Previous studies have shown that the presence of potentially charged amino acid residues within the transmembrane domains of type I integral membrane proteins can result in protein retention and, in some cases, degradation within the endoplasmic reticulum (ER). An apparent exception to this observation is the CD3- chain of the T-cell antigen receptor complex, which is relatively stable in spite of having a transmembrane aspartic acid residue. A chimeric protein (T:T) made by replacing the transmembrane domain of the Tac antigen with that of CD3- was normally transported to the cell surface, indicating that the transmembrane domain of CD3- was essentially unable to confer the phenotype of ER retention and degradation to another protein. Progressive shortening of the T:T transmembrane domain, however, resulted in increasing retention and degradation of the mutant proteins in the ER. Conversely, a mutant Tac protein containing a single aspartic acid residue in its transmembrane domain was found to be retained and degraded in the ER, but when the transmembrane domain was lengthened, ER retention and degradation of the protein were abrogated. The aspartic acid residue in the transmembrane domain of all of these mutant proteins could mediate assembly with another protein having an arginine residue in its transmembrane domain, independent of the length of the transmembrane sequence. These findings demonstrate that the length of the hydrophobic transmembrane sequence has a critical influence on the ability of potentially charged transmembrane residues to cause protein retention and degradation in the ER.

Over the past several years, research on a number of experimental systems has brought to light some general rules that relate the structure of newly synthesized proteins to their fate within the cell. In particular, a large amount of information concerning proteins that are inserted into the central vacuolar system at the level of the endoplasmic reticulum (ER) has implicated quality control systems that prevent transport of abnormally or incompletely assembled proteins through the secretory pathway (Rose and Doms, 1988; Hurttley and Helenius, 1989; Klausner, 1989). Our laboratory has been utilizing the multicomponent T-cell antigen receptor (TCR) complex as a model with which to examine the molecular basis for this quality control. These studies have shown that unassembled chains or partially assembled complexes of the TCR are in many cases retained within the ER. Some of the chains retained within the ER are subsequently degraded by a nonlysosomal pathway (reviewed by Klausner and Sipta (1990)). For efficient quality control, cells must be able to distinguish proteins destined for retention and degradation from those that will survive and be transported out of the ER. Thus, retention and degradation within the ER must be highly selective processes. Understanding this selectivity has motivated our search for sequence and/or structural characteristics that target a protein for ER retention and degradation. Identification of these putative signals could give insight into the mechanism of this cellular process that may regulate the fate of a variety of proteins within the ER in addition to serving a role in quality control.

We began the search for determinants of ER retention and degradation by analyzing the fate of normal and mutagenized forms of the TCR- chain. When expressed in T-cells that fail to synthesize other subunits of the receptor or in transfected fibroblasts, this chain is retained in the ER and subjected to degradation (Lippincott-Schwartz et al., 1988; Bonifacino et al., 1989). It was discovered that a determinant localized to the putative transmembrane domain (TMD) of the TCR- chain was responsible for targeting the protein to ER degradation and that this determinant could be transferred to an unrelated protein (Bonifacino et al., 1990a). Two positively charged amino acid residues in the putative transmembrane sequence of the TCR- chain were found to be critical for retention and degradation within the ER (Bonifacino et al., 1990b). We then demonstrated that placing a single potentially charged amino acid residue within the TMD of the Tac antigen (interleukin-2 receptor chain (Leonard et al., 1984) converted this protein from one that was normally delivered to the cell surface to one that was retained and degraded within the ER (Bonifacino et al., 1991). Either basic or acidic residues could confer this phenotype. The efficacy of such residues was exquisitely sensitive to the position of the potentially charged residue in the TMD of Tac. Despite the ability to transfer the degradation phenotype with a single charged residue to Tac, however, we did not know whether another undefined characteristic of Tac was necessary, although not sufficient, for ER degradation. This latter possibility was strengthened by the observation that another chain of the T-cell antigen receptor complex, CD3- , which contains

The abbreviations used are: ER, endoplasmic reticulum; BFA, brefeldin A; endo H, endoglycosaminidase H; TCR, T-cell antigen receptor; TMD transmembrane domain; Tac, interleukin-2 receptor a chain; FCS, fetal calf serum; PBS, phosphate-buffered saline.
an aspartic acid residue in its TMD and, thus, would be expected to undergo ER degradation was, in fact, stably retained in the ER (Bonifacino et al., 1989; Wileman et al., 1990).

