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Transmembrane helical interactions: zeta chain dimerization and functional association with the T cell antigen receptor

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Members of the ζ family of receptor subunits (ζ, η and γ) are structurally related proteins found as components of the T cell antigen receptor (TCR) and certain Fc receptors. These proteins share the ability to form disulfide-linked dimers with themselves and with other members of the family. Comparison of the amino acid sequences of ζ and γ reveals a significant degree of homology, which is highest within their membrane-spanning domains. Analysis of their transmembrane sequences on a helical wheel projection suggests that all of the identical amino acids are clustered on one face of a potential α-helix. This face contains the only cysteine residue within ζ, suggesting that this conserved region may function to mediate dimerization. Indeed, replacing the transmembrane domain of the Tac antigen (α chain of the interleukin-2 receptor) by that of the ζ chain resulted in the formation of disulfide-linked dimers of Tac. The conserved aspartic acid residue found in the ζ and γ transmembrane sequences was found to play a role in disulfide linkage. Replacing the aspartic acid with a lysine but not with an alanine or valine residue allowed formation of disulfide-linked dimers. The ability of the aspartic acid residue to support dimerization was dependent upon its position within the helix. Thus, these observations indicate that residues within the ζ transmembrane domain play a critical role in the formation of disulfide-linked dimers. Expression of ζ mutants in ζ-deficient T cells revealed that the ζ transmembrane domain is also responsible for reconstituting transport of functional TCR complexes to the cell surface and differentiated the requirements for disulfide-linked dimerization *per se* from assembly of the TCR complex.

Key words: disulfide-linked dimers/T cell antigen receptor/transmembrane domain

Introduction

Many cell surface receptors are now recognized as being composed of multiple subunits. One of the best studied examples of a complex oligomeric receptor is the antigen receptor found on the surface of T lymphocytes, known as the T cell antigen receptor (TCR) (reviewed by Clevers *et al.*, 1988; Klausner *et al.*, 1990). The TCR is currently viewed as a complex of eight transmembrane proteins (de la Hera *et al.*, 1991; Manolios *et al.*, 1991). These consist of four dimers including: (i) the clonotypic heterodimer (generally either $\alpha\beta$ or $\gamma\delta$), (ii) two CD3 dimers (CD3$\epsilon\gamma$ and CD3$\epsilon\delta$) and (iii) a ζ family dimer. The actual stoichiometry of a functional surface complex (i.e. how many ζ dimers/complex) has not been established. The ζ family of proteins has three known members: ζ, η and γ. Zeta is a 16 kDa non-glycosylated protein and is the predominant member expressed in T cells (Samelson *et al.*, 1985; Oettgen *et al.*, 1986; Weissman *et al.*, 1986, 1988b). A minor variant of ζ, called η, results from the use of an alternative last exon of the ζ gene (Baniyash *et al.*, 1988; Orloff *et al.*, 1989; Jin *et al.*, 1990). Finally, the γ chain of the Fcϵ receptor (FcϵR1-γ) is encoded by a distinct gene (Blank *et al.*, 1989; Küster *et al.*, 1990). The genes encoding ζ and γ are both found on chromosome 1 (in both human and mouse) and share a similar intron/exon structure (ζ has eight exons while γ has five, lacking the homologs of ζ exons 5-7) (Weissman *et al.*, 1988a; Baniyash *et al.*, 1989; Huppi *et al.*, 1989; Letourneur *et al.*, 1989; Küster *et al.*, 1990). In cells expressing ζ and η, both disulfide-linked homodimers and $\zeta\eta$ heterodimers are formed (Baniyash *et al.*, 1988). In addition, T cell lines have been described which express all three proteins, and all disulfide-linked dimer combinations are observed (Orloff *et al.*, 1990).

An advantage of building receptors by the assembly of multiple subunits is the ability to mix and match those subunits to generate receptor diversity. Assuming that individual subunits have characteristic functional abilities, such mixing allows a cell to construct different receptors with differing ligand specificity and/or signaling potentials. As a population, T cells possess one of two major classes of TCR complexes defined as containing either $\alpha\beta$ or $\gamma\delta$ clonotypic chains. These heterodimers provide the ligand specificity of the receptor. In either case, these subunits are part of the more complex assembled oligomer described above. A striking example of subunit diversification is in the expression of the ζ chain which is found in TCR-negative natural killer cells in addition to T cells (Anderson *et al.*, 1989; Lanier *et al.*, 1989). In the former, the ζ dimer assembles with the low affinity Fcγ receptor, FcγRIII, also known as CD16 (Lanier *et al.*, 1989). A summary of the overlapping receptor types that utilize TCR and Fc receptor subunits is shown in Figure 1.

Studies on the assembly of the TCR complex have begun to elucidate the underlying subunit interactions that are in turn most probably reflected in the structure of the mature receptor (Berkhout *et al.*, 1988; Bonifacino *et al.*, 1988; Hall *et al.*, 1991; Manolios *et al.*, 1991). We have recently described several types of interactions between TCR subunits (Manolios *et al.*, 1991). Pairwise interactions most probably form the basis for receptor assembly. Strong pairwise interactions can be observed for $\alpha\beta$ and for the two CD3 dimers, $\epsilon\gamma$ and $\epsilon\delta$. In addition, pairwise interactions can lead

to the stable assembly of individual clonotypic chains with individual CD3 chains (Manolios *et al.*, 1990, 1991). The covalent dimerization of ζ also represents a pairwise interaction, requiring the presence of no other subunits (Weissman *et al.*, 1988b). Other TCR interactions are strictly cooperative. The most striking example of this is the assembly of the ζζ homodimer with the other subunits. No interaction is observed between ζ and either CD3 dimers or αβ dimers. Only when both of the latter are present will ζ assemble. Finally, at least one inhibitory interaction prevents the formation of dimers between CD3 γ and δ. One of the most intriguing aspects of TCR subunit interactions is the ability to dissect the individual molecules to determine the domains or motifs responsible for assembly. For example, αβ assembly is mediated by the external domains of the two proteins, as is the assembly of CD3 dimers. In contrast, the interactions between some of the CD3 chains and clonotypic chains appear to be entirely accounted for by their transmembrane domains (Manolios *et al.*, 1990; Tan *et al.*, 1991). Mutations within the transmembrane domains of ζ or FcεR1-γ suggest that these regions are important for assembly interactions as well (Lanier *et al.*, 1991; Kurosaki *et al.*, 1991; Romeo and Seed, 1991).

