) and incubated for 30 min at room temperature. After several washes in tris buffer (pH 7.5) containing 0.1% Triton X-100 and 100 μM orthovanadate and a final wash in Triton-free tris buffer (pH 6.8), the immunoprecipitates were eluted in Laemmli-loading buffer, fractionated on 7.5% SDS-PAGE, and transferred to reinforced nitrocellulose (Schleicher & Schuell, Dassel, Germany) for autoradiography. In some cases, the protein bead immunocomplexes were treated with DETA/NO or DETA (1 to 100 μM in kinase buffer) for 60 min before the in vitro kinase reaction.
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38. Cell lysates were prepared in 40 mM tris buffer (pH 8.0) containing 200 μM phenylmethylsulfonyl fluoride and aprotinin, chymostatin, pepstatin A, and leupeptin (5 μg/ml each), then separated on a 7.5% SDS-PAGE followed by protein immunoblotting with a rabbit anti-mouse NOS2 IgG (*9*) using the ECL Plus detection system (Amersham Pharmacia Biotech, Freiburg, Germany).

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Interaction of Diphtheria Toxin T Domain with Molten Globule-Like Proteins and Its Implications for Translocation

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The transmembrane (T) domain of diphtheria toxin has a critical role in the low pH–induced translocation of the catalytic domain (A chain) of the toxin across membranes. Here it is shown that at low pH, addition of proteins in a partly unfolded, molten globule-like conformation converted the T domain from a shallow membrane-inserted form to its transmembrane form. Fluorescence energy transfer demonstrated that molten globule-like proteins bound to the T domain. Thus, the T domain recognizes proteins that are partly unfolded and may function in translocation of the A chain as a transmembrane chaperone.

Diphtheria toxin, a protein secreted by *Corynebacterium diphtheriae*, consists of an A chain (21 kD) and a B chain (37 kD). The A chain is the catalytic domain, and the B chain contains the transmembrane (T) and receptor-binding domains (*1*). After binding to mammalian cells and undergoing endocytosis, the toxin partially unfolds within the low pH of the endosomal lumen. This exposes hydrophobic sites, induces membrane insertion, and results in translocation of the A chain into the cytoplasm (*2–4*). Translocation is believed to involve the interaction of a transmembrane structure formed largely by the T domain with the partly unfolded A chain (*2–6*). Recent studies have shown the T domain can exist in both partially membrane-penetrating (P) and transmembrane (TM) conformations (*7, 8*). Conversion of the P to the TM conformation can be detected by the blue shift of the emission of fluorescent groups attached to single Cys residues introduced into TH8 or TH9, such as residue C^{356} (*7*). TH8 and TH9 are hydrophobic helices that become buried in the TM conformation (*7–9*).

At low pH, the addition of bovine serum albumin (BSA), human serum albumin (HSA), α-lactalbumin, apomyoglobin, or diphtheria toxin A chain to T domain bound in the P conformation to dioleoylphosphatidylglycerol (DOPG)/dioleoylphosphatidylcholine (DOPC) model membranes induced a blue shift in the fluorescence of bimane attached to C^{356} (Fig. 1A). Below pH 5 these added proteins have molten globule (MG)-like conformations, which exhibit some degree of partial unfolding and increased hydrophobicity (*4, 10–12*). In contrast, no effect on bimane fluorescence was observed when proteins that do not form an MG-like state [egg white lysozyme, ovalbumin, and an anti-dansyl immunoglobulin G (IgG)] were added (*13*) (Fig. 1B).

The ability of HSA to blue shift bimane fluorescence was not diminished by predialysis in tubing with a 10,000-kD cutoff and could not be induced by an equivalent volume of an ultrafiltrate of an HSA solution. Thus, HSA itself induced the blue shift.