In the present study, we have examined the characteristics that distinguish the TMD of CD3-ε from that of a Tac mutant containing an aspartic acid residue to assess whether other definable features, in addition to the nature of the charged amino acid and its position within the TMD, determine the assembly and fate of integral membrane proteins within the ER. The results of these experiments demonstrate that the length of the transmembrane sequence is a critical factor that determines the efficacy with which potentially charged amino acid residues cause protein retention and degradation within the ER.

MATERIALS AND METHODS

Construction and Transformation of Mutant DNAs—Mutant Tac cDNAs were constructed by annealing complementary oligonucleotides encoding TMD sequences and subcloning these fragments into a modified Tac CDNA from which the TMD region had been removed, as previously described (Cosson et al., 1991). The sequences of the TMDs of the different constructs are shown in Table I. The CD3-εR β-galactosidase fusion protein (εR-gal), constructed by polymerase chain reaction mutagenesis, was identical with the β-gal fusion protein previously described (Cosson et al., 1991), but the aspartic acid residue in position 5 of the transmembrane was replaced with an arginine residue. Mutant cDNAs were cloned into a modified version of the pCDM8 expression plasmid (Seed, 1987) as previously described (Bonifacino et al., 1990a). COS-1 cells (American Type Culture Collection, Rockville, MD) were plated on 150-mm culture dishes and transfected with 20 μg of plasmid DNA using the calcium phosphate precipitation method (Graham and Van der Eb, 1973). After 16 h, transfected cells were trypsinized and plated on 35-mm culture dishes and allowed to grow for an additional 24 h.

Metabolic Labeling and Immunoprecipitation—Transfected cells were labeled for 30 min at 37°C with 1 ml of 0.55 μCi/ml [35S]methionine (Trans35S-label, ICN Radiochemicals, Irvine, CA) in methionine-free Dulbecco’s modified Eagle’s medium containing 10% fetal calf serum (FCS), as described previously (Bonifacino et al., 1991). Labeled cells were then chased by incubating at 37°C in Dulbecco’s modified Eagle’s medium containing unlabeled methionine and 10% FCS for 0, 2, or 6 h. At each time point, cells were scraped from the culture plate, pelleted by centrifugation and frozen at -70°C before detergent lysis and immunoprecipitation (Bonifacino et al., 1990a). Brefeldin A (BFA, 5 μg/ml, Epicenter Technologies, Madison, WI) was added to the culture medium where indicated. Digestions with endoglycosidase H (endo H) (Boehringer) were done for 16 h at 37°C, as previously described (Chen et al., 1988). Immunoprecipitates were analyzed by SDS-polyacrylamide gel electrophoresis under reducing conditions. Densitometric scanning was done on autoradiographs of the pulse-chase experiments in order to quantitate and compare retention and degradation of the various mutants, as previously described (Bonifacino et al., 1991).

Immunofluorescence Microscopy—Immunofluorescence microscopy was done as previously described (Bonifacino et al., 1991). Transfected cells were grown on 12-mm coverslips for 40 h before fixation for 10 min with 2% (w/v) formaldehyde in PBS. After rinsing with PBS, cells were incubated for 15 min with 10% FCS in PBS (PBS-FCS) to block nonspecific antibody interactions. Cells were then incubated for 45 min at 35°C with 50 μl of a 1:500 dilution of the mouse monoclonal antibody 7G7 (Rubin et al., 1985) in 0.1% (w/v) saponin and 0.1% FCS in PBS. After rinsing with PBS-FCS, cells were incubated for 30 min with a 1:500 dilution of rhodamine-conjugated goat anti-mouse IgG (Cappel) plus 0.1% saponin and 0.1% FCS in PBS. Following this, cells were rinsed with PBS, and coverslips were mounted on glass microscope slides using Fluoromount G (Southern Biotechnology Associates, Birmingham, AL).

Analysis of Charge-mediated Assembly—Charge-mediated assembly of Tac mutants with CD3-εR β-galactosidase (εR-gal) was studied by methods described previously (Bonifacino et al., 1991). COS-1 cells were cotransfected with an expression plasmid containing a mutant Tac cDNA and an expression plasmid encoding εR-gal. Two days after transfection, the cells were lysed, and galactosidase activity was assayed in both the anti-Tac immunoprecipitate and the total lysate. The percentage of galactosidase activity that coprecipitated with Tac was determined. One hundred percent relative binding corresponds to a cotransfection of 5R-gal with Tac-D8. In that positive control, which was included in every experiment, approximately 15% of the total galactosidase activity coprecipitates with Tac-D8.