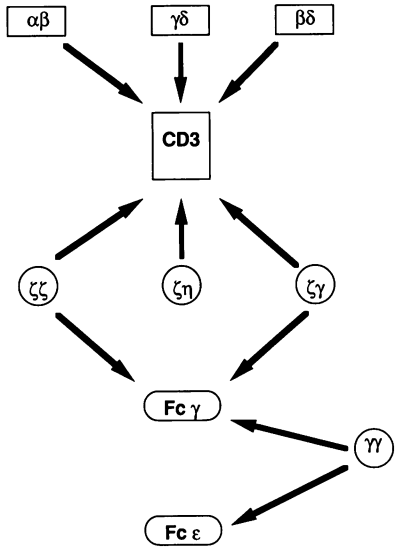
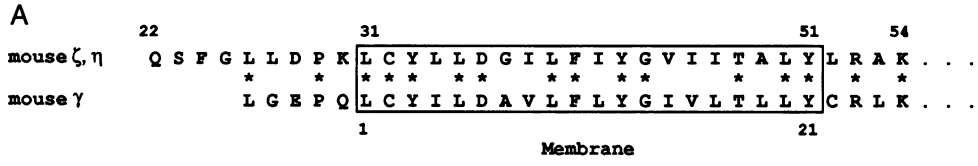


Fig. 1. Subunit diversification of TCR and Fc receptors.

The ability to identify and even to isolate defined sequences in proteins that mediate protein−protein interactions will not only help to describe specific proteins but will potentially yield useful general information on our ability to understand and to engineer protein structure. For these reasons, we have studied the ζ chain in order to determine whether the region of the protein responsible for the efficient formation of disulfide-linked dimers could be so identified. The localization of the single cysteine residue of ζ to the predicted transmembrane region, as well as the ability to form mixed ζγ dimers, led us to examine the transmembrane domain of the protein, as this region shows the greatest identity between ζ and γ. In this study, we demonstrate that it is the transmembrane domain of ζ that is responsible for dimerization and provide evidence for the surprising role of a negatively charged transmembrane residue in this process. In addition, we demonstrate the critical role of the transmembrane region of ζ for reconstituting a functional surface TCR complex.



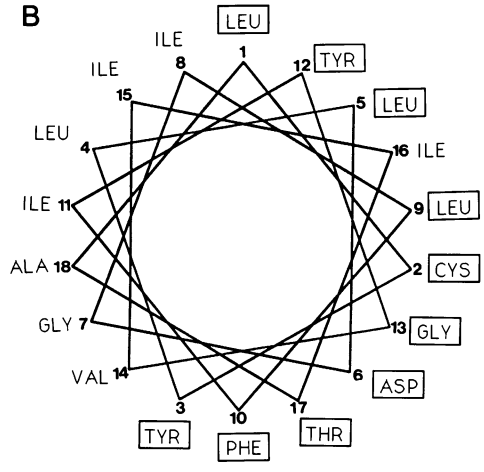


Fig. 2. Conservation of a face of the transmembrane helix in ζ family proteins. (**A**) Amino acid sequences of the transmembrane domains and adjacent segments of ζ family proteins. Numbers on top of the sequence indicate the positions of amino acid residues encoded by the mouse ζ cDNA (Weissman *et al.*, 1988a). Numbers at the bottom denote the positions of transmembrane amino acid residues with respect to the lumenal side of the membrane. (**B**) Helical wheel representation of the mouse ζ transmembrane sequence. Conserved residues between mouse ζ and mouse FcεR1/γ are indicated by boxes.

Results

Helical analysis of ζ and γ transmembrane domains

Zeta family proteins are all type I integral membrane proteins containing N-terminal leader sequences, very short (5–9 amino acids) extracellular domains, a single transmembrane domain followed by a variable length cytoplasmic domain. None of the members of this family are glycosylated and their molecular masses are 7, 16 and 21 kDa for γ, ζ and η respectively. Their transmembrane domains are predicted to contain 21 amino acid residues and are notable for the presence of a cysteine residue at the membrane–extracellular interface which is responsible for disulfide linkage and an aspartic acid residue found four residues C-terminal to the cysteine (Figure 2A). Zeta and γ demonstrate their highest degree of homology within their transmembrane domains (Figure 2A). Twelve and 13 of the 21 amino acid residues in the hydrophobic spans of the two proteins are identical when murine and human sequences respectively are compared. Analysis of this region on a helical wheel structure, a two-dimensional representation of an α-helix, demonstrates that the identical residues are clustered on one face of the helix (Figure 2B). Only one non-identical residue (isoleucine at position 16) is found on the conserved face of the murine ζ and γ chains. Human ζ, however, possesses a leucine residue at this position, completing the helical face identity. This conservation pattern strongly suggests that this region does indeed exist as an α-helix. Not only do ζ and