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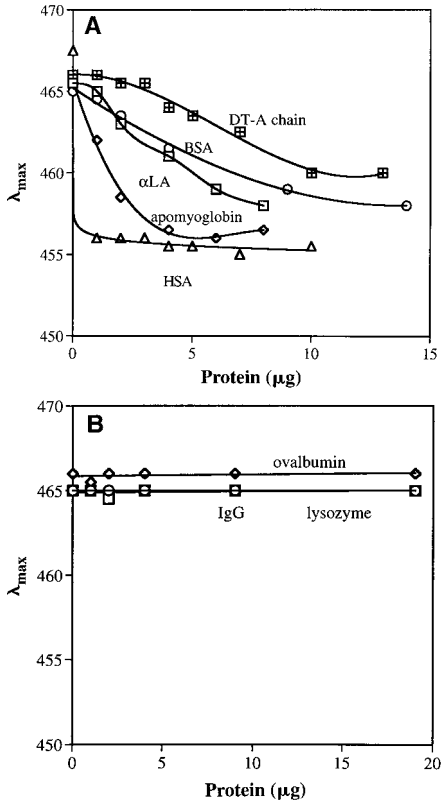

Fig. 1. Interaction of membrane-inserted T domain with various proteins. (**A**) Effect of proteins on the wavelength of maximum fluorescence emission of membrane-inserted bimane-labeled A356C T domain. ⊞, A chain; ○, BSA; Δ, HSA; ◇, apomyoglobin; □, α-lactalbumin. (**B**) Same as (A). □, IgG; ○, lysozyme; ◇, ovalbumin. Samples contained bimane-labeled A356C T domain (1.7 μg/ml) incorporated into small unilamellar vesicles prepared by ethanol dilution (*7*) and composed of 200 μM DOPG/DOPC (3:7 mol/mol) (Avanti Polar Lipids) at pH 4.5 (*7*, *22*) (total volume, 600 μl) to which small aliquots of BSA; horse apomyoglobin; bovine milk lactalbumin type III, Ca^{2+}-depleted (Sigma Chemical); HSA; ovalbumin (Worthington Biochemical); egg white lysozyme (Fluka Chemical); or anti-dansyl IgG (Molecular Probes) dissolved in water was added. The purity of these proteins was confirmed by SDS gel electrophoresis. Fluorescence was measured as described (*7*) 15 min after addition of each aliquot. Results shown are averages from two experiments. Values were reproducible to within ±1 to 2 nm in all cases.

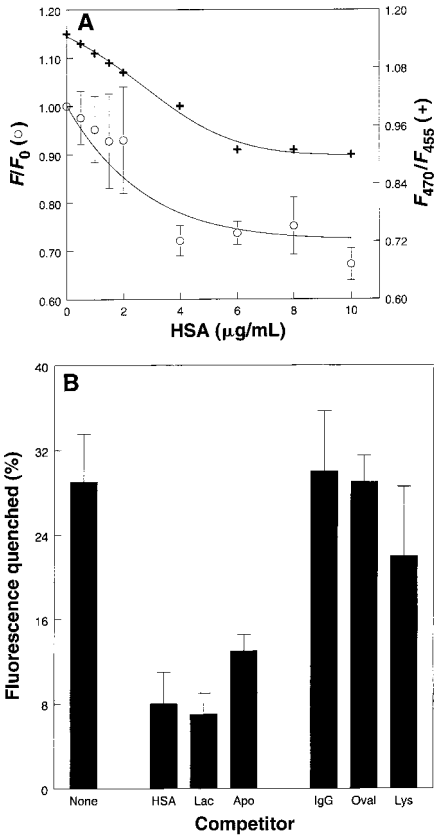

Fig. 2. Detection of T domain–HSA association by energy transfer. (**A**) Effect of addition of rhodamine-labeled HSA on fluorescence of bimane-labeled A356C T domain at pH 4.1. ○, F/F_0 fluorescence in the presence of rhodamine-labeled HSA divided by that in the presence of unlabeled HSA; +, ratio of fluorescence intensity at 470 nm relative to that at 455 nm in samples containing unlabeled HSA (*22*). Samples contained 200 μM DOPG/DOPC (3:7 mol/mol) sonicated small unilamellar vesicles (*7*) in 167 mM sodium acetate, 6.7 mM tris-Cl, 150 mM NaCl (pH 4.1) mixed well with a 1- to 1.5-μg aliquot of bimane-labeled A356C dissolved in 7 to 15 μl of 20 mM tris-Cl containing about 250 mM NaCl (pH 8) (final volume, 800 μl). Bimane emission was measured (excitation 375 nm) 1 to 2 min after 3- to 12-μl aliquots of HSA in 10 mM tris-Cl (pH 8) were added (final pH 4.1). Average values from three samples and standard deviations are shown. (**B**) Effect of inclusion of unlabeled proteins on energy transfer between rhodamine-labeled HSA and A356C T domain. Samples contained 200 μM DOPG/DOPC (3:7 mol/mol) sonicated vesicles with bimane-labeled A356C T domain (pH 4.1) prepared as described in (A). A small aliquot of unlabeled protein [1-mg/ml stock solutions, 0.5 mg/ml for apomyoglobin, in 10 mM tris-Cl (pH 8)] containing 16 μg of anti-BODIPY IgG (*23*), α-lactalbumin, lysozyme, ovalbumin, or apomyoglobin or 8 μg of HSA was added. Bimane fluorescence was measured before (to normalize to equal protein concentrations) and after 3.2 μg of rhodamine-labeled or unlabeled HSA was added (from 1-mg/ml stocks in tris-Cl). % quenching = [1 − (fluorescence in sample with rhodamine-labeled HSA)/(fluorescence in sample with unlabeled HSA)] × 100. Average values ± SD from three samples are shown. Lac, α-lactalbumin; Apo, apomyoglobin; Oval, ovalbumin; Lys, lysozyme; IgG, anti-BODIPY IgG.