RESULTS

Stability of the CD3-ε Chain—Previous studies have implicated the presence of negatively charged amino acid residues within transmembrane domains in causing protein retention and degradation within the ER (Bonifacino et al., 1991; Kurosaki et al., 1991). Interestingly, the CD3-ε chain of the TCR complex contains a transmembrane aspartic acid residue, but the protein is relatively stable when expressed by transfection into fibroblast cell lines (Bonifacino et al., 1989; Wileman et al., 1990). In order to examine whether the luminal or cytoplasmic domains of CD3-ε interfered with retention/degradation mediated by its TMD, we analyzed the fate of a chimeric protein (TcT) made by replacing the TMD of the Tac antigen by that of the CD3-ε chain. Pulse-chase experiments of the TcT construct (Table I) showed that this chimeric protein escaped the ER and underwent Golgi processing as measured by the appearance of an endo H-resistant form at 2 h of chase (Fig. 1c). This protein was also stable in the presence of BFA (Fig. 1c), a drug that blocks secretion from the ER-Golgi system but not ER degradation (Lippincott-Schwartz et al., 1989), indicating that it is resistant to degradation in the ER. This is in contrast to Tac-D8 (Table I), a mutant of the Tac antigen having an aspartic acid residue at position 8 of the TMD, which showed retention in the ER (i.e. no resistance to endo H) and degradation in the absence or presence of BFA (Fig. 1b). This retention and degradation is dependent on the presence of the aspartic acid residue in the TMD.

Table I

| Transmembrane domains and adjacent sequences of constructs used in this study |
|---------------------------------------------------------------|
| The predicted transmembrane amino acid residues are underlined. |
| The positions of the transmembrane aspartic acid residues are boldface. |

- Tac
- Tac-D2
- Tac-D5
- Tac-D8
- Tac-D10
- Tac-D12
- Tac-D14
- Tac-D17
- Tac-T
- Tac-D8
- Tac-D14
- Tac-D17
- Tac-T
- Tac-D8
- Tac-D14
- Tac-D17
- Tac-T
- Tac-D8
- Tac-D14
- Tac-D17
- Tac-T
- Tac-D8
- Tac-D14
- Tac-D17
- Tac-T
- Tac-D8
- Tac-D14
- Tac-D17
- Tac-T
- Tac-D8
- Tac-D14
- Tac-D17
- Tac-T
- Tac-D8
- Tac-D14
- Tac-D17
- Tac-T
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Although it is difficult to define the absolute length (in number of amino acids) of TMDs, it appears that the hydrophobic stretch comprising the CD3-ε TMD is 6 amino acids longer than that of Tac (see Table I). Therefore, we decided to test if shortening the CD3-ε TMD by removing hydrophobic residues would result in retention and/or degradation in the ER. Table II summarizes the effects of shortening the CD3-ε TMD by 2 (TtT-6), 4 (TtT-4), or 6 (TtT-6) amino acid residues (see Table I for sequences). We observed that as the TMD was shortened, the protein demonstrated greater levels of retention (as measured by the percentage of endo H-resistant, mature protein at 2 h), with the TtT-6 construct having nearly complete retention and slow degradation in the presence of BFA. Thus, we were able to progressively change the TtT phenotype by shortening its TMD to a length equivalent to that of Tac.

Although the TtT-6 construct looked more like Tac-D8 in terms of charge-mediated ER retention and degradation, its degradation was relatively slow (Fig. 2a). Since the position of the charge within a given TMD can have a profound effect on the phenotypic characteristics of the proteins (Bonifacino et al., 1991), the charge in the TtT-6 construct was moved to a position more central in the predicted TMD (TtT-6-D10; Table I). Analysis of this mutant by pulse-chase metabolic labeling revealed that it was more efficiently degraded in the ER (Fig. 2b). Removing the charge from the TtT-6 TMD (TtT-6-L8; Table I) abrogated retention and rapid degradation, indicating that the aspartic acid residue is, indeed, essential for this effect (Table II).

To confirm the biochemical observations on the effects of these TMD changes on intracellular localization, we performed immunofluorescence microscopy on cells expressing the different mutant proteins (Fig. 3). In agreement with the pulse-chase experiments, the Tac and TtT proteins (Fig. 3, A and B, respectively) were detected at the plasma membrane. In contrast, the Tac-D8 and the TtT-6-D10 constructs (Fig. 3, C and D, respectively) both showed a reticular pattern characteristic of the ER. Thus, proteins retained and degraded by this pathway appear localized to the ER system.