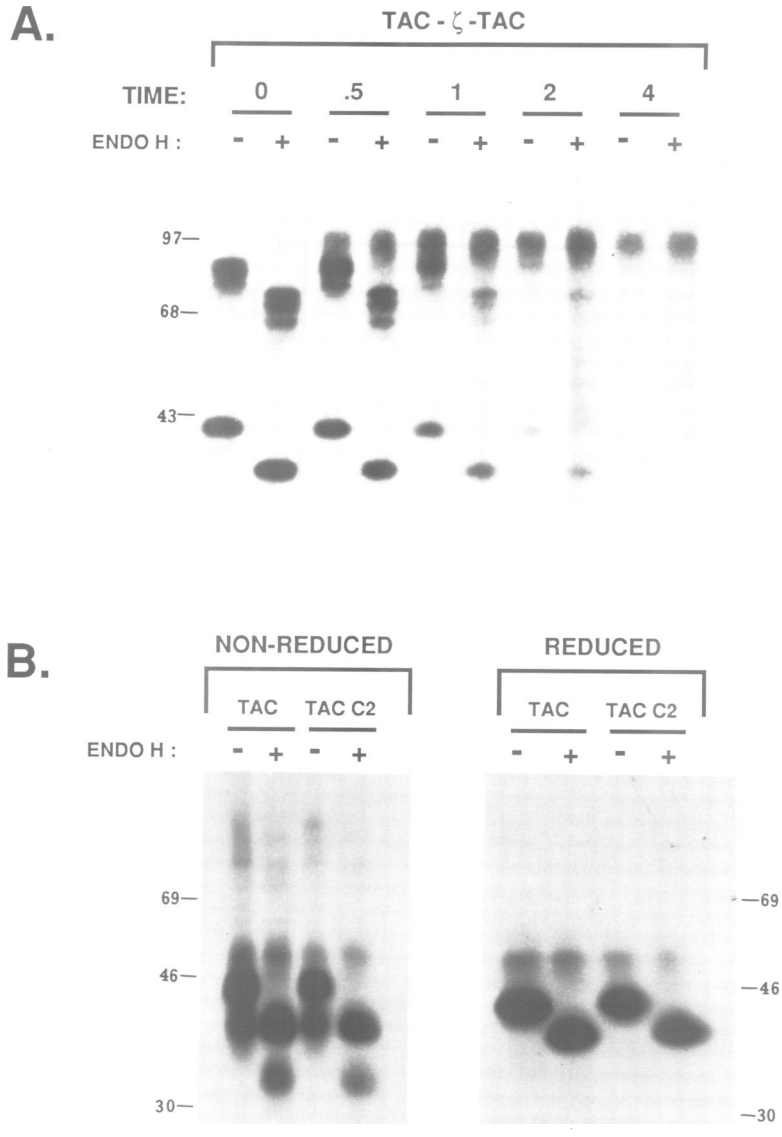


Fig. 3. Zeta transmembrane domain mediates formation of disulfide-linked dimers. (**A**) COS-1 cells transiently transfected with a plasmid encoding Tac-ζ-Tac were pulse-labeled with [^{35}S]methionine for 30 min at 37°C (time 0) and chased for 0.5, 1, 2 or 4 h. The Tac-ζ-Tac proteins were isolated with the anti-Tac monoclonal antibody, 7G7 and immunoprecipitates were either not treated (−) or treated with endo H (+) before analysis by non-reduced SDS–PAGE on 11% acrylamide gels. The positions of mol. wt (M_r) markers, expressed as $10^{-3} \times M_r$, are indicated on the left. (**B**) COS-1 cells were transiently transfected with plasmids encoding normal Tac or Tac C2 and analyzed as described in (A), except that proteins were separated by SDS–PAGE under either reducing or non-reducing conditions. The positions of mol. wt (M_r) markers, expressed as $10^{-3} \times M_r$, are indicated on the left and right.

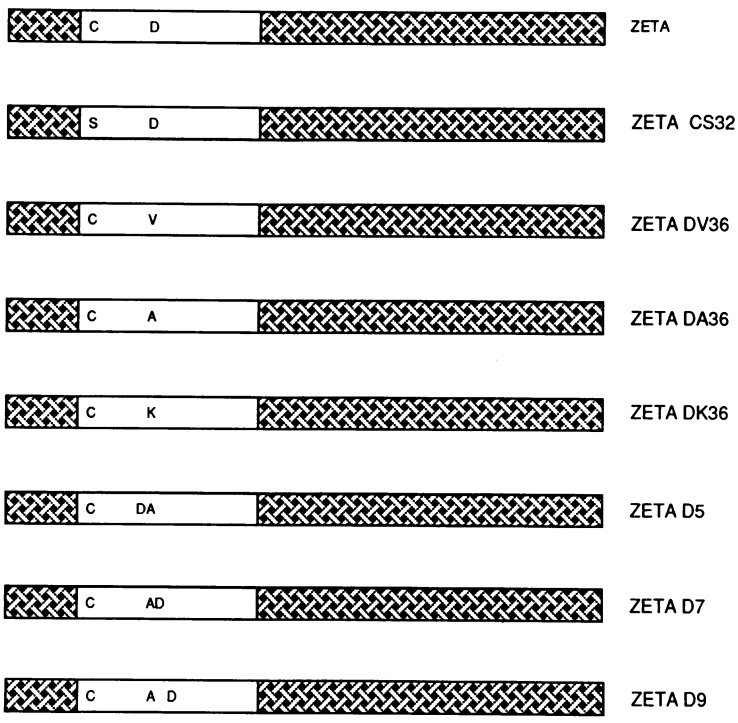


Fig. 4. Schematic representation of ζ transmembrane mutants. Extracellular and cytoplasmic domains of ζ are indicated by hatched boxes. Positions of Cys^{32} and Asp^{36} in the transmembrane domain are indicated. Mutations of these residues in ζ constructs CS32, DV36, DA36 and DK36 are indicated. In D5, D7 and D9, Asp^{36} was replaced by alanine. D5, D7 and D9 introduce an aspartic acid at the fifth, seventh and ninth positions in the transmembrane domain.

γ share the ability to associate with the same multisubunit perceptors, but both proteins are also able to form disulfide-linked homo- and heterodimers. As shown in Figure 2B, the only cysteine residue in the ζ protein is located within the conserved face of the α-helix, at the lumenal boundary of the transmembrane domain, suggesting that this region may also function to mediate disulfide dimerization.

Zeta transmembrane domain is sufficient to mediate formation of disulfide-linked dimers

In order to test whether the ζ transmembrane domain was sufficient to mediate the formation of disulfide-linked dimers, this sequence was placed in the context of an unrelated protein sequence. To this end, a Tac-ζ-Tac chimeric protein consisting of the interleukin-2 (IL-2) receptor α chain (the Tac antigen) with its transmembrane domain replaced by the ζ transmembrane domain was constructed. To assess the ability of the ζ transmembrane domain to induce formation of disulfide-linked dimers, this construct was expressed by transient transfection into COS-1 cells and compared with normal Tac. After pulse−chase metabolic labeling, the cells were lysed and proteins immunoprecipitated with the anti-Tac monoclonal antibody, 7G7, and analyzed on non-reduced SDS−PAGE after treatment with and without endo H. As shown in Figure 3A, the Tac-ζ-Tac chimeric protein forms a disulfide-linked dimer. In several experiments, between 56 and 80% of the chimera were found to be dimerized. Pulse−chase studies demonstrated that only the dimer is capable of leaving the endoplasmic reticulum (ER), as assessed by the acquisition of endo H resistance (Figure 3A). In addition, the dimer appears to be more stable than the Tac-ζ-Tac monomer over the 4 h chase period (Figure 3A). Thus, the presence of a ζ transmembrane domain results in the ER retention of the monomeric chimeric protein. Dimerization abrogates this retention and allows efficient transport to the cell surface. In contrast to the chimeric protein, minimal disulfide-linked dimer formation is observed for the native Tac protein (Figure 3B, 0.7% by densitometry) and the native Tac monomer rapidly exits the ER, as demonstrated by its efficient acquisition of endo H resistance during the chase (data not shown).

As mentioned above, the single cysteine residue in ζ is predicted to be located at the lumenal end of the transmembrane domain. To evaluate whether this position is particularly susceptible to intermolecular disulfide bonding, a mutant Tac protein, Tac-C2, with a cysteine residue in the analogous position as in the ζ chain was generated using M13 based mutagenesis. This construct was then analyzed for its ability to form disulfide-linked homodimers. As seen in Figure 3B, the Tac-C2 mutant is indistinguishable from the normal Tac protein in terms of the low level of dimers formed. Thus, the motif in the ζ transmembrane domain leading to the formation of disulfide-linked dimers most likely consists of more than a critically located cysteine residue. Most probably, other amino acid residues present in the ζ transmembrane domain must interact and correctly orient the transmembrane cysteine residue to allow disulfide bond formation to occur.

