The EMBO Journal vol.10 no.10 pp.2783-2793, 1991

Role of potentially charged transmembrane residues in targeting proteins for retention and degradation within the endoplasmic reticulum

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Communicated by M.Neuberger

The selective breakdown of newly synthesized proteins retained within the endoplasmic reticulum (ER) is probably mediated by the specific recognition of structural features of protein substrates by components of a degradative system. Within the α chain of the multisubunit T-cell antigen receptor (TCR) complex, a transmembrane sequence containing two basic amino acid residues has been shown to act as a determinant for retention and rapid degradation in the ER. We now demonstrate that single basic or acidic amino acid residues can cause targeting for retention and degradation in the ER when placed within the transmembrane domain of an integral membrane protein normally destined for the cell surface. The effect of such potentially charged residues is dependent on their relative position within the transmembrane sequence and on the nature of the amino acid side chains. The phenotypic changes induced by potentially charged transmembrane residues occur without apparent alterations of the global folding or transmembrane topology of the mutant proteins. These observations test the hypothesis that potentially charged residues within transmembrane domains can provide the basis for a motif for ER degradation and explain the selective breakdown of some proteins retained within the ER.

Key words: degradation signals/membrane proteins/membrane transport/protein turnover

Introduction

One of the major advances in understanding the molecular bases of the regulated turnover of cellular proteins has been the realization that the metabolic stability of proteins is intimately linked to their structure. The specific recognition of certain features of proteins by the cell's multiple degradative pathways probably explains the vastly different rates at which proteins are degraded. The ability to degrade proteins in a selective fashion provides a central means of regulating cellular functions. Two physiologically essential processes that involve selective proteolysis are the destruction of structurally abnormal proteins and the controlled breakdown of key regulatory proteins to accommodate specific metabolic needs. The greatest progress over the last several years has been made in describing the nature of cytosolic pathways of protein turnover, the best characterized of which involves an ATP-dependent conjugation of ubiquitin to a protein prior to its degradation (reviewed by Rechsteiner, 1987; Hershko, 1988; Ciechanover and Schwartz, 1989). Addition of ubiquitin has been proposed to be dependent upon the identity of the NH_2-terminal amino acid residue and the presence of a lysine residue or other specific sequences at particular internal positions within the molecule (Bachmair *et al.*, 1986; Chau *et al.*, 1989; Glotzer *et al.*, 1991). In contrast to this pathway, very little is known about the mechanism of other processes involved in the regulated turnover of cellular proteins and the nature of the structural information that underlies their selective targeting for destruction.

Recent studies have demonstrated the existence of a novel degradative pathway for newly synthesized proteins retained within the endoplasmic reticulum (ER) (reviewed by Klausner and Sitia, 1990). Substrates for this degradative process include some resident ER proteins, subunits of multicomponent complexes and mutant proteins retained in the ER, and even apparently normal proteins whose lifespan in the ER is subject to regulation. Although at first impression it may seem surprising that the organelle in which membrane and secretory proteins are synthesized can also be a degradative compartment, the evolving picture of the ER as a site of quality control makes sense of this observation. The correct functioning of the ER in its dual role as a synthetic and a degradative organelle, however, requires the ability to discriminate between proteins destined for stable retention or export and those targeted for destruction.

Studies on the fate of newly synthesized chains of the multicomponent T-cell antigen receptor (TCR) have begun to unravel the complex relationship between protein structure and targeting for degradation within the ER (reviewed by Klausner *et al.*, 1990). The TCR is composed of at least eight transmembrane chains $\alpha\beta\gamma\delta\epsilon_2\zeta_2$, Koning *et al.*, 1990; de la Hera *et al.*, 1991) which must be correctly assembled in the ER for efficient transport to the plasma membrane. Most partially assembled complexes and unassembled chains are unable to exit the ER and, in some cases, undergo rapid degradation within this organelle. Like other intracellular processes of protein breakdown, this pathway exhibits a striking selectivity with respect to substrates. Indeed, whereas the α, β, δ, and murine γ chains are rapidly degraded, ϵ and ζ have relatively long half lives (Chen *et al.*, 1988; Bonifacino *et al.*, 1989; Wileman *et al.*, 1990a).