To confirm that a change in T domain structure had occurred, we measured the binding of 4,4-difluoro-4-bora-3a,4a-diaza-s-indicine antibodies (anti-BODIPY) to T domain carrying a BODIPY-labeled Cys residue. The fluorescence of a BODIPY-labeled Cys residue exposed to the external solution is quenched by about half upon binding of anti-BODIPY (*8*). Binding decreased upon formation of the TM conformation (Table 1). The proteins that induced a blue shift in bimane fluorescence also inhibited anti-BODIPY binding to BODIPY-labeled Ala^{356} to C (A356C) T domain (*14*), whereas those proteins that had no effect on bimane fluorescence had no effect on anti-BODIPY binding (Table 1).

These changes in fluorescence and antibody binding could result from the occlusion of T domain residues from interaction with solvent by bound proteins rather than from deep T domain insertion. Therefore, both the depth of bimane groups, monitored by quenching of bimane fluorescence by lipids carrying nitroxide labels (*7*), and the degree of blue shift were measured for T domain mutants labeled on various single Cys residues within TH8 and TH9 (Table 2). Residues within these helices (344, 356, and 361) underwent a blue shift upon addition of HSA [a decrease in the F_{470}/F_{455} ratio (*7*)], consistent with their movement into a nonpolar environment. They also inserted more deeply upon addition of HSA, as judged by the increased quenching by lipids with a deeply buried nitroxide group (12SLPC), relative to that by lipids with a shallow nitroxide (TempoPC) (that is, a decreased $F_{12SLPC}/F_{TempoPC}$ ratio). In contrast, residues in the hydrophilic sequences flanking TH8 and TH9 (322, 378) did not show significantly deeper insertion or a blue shift upon addition of HSA. Thus, helices TH8 and TH9 membrane-inserted more deeply upon addition of HSA (*15*).

To show whether a complex forms between MG-like proteins and the T domain, we measured fluorescence energy transfer from bimane-labeled T domain to rhodamine-labeled HSA (*16*). We observed up to 30% quenching of bimane fluorescence upon addition of rhodamine-labeled HSA (Fig. 2A). There was no quenching when we added unlabeled HSA. We also found that most quenching induced by rhodamine-labeled HSA was abolished when unlabeled HSA or other MG-forming proteins were present with rhodamine-labeled HSA but not when the non-MG proteins were present (Fig. 2B). Thus, partly unfolded HSA and other MG-like proteins competed for binding to the T domain. This competition would not have been expected if quenching were due to HSA bound nonspecifically to lipid but in the proximity of the T domain (*17*). Finally, we performed energy transfer experiments with pure DOPC vesicles, in the presence of which partial unfolding of HSA is more pronounced after preincubation with 5 mM dithiothreitol (DTT) (*12*). In this case, quenching by reduced HSA (18.7% ± 4.5%; $n = 3$) was significantly stronger than that by unreduced HSA (6.7% ± 1.5%).

We conclude that proteins in an MG-like conformation both associate with the T domain and trigger its conversion into the TM con-

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Table 1. Exposure of BODIPY-labeled A356C to external solution. Samples were prepared at pH 4.1 as described in Fig. 1, with or without 5 μg of HSA, BSA, A chain, apomyoglobin, or lactalbumin, or 10 μg of ovalbumin, anti-dansyl IgG, or lysozyme, dissolved in water. % quenching = {1 − [BODIPY fluorescence after addition of a 20-μl (about 160 μg) aliquot of anti-BODIPY/BODIPY fluorescence before antibody addition]} × 100 (*7, 8, 23*). % inhibition = [1 − (% quenching in the presence of protein shown/% quenching with T domain alone)] × 100. Averages ± SD from four samples are shown.