Mutational analysis of zeta transmembrane domain

To analyze the role of other residues in the ζ transmembrane domain in the formation of disulfide-linked dimers, mutagenesis of the ζ chain was performed (Figure 4). As shown in Figure 5, >80% dimerization is seen when normal ζ is transfected into 2M2 cells (murine T hybridoma cells

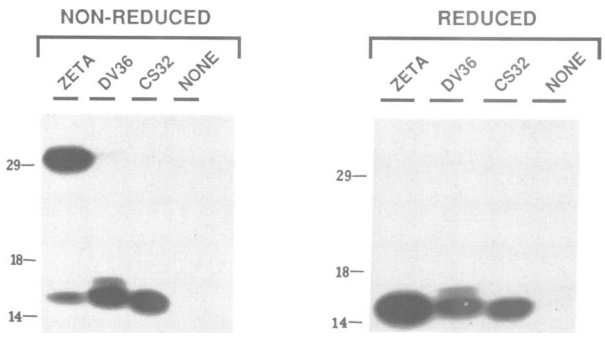


Fig. 5. Mutation of transmembrane cysteine and aspartic acid residues alters ζ disulfide dimerization. 2M2 cells were transiently transfected using DEAE/dextran with plasmids encoding ζ, DV36, CS32 or with no insert. Cells were metabolically pulse-labeled with [^{35}S]methionine for 30 min at 37°C. Zeta proteins were isolated with a polyclonal anti-ζ peptide antisera (no. 386), and immunoprecipitates were analyzed by non-reducing and reducing SDS−PAGE on 13% acrylamide gels. The positions of mol. wt (M_r) markers, expressed as $10^{-3} \times M_r$, are indicated on the left.

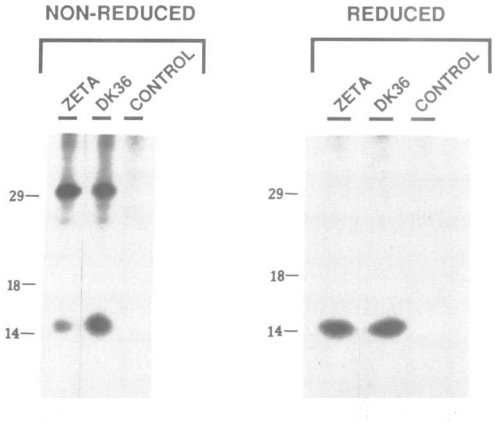


Fig. 6. A positively charged transmembrane residue is able to mediate formation of ζ disulfide-linked dimers. 2M2 cells were transiently transfected using DEAE/dextran with plasmids encoding normal ζ, DK36 or Tac (control). Cells were metabolically pulse-labeled with [^{35}S]methionine for 45 min at 37°C. Zeta proteins were isolated with a polyclonal anti-ζ peptide antisera (no. 386), and immunoprecipitates were analyzed by non-reducing and reducing SDS−PAGE on 13% acrylamide gels. The positions of mol. wt (M_r) markers, expressed as $10^{-3} \times M_r$, are indicated on the left.

expressing no endogenous ζ). This dimer is completely reducible by 3% 2-mercaptoethanol. As expected when Cys^{32} is replaced by a serine residue (to produce the construct termed CS32), no disulfide-linked dimers are formed. Perhaps the most striking characteristic of the transmembrane domain of ζ is the presence of a negatively charged amino acid residue (Asp^{36}). Importantly, all of the invariant chains of the TCR complex contain similarly placed acidic residues, while the clonotypic chains possess basic transmembrane residues. We have shown that these residues can explain some of the assembly interactions between TCR subunits (Cosson *et al.*, 1991). Using an immunoprecipitation assay, we have demonstrated that a charge pair can mediate the stable assembly of two proteins when charged residues are placed within the hydrophobic stretch of amino acids that define the transmembrane domains of the proteins. In these studies, we failed to see stable assembly when two identically charged residues were present rather than a charge pair. Based upon this observation, we expected that removal of the Asp^{36} residue would not abrogate dimerization, and might even enhance it. Surprisingly, when this residue was mutated to valine (DV36), little, if any disulfide dimerization was observed (Figure 5). There is a small quantity of the DV36 protein present in a dimerized form; however, this complex does not represent a normal disulfide-linked dimer, in that it is not reducible by 2-mercaptoethanol. Analysis of normal ζ, and the mutants CS32 and DV46 in pulse−chase experiments demonstrated that all three proteins are stable over a 6 h chase period (data not shown). Expression of the DV36 mutant results in the additional presence of a more slowly migrating form of the monomeric protein. What this increase in protein size corresponds to is unclear, although it is occasionally seen with the native ζ protein.

Role of the transmembrane charged residue in disulfide dimerization

To evaluate further the role of the transmembrane charged residue in ζ disulfide-linked dimerization, additional ζ transmembrane mutants were constructed as depicted in Figure 4). The transmembrane charged residue was first mutated to another neutral amino acid residue (alanine) and, like DV36, the mutant DA36 failed to dimerize, when transiently transfected into 2M2 cells (data not shown). We next examined the consequences of replacing the aspartic acid residue with a lysine (mutant DK36). Strikingly, transient transfection of the DK35 cDNA into 2M2 cells demonstrated ~50−60% disulfide-linked dimer formation (Figure 6). Although the efficiency of DK36 dimer formation was somewhat reduced compared to normal ζ, this result suggests that it is the presence of a charged residue at this position rather than the aspartic acid *per se* that is required for efficient disulfide dimer formation.

We next asked whether the exact position of the aspartic acid residue would affect dimerization. To evaluate this, the aspartic acid residue was moved around the transmembrane α-helix (Figure 4). In the mutants D7 and D4, the aspartic acid residue is positioned outside of the conserved helical face, while in D5 and D9 the transmembrane charge is retained within this region. In D5, the aspartic acid residue is placed in an analogous position on the other side of Cys^{32}. Figure 7 shows a [^{35}S]methionine metabolic labeling and anti-ζ immunoprecipitation study of these constructs transiently transfected into COS-1 cells, analyzed under reducing and non-reducing conditions. Densitometric analysis of these data demonstrates that each construct has a differential capacity for disulfide dimer formation. D4 and D5 give a disulfide dimerization efficiency essentially identical to wild-type ζ (74 and 83% versus 89% respectively), while both D7 (18%) and D9 (6.4%) are significantly impaired in this capacity (Figure 7). There is consistently less D9 protein present, perhaps because of degradation of the protein induced by the placement of the aspartic acid residue at position 9 (Bonifacino *et al.*, 1991).