Molecular dissection of the α chain of the TCR has allowed us to localize the structural information for rapid degradation within the ER to its single membrane-spanning domain (Bonifacino *et al.*, 1990a). Similar studies of the TCR-β chain have demonstrated the existence of a determinant for degradation within a COOH-terminal segment, comprising the transmembrane domain and short cytoplasmic tail of the protein (Bonifacino *et al.*, 1990b; Wileman *et al.*, 1990b). The transmembrane domains of the TCR-α and -β chains are unusual in that they contain two

and one basic amino acid residues, respectively. Mutational analysis of chimeric proteins containing the TCR-α transmembrane domain revealed that these residues were essential for rapid degradation (Bonifacino *et al.*, 1990b). These findings led us to explore the role of potentially charged amino acid residues within transmembrane domains as putative determinants for ER degradation. The results of this effort, reported here, demonstrate that placement of either basic or acidic amino acid residues at defined positions within the transmembrane domain of an integral membrane protein can lead to its retention and rapid degradation in the ER. Targeting for degradation is strictly dependent on the relative position and characteristics of the charged amino acid residue. Thus, these observations define the nature of a structural motif involved in the selective targeting of proteins for retention and destruction within the ER and delineate some of the requirements for its function. The identification of this structural determinant permits us to propose an explanation for a number of examples of selective degradation of newly synthesized proteins retained in the ER.

Results

Fate of Tac transmembrane mutants

In order to examine the role of potentially charged amino acid residues within transmembrane domains in determining the fate of integral membrane proteins, oligonucleotide-directed mutagenesis of the Tac antigen (interleukin-2 receptor α chain, Leonard *et al.*, 1984) was performed. The Tac antigen is a type I integral membrane protein which in its mature form is comprised of a 219-amino acid NH_2-terminal ectodomain, a single membrane-spanning domain of 19 predominantly hydrophobic amino acids, and a 13-amino acid COOH-terminal cytoplasmic tail (Leonard *et al.*, 1984). Tac is synthesized in the ER as a 48 kDa precursor containing two high mannose N-linked oligosaccharide chains sensitive to endoglycosaminidase H (endo H) (Figure 1a, lanes 1 and 2). Upon transport into the Golgi system, these oligosaccharide chains are processed into complex carbohydrates, resulting in both a shift in the migration of Tac to 55 kDa and acquisition of resistance to endo H (Figure 1a, lanes 3–6). The fully processed form of the Tac antigen is then delivered to the cell surface, from where it is partially shed into the culture medium as a proteolytically cleaved, soluble species of ~40 kDa (Rubin *et al.*, 1985b; Robb and Kutny, 1987; Cullen *et al.*, 1988). Localization of Tac to the cell surface at steady state can be demonstrated by immunofluorescence microscopy of transfected fibroblasts using a specific polyclonal antibody (Figure 2a). Addition of the drug brefeldin A, which redistributes Golgi proteins into the ER (Lippincott-Schwartz *et al.*, 1989; Doms *et al.*, 1989), prevents further movement of newly synthesized Tac through the secretory pathway (Bonifacino *et al.*, 1990a,b). Consequently, newly synthesized Tac accumulates within the ER, where it has a long half life of >360 min (Figure 1a, lanes 7–9). The progressive decrease in electrophoretic migration observed upon addition of brefeldin A is due to modification of carbohydrate chains by Golgi enzymes relocated to the ER, as previously described (Lippincott-Schwartz *et al.*, 1989).

A single amino acid substitution that placed a basic R residue at position 10 of the predicted transmembrane domain of Tac resulted in a dramatic change in the intracellular fate of the newly synthesized, endo H-sensitive protein (R^{10}, Figure 1b). The R^{10} mutant protein rapidly disappeared from the cells ($t_{1/2}$ 10–20 min) as an endo H-sensitive species (Figure 1b, lanes 1–6), without being released into the culture medium (not shown). Disappearance of R^{10} was not prevented by addition of brefeldin A (Figure 1b, lanes 7–9). Immunofluorescence microscopy of COS-1 cells expressing R^{10} revealed localization of the mutant protein to an extensive cytoplasmic network characteristic of the ER (Figure 2b). Interestingly, placement of an acidic D residue at the same position within the transmembrane domain of Tac resulted in a mutant protein (D^{10}) with similar subcellular distribution and fate as R^{10} (Figures 1c and 2c). Thus, the rapid loss of newly synthesized R^{10} and D^{10} resembled the previously described pathway for the degradation of proteins retained within the ER (reviewed by Klausner and Sitia, 1990).