Addition	Quenching by anti-BODIPY (%)	Inhibition of antibody binding (%)
None	39 ± 2	
Ovalbumin	39 ± 3	0
Lysozyme	40 ± 3	−3
IgG	41 ± 4	−5
α-Lactalbumin	21 ± 4	46
A chain	25 ± 3	36
Apomyoglobin	17 ± 4	56
BSA	22 ± 6	44
HSA	23 ± 5	41

Table 2. Effect of HSA on depth and emission of bimane-labeled T domain residues. T domain mutants with Cys at the numbered residue were prepared as described (*7*). Samples contained vesicles composed of 200 μM DOPG/(DOPC ± 15% nitroxide-labeled PC) (3:7 mol/mol) in 167 mM sodium acetate, 6.7 mM tris-Cl, 150 mM NaCl (pH 4.1) (*7*) mixed with a small aliquot containing T domain (1 to 1.5 μg/ml) (final pH 4.1). After fluorescence was measured (excitation 375 nm, emission 455 nm), 2.8 μg of HSA (from a 1-mg/ml solution) was added and fluorescence was remeasured after 30 to 60 s. F/F_0 is fluorescence intensity in vesicles containing 15% nitroxide-labeled PC divided by that in vesicles lacking nitroxide-labeled PC. Averages of quadruplicate experiments (±SD) are shown. Because the maximal formation of the deep conformation appears to be 50% (*8*), values corrected to 100% deep conformation are shown in parentheses. These were calculated by the formula $F/F_{0\ deep\ conformation} = [(1 + n)/n][F/F_{o\ +\ HSA} - [1/(1 + n)]F/F_{0\ -\ HSA}]$, where $n = [2F_{0\ +\ HSA} - F_{0\ -\ HSA}]/F_{0\ -\ HSA}$ (derivation not shown). $F_{12SLPC}/F_{TempoPC}$ is the ratio of corrected F/F_0 values. F_{470}/F_{455} (see Fig. 2) was measured in F_0 samples.

Mutant	$F_{TempoPC}/F_0$	F_{12SLPC}/F_0	$F_{12SLPC}/F_{TempoPC}$	F_{470}/F_{455}
322	0.60 ± 0.02	0.71 ± 0.04	1.18	1.31
322 + HSA	0.64 ± 0.03 (0.67)	0.73 ± 0.04 (0.75)	1.12	1.29
344	0.59 ± 0.01	0.78 ± 0.09	1.32	1.43
344 + HSA	0.47 ± 0.005 (0.43)	0.56 ± 0.02 (0.48)	1.11	1.10
356	0.47 ± 0.01	0.64 ± 0.08	1.36	1.35
356 + HSA	0.56 ± 0.03 (0.61)	0.61 ± 0.07 (0.59)	0.97	1.11
361	0.54 ± 0.04	0.61 ± 0.03	1.12	1.00
361 + HSA	0.53 ± 0.03 (0.53)	0.49 ± 0.02 (0.45)	0.85	0.95
378	0.50 ± 0.01	0.55 ± 0.02	1.10	1.35
378 + HSA	0.57 ± 0.03 (0.65)	0.67 ± 0.05 (0.80)	1.23	1.39

formation. These interactions suggest a translocation model in which the T domain recognizes the A chain when the latter is in a partly unfolded, hydrophobic MG-like state. The T domain may act like a transmembrane chaperone, forming a "sticky pore" that binds a hydrophobic surface on the A chain and helps to maintain it in an unfolded state during translocation. Thus, the partly unfolded hydrophobic conformation of the A chain would function to allow its interaction with the T domain rather than, or in addition to, its interaction with lipid.

This behavior could allow translocation to proceed by a series of transient association-dissociation events between T domain and A chain without requiring the T domain to recognize a specific amino acid sequence (*18*). The observation that introducing disulfide bonds in the A chain can stop translocation at an intermediate stage (*4, 5*) is consistent with such a multistep translocation process. Furthermore, the apparently weak nature of T domain interaction of A chain relative to that with other MG proteins (Fig. 1A) is consistent with the requirement of efficient translocation for rapid dissociation as well as association (*19*).