A cysteine and a charged amino acid residue are necessary but not sufficient to mediate disulfide dimerization

To determine whether appropriately placed cysteine and aspartic acid residues were sufficient to mediate disulfide-

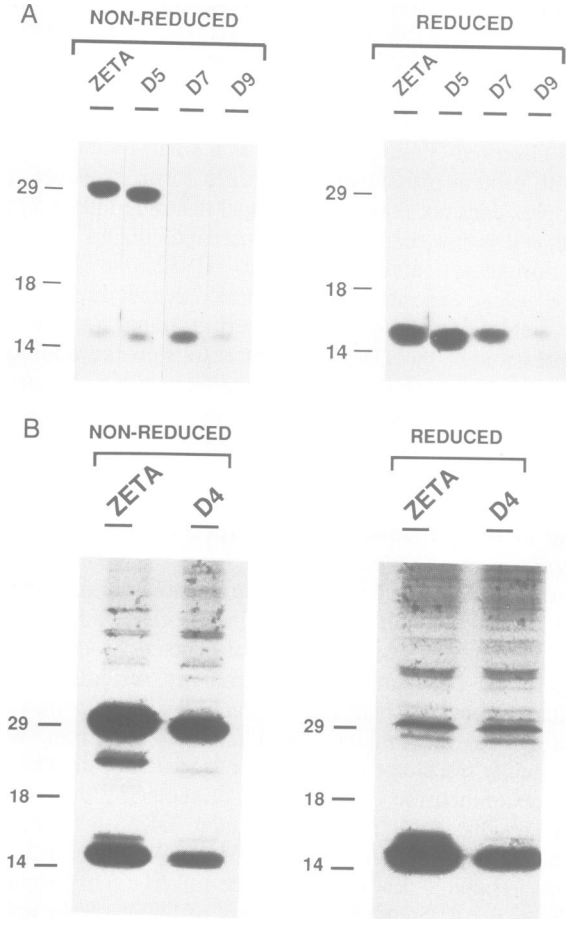


Fig. 7. Spatial location of the transmembrane charged residue affects its ability to mediate formation of disulfide-linked dimers. (**A**) COS-1 cells were transiently transfected with plasmids encoding normal ζ, D5, D7 or D9. Cells were metabolically pulse-labeled with [^{35}S]methionine for 45 min at 37°C. Zeta proteins were isolated with an affinity-purified anti-ζ peptide antisera (no. 386), and immunoprecipitates were analyzed by non-reduced and reducing SDS−PAGE on 13% acrylamide gels. The positions of mol. wt (M_r) markers, expressed as $10^{-3} \times M_r$, are indicated on the left. (**B**) Disulfide linkage of the D4 mutant in comparison with normal ζ was analyzed in a separate experiment, as described in (A).

linked dimerization, mutations were introduced into the Tac transmembrane domain using an M13 based method which placed a cysteine/aspartic acid pair at positions analogous to those seen in the native ζ protein (construct Tac C2D6) Additionally, the endogenous cysteine residue at transmembrane position 8 in the Tac protein was replaced with an alanine residue. A control construct, Tac CA8, with only the latter mutation, was also generated. These constructs, plus Tac-ζ-Tac, were transiently transfected into BW5147 cells, a thymoma cell line. Cells were then metabolically labeled with [^{35}S]methionine for 30 min, chased for different periods, lysed and immunoprecipitated with the anti-Tac monoclonal antibody, 7G7, and analyzed after treatment with or without endo H on non-reducing SDS−PAGE (Figure 8). Lanes 5 and 6 demonstrate the usual extent of disulfide dimerization seen in cells transfected with the Tac-ζ-Tac construct (~50%). As mentioned above, only the dimeric form achieves endo H resistance, indicating that it is exiting the ER and reaching the Golgi apparatus. In cells transfected with either Tac CA8 or Tac C2D6, lanes 1−4,

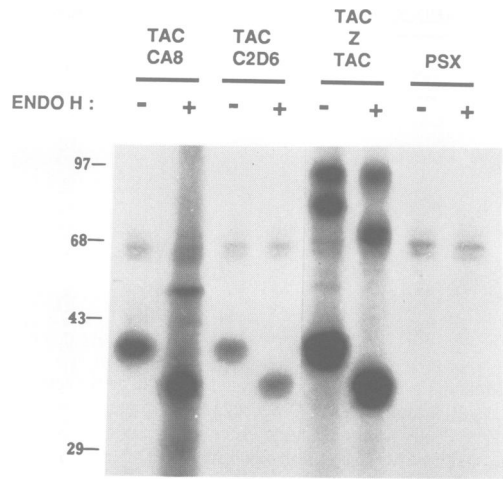


Fig. 8. A cysteine/aspartic acid pair is not sufficient to mediate formation of disulfide-linked dimers. BW5147 cells were transiently transfected with plasmids encoding Tac CA8, Tac C2D6, Tac-ζ-Tac or no insert (pSX) using DEAE/dextran. Transfected cells were metabolically pulse-labeled for 30 min at 37°C. Tac proteins were isolated with the anti-Tac monoclonal antibody, 7G7, and immunoprecipitates were either not treated (−) or treated with endo H (+) before analysis by non-reducing SDS−PAGE on 11% acrylamide gels. The positions of mol. wt (M_r) markers, expressed as $10^{-3} \times Mr_r$, are indicated on the left.

no disulfide-linked dimers are detectable. Additionally, no endo H resistant proteins are seen suggesting that these constructs are defective in traversing the secretory pathway. To assess whether these constructs can reach the cell surface, flow cytometry was used. Figure 9 shows a fluorescence activated cell sorting (FACS) profile of the transfected cells described above. A control transfectant of vector without insert was also performed and its negative receptor profile is shown in bold face. In the cells transfected with Tac-ζ-Tac, cell surface expression of the chimeric protein is detected with 7G7 antibody staining (Figure 9C). There is also a cell surface positive population present in Tac CA8 (Figure 9A), although the acquisition of endo H resistance was not demonstrated in the metabolic pulse labeling. However, the presence of this construct on the cell surface demonstrates that, as a monomer, Tac CA8 is competent to transverse the ER and Golgi apparatus. In cells transfected with Tac C2D6, cell surface receptor expression does not occur (Figure 9B). The FACS profile of the negative control transfectant superimposes the Tac C2D6 FACS profile, indicating that the cysteine/aspartic acid pair in the Tac transmembrane domain has prevented cell surface receptor expression and has introduced a retention determinant in the normally cell surface bound Tac protein. These results indicate that a transmembrane cysteine/aspartic acid pair is necessary but not sufficient for disulfide dimerization. Additional residues in the transmembrane domain appear to be necessary to complete the ζ transmembrane dimerization motif.