Effects of Lengthening the Tac TMD—To further test the role of the length of the TMD in targeting for ER retention and degradation, we examined the effect of lengthening the TMD of Tac-D8 (see Table I). Table II summarizes the data from lengthening the Tac-D8 TMD by 2 (Tac+4-D8) and by
placed in positions immediately flanking this window confers by pulse-chase metabolic labeling as described in the legend to Fig. 1. The positions of molecular weight markers (expressed as $10^{-3} \times M_r$) are indicated.

4 (Tac$^{4+}$-D8) amino acids. As the TMD is lengthened, retention and degradation in the presence of BFA progressively disappear (Fig. 4a and b). To be sure that we had altered the phenotype of the TacD8 protein by making its TMD 4 amino acids longer (Tac$^{4+}$-D8), we also tested the effect of changing the position of the charge (see Table I) on the fate of this mutant (Table II and Fig. 5). Although Tac$^{4+}$-D10 did show ER retention and degradation, Tac$^{4+}$-D12, Tac$^{4+}$-D14, and Tac$^{4+}$-D17 showed little or no ER retention or degradation (Table II). In a previous study, we showed that placement of negatively charged residues within the transmembrane domain of Tac defines a large “window” of rapid degradation spanning from positions 8 to 15 (Bonifacino et al., 1991; reproduced in Fig. 5a for comparison). An aspartic acid residue placed in positions immediately flanking this window confers ER retention. For Tac$^{4+}$, the window of rapid degradation, as well as the window of retention are dramatically reduced (Fig. 5b). In addition, even when degradation is observed, it is much slower than observed with the Tac constructs. Thus, it appears that TMD length is the primary difference between the CD3-ε and the Tac TMDs and suggests that length is a critical factor in charge-mediated degradation in the ER.

Role of TMD Length in Charge-mediated Assembly—Previous data had demonstrated that Tac mutants containing single positively charged residues in their TMDs could assemble in a position-dependent manner with CD3-δ, which contains a negatively charged transmembrane residue (Cosson et al., 1991). We therefore decided to test whether the potentially charged residue in CD3-ε could mediate assembly despite the fact that it has no effect on the intracellular fate of the protein. To do this, we first made a mutant of CD3-δ containing a positively charged, arginine residue within its TMD and fused Escherichia coli β-galactosidase to its cytoplasmic tail as a reporter enzyme. The results of these experiments demonstrated that δR-gal could assemble with Tac mutants containing single negative charges in a position-dependent manner (Table III). Maximum binding occurred when the aspartic acid residue was approximately at the same predicted level as the arginine residue in δR-gal (position 8). This pattern is similar to the pattern of assembly of CD3-δ and Tac mutants containing positive TMD charges (Cosson et al., 1991). We then co-expressed δR-gal with mutants of Tc/T containing the negatively charged residue at different positions in the membrane and found they could also assemble in a position-dependent fashion (Table III). We next tested all the other TMD mutants and found they all could assemble by potential charge-pair formation irrespective of their intracellular fates (Table III). As a general rule, mutants with longer TMD seem to be more competent for assembly. Hence, a charged residue that is not able to target a protein for retention and degradation is nonetheless available to mediate charge-pair formation in the membrane.

**DISCUSSION**

Transmembrane domains of type I integral membrane proteins are predominantly composed of hydrophobic amino acid residues. Potentially charged transmembrane residues are only found in a relatively small number of these proteins, including subunits of the TCR (Clevers et al., 1988; Klausner et al., 1990). In many of these cases, the potentially charged transmembrane residues have been shown to play important roles in the assembly, intracellular fate, and biological function of the proteins. Previous studies have pointed to the ability of potentially charged transmembrane residues to affect the subcellular localization and fate of newly synthesized proteins (Adams and Rose, 1985; Davis and Hunter, 1987; Bonifacino et al., 1990b, 1991). Two basic amino acid residues contained within the TMD of the TCR-α chain were implicated in retention and degradation of the unassembled protein within the ER system (Bonifacino et al., 1990b). The transfer of the phenotype of retention and degradation within the ER to another integral membrane protein by simply placing a potentially charged amino acid residue near the middle of the transmembrane domain suggested that the presence of such a residue could be sufficient to alter the fate of newly synthesized proteins (Bonifacino et al., 1991).