It is also possible that the T domain is representative of other proteins that act as transmembrane chaperones and may illustrate a mechanism that is used in other protein translocation processes. In addition, the structural similarity between T domain and the Bcl protein family, which has an important role in apoptosis, is intriguing (*20*), as it suggests that there could be a similar basis for their recognition of other proteins. In any case, T domain behavior may suggest new approaches for using diphtheria toxin to deliver proteins into the cell cytoplasm (*21*).

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12. We confirmed that there was partial unfolding of α-lactalbumin, apomyoglobin, and HSA by both circular dichroism and fluorescence. Partial unfolding can be detected for apomyoglobin by the decrease in the (absolute values of) ellipticity at 222 nm [F. M. Hughson, D. Barrick, R. L. Baldwin *Biochemistry* **30**, 4113 (1991)], for α-lactalbumin by the decrease in ellipticity at 280 nm [J. J. Ewbank and T. E. Creighton, *Nature* **350**, 518 (1991)], and for HSA by an unusual blue shift in Trp emission [J. Y. Lee and M. Hirose, *J. Biol. Chem.* **267**, 14753 (1992)]. Relative to the signal in solution at neutral pH, we found that in the presence of DOPG/DOPC vesicles at pH 4.1 there was a 20% decrease in the absolute value of the ellipticity of apomyoglobin at 222 nm, a 33% reduction of α-lactalbumin ellipticity at 280 nm, and a 13-nm blue shift in HSA Trp emission wavelength. We also found that in the presence of DOPC vesicles, the blue shift in HSA fluorescence was 5 nm greater upon reduction of HSA by DTT.
13. Ovalbumin and lysozyme remain folded at moderately low pH and tend to undergo unfolding transitions without MG intermediates [F. Ahmad and A. Salahuddin, *Biochim. Biophys. Acta* **576**, 333 (1979); P. Haezebrouck *et al., J. Mol. Biol.* **246**, 382 (1995)]. Retention of tight dansyl binding demonstrated that anti-dansyl IgG remained folded at low pH (M. Rosconi and E. London, data not shown).
14. There is residual antibody binding because it appears that, at most, half of the T domain molecules convert to the TM state (*7, 8*).
15. We found that the T domain acquires the TM conformation in large unilamellar vesicles (LUV) (J. Sharpe and E. London, unpublished observations). Thus, the possibility that fusion of small unilamellar vesicles (SUV) into LUV by MG proteins affected T domain conformation was of concern. However, although Sepharose 4B-CL size fractionation of samples containing SUV to which T domain and apomyoglobin were added indicated some vesicle fusion (or aggregation), T domain molecules remaining in SUV-containing fractions still appeared to exhibit the blue shift in bimane fluorescence characteristic of the TM state (data not shown).
16. For labeling, 3 mg of HSA and 12 mg of lissamine rhodamine B–SO_2Cl (10% adsorbed on Celite) (Molecular Probes) was dissolved in l ml of 50 mM $NaCO_3$ (pH 9.2) and mixed for 45 min. After pelleting celite at low speed, we chromatographed the supernatant on a Sephadex G-50 column (1 × 23 cm) to remove free rhodamine. The absorbance of the HSA-containing fractions [1 mg/ml by Coomassie assay (Pierce Chemical)] indicated labeling at about two rhodamines per HSA with ε_{576} = 93,000 cm^{-1} M^{-1}.
17. This was of concern because MG proteins bind to lipids at low pH (*11*). The absence of nonspecific quenching was confirmed by experiments that showed no significant energy transfer from a membrane-inserted, bimane-labeled polyleucyl peptide [J. Ren, S. Lew, Z. Wang, E. London, *Biochemistry* **36**, 10213 (1997)] to rhodamine-labeled HSA (data not shown). It is also noteworthy that addition of unlabeled MG proteins did not result in dissociation of the rhodamine-labeled HSA from the membrane (data not shown).
18. Comparing the sequence of the A chain to that of other proteins that affect T domain revealed no strong similarities (data not shown).
19. These experiments have not defined the maximum degree of unfolding (or the minimum) that would still allow recognition by the T domain. In this regard it is interesting that we have found that a 25-residue polyalanyl polypeptide that is only partly helical in solution [L.-P. Liu, S.-C. Li, N. K. Goto, C. M. Deber, *Biopolymers* **39**, 465 (1996)] can trigger the P to TM conformational change.
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