A transmembrane charged residue is also required for TCR surface expression

Besides sharing the ability to form disulfide-linked dimers, the ζ gene family proteins can also associate with the TCR complex (Orloff *et al.*, 1990). To analyze the effects of

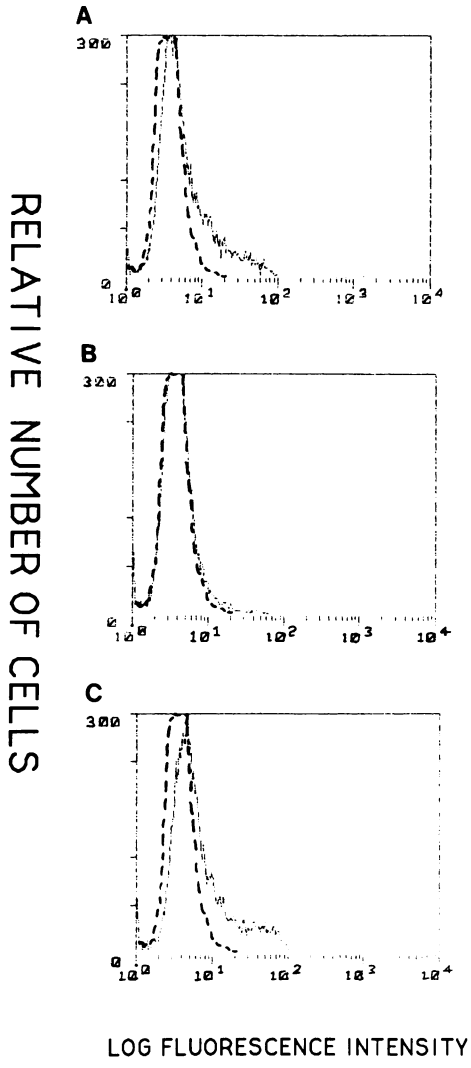


Fig. 9. Placement of a cysteine/aspartic acid pair prevents cell surface expression of the Tac protein. BW5147 cells were transiently transfected with plasmids encoding Tac CA8 (**A**), Tac C2D6 (**B**), Tac-ζ-Tac (**C**) or no insert using DEAE/dextran. Transfected cells were analyzed for surface protein expression using the monoclonal antibody 7G7, followed by a fluoresceinated goat anti-mouse second antibody. The surface negative profile for the no insert control transfection is indicated in each panel by the thick dashed line.

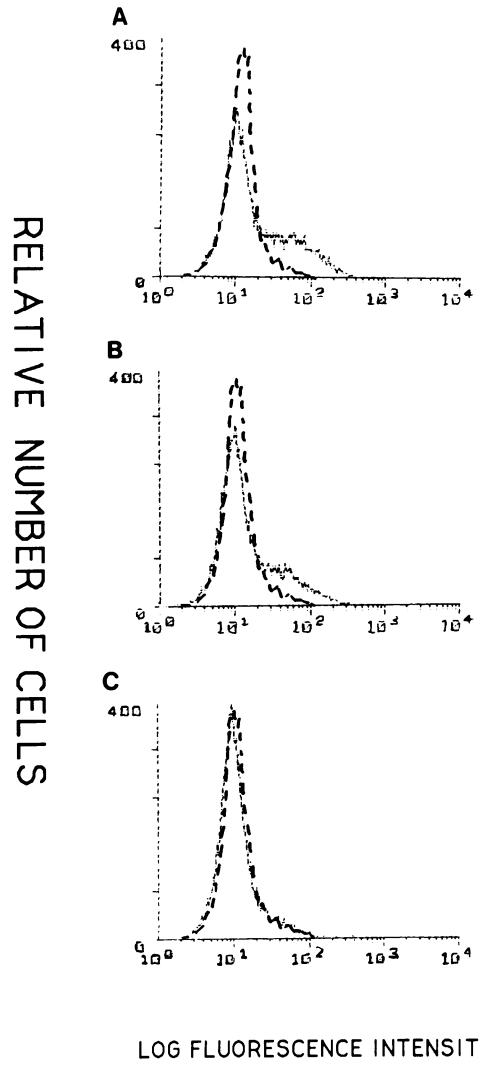


Fig. 10. The cysteine-negative mutant, but not the chargeless mutant can mediate TCR surface expression. 2M2 cells were transiently transfected using DEAE/dextran with plasmids encoding normal ζ (**A**), CS32 (**B**), DV36 (**C**) or no insert. Transfected cells were analyzed for TCR surface expression using the monoclonal anti-TCR antibody, A2B4-2, followed by a fluoresceinated goat anti-mouse second antibody. The surface negative profile for the no insert control transfection is indicated in each panel by the thick dashed line.

mutations of the ζ transmembrane domain on TCR surface expression, cDNAs for normal ζ, CS32 and DV36 were transiently transfected into the 2M2 ζ-deficient variant. The transfected cells were then analyzed by flow cytometry with the anti-α monoclonal antibody, A2B4, to assess TCR surface expression (Figure 10). Transfection efficiencies of ~20% were attained using this method. FACS analysis of transfected cells demonstrated that ζ is able to restore TCR surface receptor expression in 2M2 cells (Figure 10A), as previously reported (Weissman *et al.*, 1989). The CS32 construct, lacking a transmembrane cysteine residue, was capable of mediating comparable cell surface receptor expression (Figure 10B). Thus, the formation of a disulfide-linked dimer is not necessary for TCR assembly or surface expression. However, a requirement for non-covalent dimers has not been excluded. Additionally, ζ and CS32 transfectants were analyzed for their ability to respond to activating ligands; both constructs produced receptor complexes that were responsive to antigen, anti-Thy-1 and anti-receptor antibody stimulation. The amount of IL-2 produced as well as their dose–response curves were comparable in ζ and CS32 transfectants (data not shown). Interestingly, 2M2 cells transfected with DV36, lacking the negatively charged transmembrane residue, did not demonstrate any increase in cell surface receptor expression (Figure 10C). Whether a primary defect exists in DV36's association with the other TCR components or there is a secondary inability of the completely assembled receptor complex to negotiate the secretory pathway has not been analyzed. The inability of the DV36 construct to mediate TCR surface expression could result solely from its failure to dimerize. To investigate this possibility, mutant ζ constructs that were capable of dimerizing were analyzed for their ability to mediate TCR surface expression. DK36, D5, D7 and D9 were transfected into 2M2 cells and analyzed for cell surface receptor expression by FACS analysis with

an anti-α monoclonal antibody. None of the above listed constructs was able to increase cell surface receptor expression in 2M2 cells (Figure 11), indicating that despite their ability to disulfide dimerize to varying levels, no productive associations with the other components of the TCR occurred.