Extensive mutagenesis analysis of the Tac antigen TMD identified two factors which determine the effect of placing charged amino acid residues: the nature of the amino acid residue and its position within the transmembrane sequence. Arginine and aspartic acid were considerably more effective than lysine, glutamic acid, and histidine residues (Bonifacino et al., 1991). Each of these potentially charged residues was able to cause protein retention and/or degradation when placed at a characteristic position of the membrane-spanning domain, centered around the middle of the transmembrane sequence (Bonifacino et al., 1991). Despite their potential for predicting the fate of other integral membrane proteins, however, these observations failed to explain the stability of the CD3-ε chain. Indeed, CD3-ε has a relatively long half-life in the ER when expressed alone in fibroblasts (Bonifacino et al., 1989, Wileman et al., 1990), even though it contains an aspartic acid residue within its transmembrane domain (Gold et al., 1986). The experiments reported here demonstrate that the inability of this aspartic acid residue to target proteins for degradation is not due to an inhibitory effect of the CD3-ε extracellular or cytoplasmic domains or to the specific amino acid composition of the transmembrane sequence but to the length of the TMD.

How might the length of the transmembrane sequence affect charge-mediated retention or degradation within the ER? We envision two types of explanations as potentially
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FIG. 3. Immunofluorescence microscopy of cells expressing Tac and TtT constructs. COS-1 cells expressing normal Tac (A), TtT (B), Tac-D8 (C), or TtT-D10 (D) were fixed, permeabilized, and stained with a monoclonal anti-Tac antibody (7G7) and a rhodamine-conjugated goat anti-mouse IgG, as described under “Materials and Methods.”

FIG. 4. Effects of lengthening the TMD on the fate of Tac-D8 constructs. TMD of Tac-D8 mutant was lengthened by two (Tac<sup>+</sup>-D8), (a) or four amino acid residues (Tac<sup>+</sup>4-D8) (b), and fate of the mutated proteins was examined by pulse-chase metabolic labeling as described in the legend to Fig. 1.

underlying this phenomenon. The first assumes that these transmembrane domains are directly “recognized” by an as yet unknown cellular machinery that targets the protein for ER retention and degradation. We have previously shown that the presence of a potentially charged residue within a TMD can be recognized by a residue within a second TMD capable of assuming the opposite charge, and that such a potential charge pair can be sufficient for the interaction of the two proteins (Cosson et al., 1991). Furthermore, the relative positions of these residues within their TMDs had a profound effect on their ability to interact. Likewise, the same potentially charged residue can be responsible for targeting the protein for ER retention or degradation, and the position of the charged residue can have a major impact on this targeting (Bonifacino et al., 1991). Even if the specific recognition of a TMD charged residue via such an interaction is an important part of the retention and/or degradation produced by these residues, our results now show that the presence of such polar residues is not sufficient for altering the protein’s fate within the ER. This is true despite the continued availability of these potentially charged residues for pairing. Thus, our previous results with the recognition of potentially charged residues within TMDs provides no prediction of the profound effect of the length of the TMD in which these residues are found.

The second type of explanation of the induction of ER
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FIG. 5. Lengthening the TMD of Tac reduces the retention/degradation window. The graphs show the position-dependence of degradation induced by aspartic acid residues within the TMD of normal Tac (a) or Tac plus an additional 4 amino acid residues (Tac**) (b). The extent of Golgi processing at 2 h of chase (open squares) and half-lives of the different Tac constructs in the presence of BFA (closed circles) were plotted against the position of these residues within the transmembrane domain. Data corresponding to the half-lives of Tac-D mutants (a) were replotted from the study by Bonifacino et al. (1991).

TABLE III

| Construct     | Relative assembly % |
|---------------|---------------------|
| Tac           | 9                   |
| Tac-D2        | 55                  |
| Tac-D6        | 138                 |
| Tac-D8        | 100                 |
| Tac-D10       | 55                  |
| Tac-D13       | 40                  |
| Tac-D15       | 26                  |
| Tac**-D8      | 274                 |
| Tac**-D8      | 131                 |
| TtT-D17       | 177                 |
| TtT-D11       | 348                 |
| TtT-D14       | 421                 |
| TtT-D17       | 195                 |
| TtT**-        | 200                 |
| TtT-4         | 164                 |
| TtT-5         | 142                 |

Tac mutants were expressed with δR-gal. As described under "Materials and Methods," the assembly levels were determined and expressed as a percentage of a positive control (Tac-D8) included in every experiment. (Aoki et al., 1991; Aoki et al., 1992). Other characteristics of TMDs such as length or amino acid composition might explain why similar structural features result in targeting to different membrane compartments.

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