Discussion

Conservation of amino acid residues between families of related proteins often delineates regions important for shared functions. The identical distribution of amino acid residues on one face of the transmembrane domains of the ζ gene family of proteins is likely to be significant for their ability to form disulfide-linked dimers or for their interaction with CD16 or the TCR. Evidence already exists that within this region there are critical residues for CD16 association (Lanier *et al.*, 1991; Kurosaki *et al.*, 1991; Romeo and Seed, 1991). Murine ζ, a natural transmembrane mutant, illustrates this point (Kurosaki *et al.*, 1991). Human, but not murine, ζ is capable of mediating CD16 cell surface receptor expression. When the non-conserved isoleucine in murine ζ is mutated to a leucine residue, murine ζ's ability to mediate CD16 cell surface receptor expression is restored, localizing critical interactions with CD16 to this transmembrane residue. This leucine is not the only transmembrane residue necessary for ζ's association with CD16. Recent studies (Lanier *et al.*, 1991; Romeo and Seed, 1991) have demonstrated a dependence upon a transmembrane aspartic acid residue in mediating interactions with CD16. In a chimeric protein system, utilizing the CD4 extracellular domain fused to the ζ transmembrane and cytoplasmic domains, CD16 association and cell surface receptor expression occur in the presence of the chimeric protein. However, mutation of the transmembrane aspartic acid to a neutral amino acid residue in the chimera abrogated CD16 surface expression. Similar mutations in the ζ chain have also been shown to decrease CD16 cell surface receptor expression.

In this study, we have demonstrated that the ζ transmembrane domain is responsible for its ability to form disulfide-linked dimers. By creating a chimeric Tac-ζ-Tac construct, we have shown that the transmembrane domain is sufficient to mediate disulfide dimerization and that this phenotype is transferable to other proteins, solely via the transmembrane domain. The cysteine at position 32 is the only cysteine residue present in the protein and, therefore, must mediate the disulfide linkage. However, as with other proteins, disulfide linkage presumably occurs subsequent to protein–protein interactions mediated by other specific non-covalent interactions. The most unusual and striking feature of the transmembrane domain of ζ is the aspartic acid residue at position 36. Such a residue is quite uncommon in most membrane spanning domains, but is characteristic of the invariant chains of the TCR (Clevers *et al.*, 1988). Although we have shown that potential charge pairs within the transmembrane domains of single membrane-spanning proteins can mediate stable protein assembly (Cosson *et al.*, 1991), we did not expect that the aspartic acid residue would aid in the dimerization of ζ. In fact, we wondered whether removal of this residue might even enhance dimerization. Thus we were surprised that replacing it with either of two hydrophobic residues resulted in essentially complete loss of dimerization. Perhaps the side chain of this residue

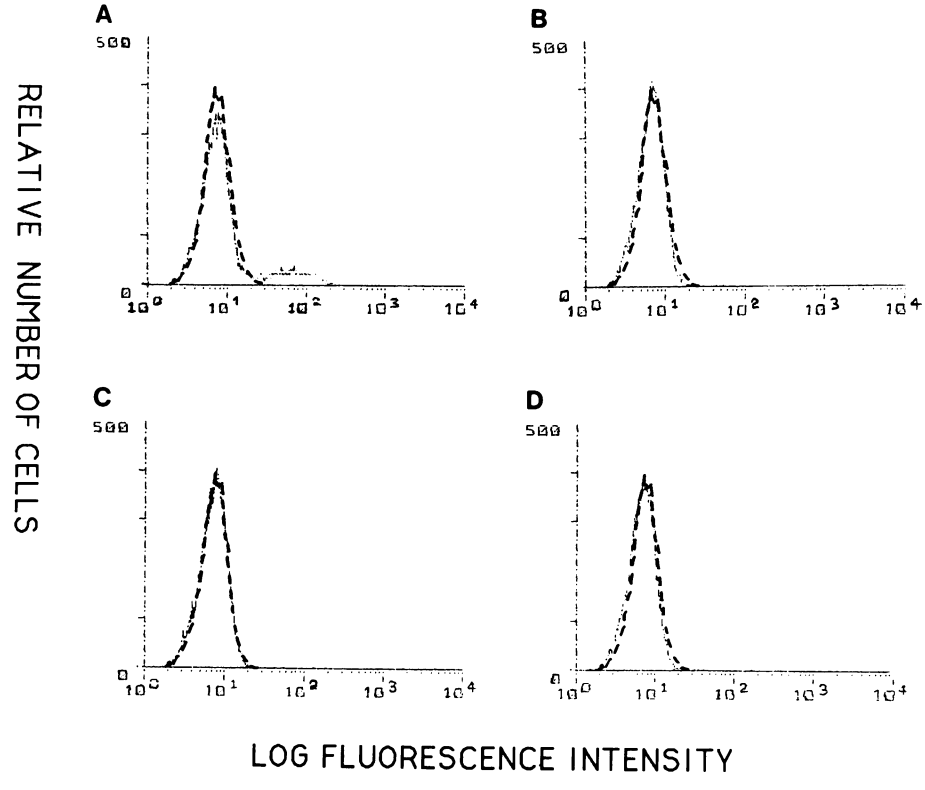


Fig. 11. Transmembrane charge requirements for surface expression of the TCR complex. 2M2 cells were transiently transfected using DEAE/dextran with plasmids encoding normal ζ (**A**), D7 (**B**), D5 (**C**), DK36 (**D**) or no insert. Transfected cells were analyzed as described in the legend to Figure 10.

possesses some specific interactions with the ζ transmembrane helix to promote dimer formation. Although we cannot rule this out, two observations mitigate against this view. First, the aspartic acid residue can be moved to either transmembrane position 4 or 5 with little loss of dimerization and, second, it can be replaced with a lysine residue with preservation of ζ dimerization. The ability to replace the aspartic acid with a lysine residue suggests that it is the location of a potentially charged residue within the ζ helix that is essential for dimerization.

As mentioned earlier, the transmembrane domain is most likely, on first principles, to exist as an α-helix (Singer, 1990). The conservation of one face of a proposed α-helix between ζ and γ provides an additional argument for such a conformation for this domain. Given the likelihood of an α-helix, the location of the cysteine residue orients the conserved face of the helices as the dimerization face. How might we rationalize the role of the aspartic acid residue at transmembrane position 6 in the dimerization process? It seems likely that the presence of an aspartic acid residue within the putative transmembrane domain would be disruptive to the interactions between the lipid bilayer and the otherwise hydrophobic protein domain. One could imagine that the energetic cost of burying these residues within the bilayer could be offset if they resulted in a disruption of the local lipid structure, perhaps allowing some water to penetrate or if they resulted in the repositioning of the transmembrane domain such that these residues were displaced towards the membrane interface (Singer, 1990). The result of either of these perturbations might be to place the critical cysteine in a more polar environment where the requisite formation of the cysteinyl anion intermediate for disulfide bonding would be more favorable. Thus, aspartic acids at positions 4, 5 or 6 could all promote dimerization, while placement of the acidic residue beyond position 6 may be too far removed from either the cysteine or the lipid−aqueous interface to be effective.

While the presence of a suitably placed charged residue is important for dimerization, the inability to reproduce such dimerization by the presumably identical placement of both an aspartic acid and a cysteine in the transmembrane domain of Tac points to additional specificity of the transmembrane sequence of ζ in dimerization. Although this may be true, the nature of these putative interactions is obscure. These residues have, for the most part, hydrophobic side chains. Such hydrophobic residues have been shown to be the essential elements of protein dimerization motifs in soluble proteins (Johnson and McKnight, 1989). In this case (i.e. the leucine zipper), it is through hydrophobic interactions that are relevant in the aqueous environment. In the ζ transmembrane domain, these residues would be expected to be in contact with lipid which might obscure protein−protein interactions. Presumably, successful packing of the specific side chains in ζ would provide additional stabilization and even help with the entropic problem of packing lipid acyl chains onto the irregular surface of the protein (Engelman *et al.*, 1986). Whatever the details of the biophysics of this transmembrane dimerization, the identification of this sequence as a transplantable motif for the production of efficient covalent dimers in a physically distinct domain of transmembrane proteins provides the first example of the ability to engineer the dimerization of membrane proteins predictably. The introduction of such a motif into other proteins would be of use in assessing the role of dimerization in signal transduction and the production of novel heterodimers.

In addition to dimerization, the transmembrane domain of ζ is clearly important in its assembly with the rest of the TCR complex and in allowing the full complex to be successfully transported to the plasma membrane. These results are not surprising and are in keeping with previous results on the assembly of ζ and γ with Fc receptor subunits (Kurosaki *et al.*, 1991; Lanier *et al.*, 1991; Romeo and Seed, 1991). Our results suggest that, in relation to the transmembrane aspartic acid residue, the requirements for successful assembly of a surface TCR complex are much more stringent than for dimerization. Of all the transmembrane mutants described in this study, only normal ζ and CS32 will assemble with the other components of the TCR complex in a manner that results in transport to the cell surface. We suspect that the CS32 mutant exists as a non-covalent dimer but we have no direct data on this point. From our previous studies, we would expect that the aspartic acid at position 36 might interact with the basic residues present within the transmembrane domains of the α and β chains. However, experiments to examine this directly have shown that ζ cannot assemble to produce stable interactions with either α, β or αβ pairs (Manolios *et al.*, 1991). Thus, the aspartic acid residues in the ζ dimers are not directly available for transmembrane charge pair mediated assembly. If the aspartic acid residue interacts with the basic residues of the clonotypic chains, it may be that the cooperative interactions necessary for ζ assembly into the complex are required to form these charge pairs.

Materials and methods

Cell lines and antibodies

COS-1 cells, green monkey fibroblasts, obtained from the American Type Culture Collection, Rockville, MD, were grown in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum and 0.15 mg/ml gentamicin. 2M2 cells are a ζ/η negative variant of the pigeon cytochrome *c*-specific hybridoma, 2B4, obtained as originally described by Sussman *et al.* (1988). The BW5147 cell line is a murine T cell thymoma (White *et al.*, 1989). 2M2 and BW5147 cells were grown in RPMI 1640 medium supplemented with 8% fetal calf serum. A2B4 is a mouse monoclonal antibody against a clonotypic determinant present in the Vα domain of the 2B4 α chain (Samelson *et al.*, 1983). Anti-ζ peptide antisera were generated against cytoplasmic sequences, as described originally by Orloff *et al.* (1989). The 7G7 antibody is a mouse monoclonal antibody against an epitope localized to the extracellular domain of the human Tac antigen (Rubin *et al.*, 1985).

Recombinant DNA procedure

A mutant Tac protein was constructed to introduce a *Bgl*II site at the N-terminal end of the transmembrane domain. This mutant Tac cDNA, cloned into a modified version of the expression vector pCDM8 (Seed, 1987; Bonifacino *et al.*, 1990), was digested with *Bgl*II and *Xba*I, removing sequences encoding the Tac transmembrane and cytoplasmic domains. Phosphorylated oligonucleotides encoding the ζ transmembrane domain fused to the Tac cytoplasmic tail were ligated into the cut pCDM8-Tac vector. For T cell studies, the Tac-ζ-Tac construct was cloned into the expression vector pCDL-SRα as described by Takebe *et al.* (1988). Mutations in the transmembrane domain of ζ and Tac (CS32, DV36, DK36, D5, D7, D9, Tac C2, Tac CA8, Tac C2D6) were made using oligonucleotide-directed mutagenesis on M13 vectors (Kunkel, 1985). All of these constructs were subcloned into the expression plasmid pCDL-SRα.

Transfections

Transient transfection studies on COS-1 cells were performed using the calcium phosphate precipitation method (Graham and Van der Erb, 1973), as described by Bonifacino *et al.* (1989). Transient transfections into T cell

lines were performed using the DEAE/dextran method as described by Selden *et al.* (1991).

Radiolabeling, immunoprecipitation and electrophoresis

Transfected COS-1, BW5147 or 2M2 cells were metabolically labeled with 5 ml of 0.5 mCi/ml [^{35}S]methionine for 30–45 min at 37°C. Whenever indicated, pulse-labeled cells were chased for different periods in regular culture medium. Cells were solubilized in 1 ml lysis buffer [0.5% (w/v) Triton X-100, 0.3 M NaCl, 50 mM Tris–HCl buffer (pH 7.4)] and proteins immunoprecipitated by protein A-bound antibodies at 4°C. When indicated, immunoprecipitates were treated with endoglycosidase H (endo H, Genzyme, Boston, MA) as described previously by Chen *et al.* (1988). Proteins were resolved by one-dimensional SDS–PAGE. Densitometric scanning of autoradiograms was performed using an LKB Ultroscan enhanced laser densitometer.

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