The possibility that the disappearance of R^{10} and D^{10} was due to loss of a conformational epitope or to the formation of species insoluble in 0.5% (w/v) Triton X-100 at 4°C was addressed by solubilizing cells after pulse–chase with a variety of treatments, including: (i) 0.5% SDS (w/v) at 22°C, (ii) 1% (w/v) SDS at 95°C, (iii) 1% (w/v) SDS, 2% (v/v) β-mercaptoethanol at 95°C, (iv) 8 M urea, 0.1% (w/v) SDS at 22°C, and (v) 10 μg/ml proteinase K, 1% (w/v) Triton X-100 at 4°C. The solubilized Tac products generated by the different treatments were immunoprecipitated using the polyclonal antibody R3134 (Sharon *et al.*, 1986), which recognizes both native and denatured forms of the Tac antigen. Under all conditions, Tac species were recovered from pulse-labeled cells but not from cells chased for 3 h after labeling (data not shown). Thus, the disappearance of the R^{10} and D^{10} mutant proteins is not likely due to the loss of a conformational epitope or to conversion into an insoluble species. Rather, this phenomenon is best explained by their rapid degradation after synthesis.

Probing the folding of Tac transmembrane mutants

Although the tertiary structure of proteins may often be tolerant of amino acid substitutions (Bowie *et al.*, 1990), there are instances in which even a single amino acid change results in profound alteration of overall protein conformation (Yu and King, 1984; Doms *et al.*, 1987). Proteins within the secretory pathway that fail to fold into conformations that cells recognize as 'normal' are frequently retained in the ER (reviewed by Rose and Doms, 1988; Hurtley and Helenius, 1989). Due to their nonconservative nature, mutations that place strongly polar amino acid residues within the transmembrane domains of integral membrane proteins could potentially result in abnormal folding and, as a consequence, altered intracellular transport and increased susceptibility to proteolytic breakdown.

In order to determine whether misfolding could explain the phenotypic characteristics observed for the Tac transmembrane mutants, the folding state of selected mutants was analysed by limited proteolysis with *Tritirachium album* proteinase K and separation of the partial digestion products by SDS–PAGE. This method relies on the fact that only a fraction of all the potential cleavage sites are accessible to proteolysis in the folded molecule (Miedel *et al.*, 1988). Treatment of newly synthesized Tac and the R^{10} and D^{10} mutants with proteinase K revealed that that three proteins were equally sensitive to digestion over a broad range of

enzyme concentrations (Figure 3a). In addition, the labeled partial digestion products were discrete and essentially the same for the normal and mutant forms of Tac (Figure 3a). As a control for the efficacy of this method, the same three proteins were treated with 100 μg/ml proteinase K after denaturation in 1% (w/v) SDS and 2% (v/v) β-mercaptoethanol. The exposure of additional sensitive sites induced by molecular unfolding led to their complete degradation (Figure 3b, lanes 3).

The correct arrangement of intrachain disulfide bonds depends on the spatial positioning of cysteine residues and can hence be used as an additional measure of protein conformation. Comparison of the fragments resolved by nonreducing and reducing SDS–PAGE after digestion with 100 μg/ml proteinase K permitted a partial analysis of the pattern of disulfide bonding in Tac, R^{10} and D^{10} (Figure 3b). Under nonreducing conditions, the three proteins generated two poorly separated labeled species of ~26–27 kDa. Upon reduction with β-mercaptoethanol, the higher molecular weight species was decomposed into three smaller fragments, implying that at least two intramolecular disulfide bonds were normally formed in the mutant proteins (Figure 3b).

Taken together, these observations suggest that placement of a basic or acidic amino acid residue within the transmembrane domain of Tac does not lead to major changes in the folding of the newly synthesized proteins. Thus, retention and degradation in the ER are not likely to be a

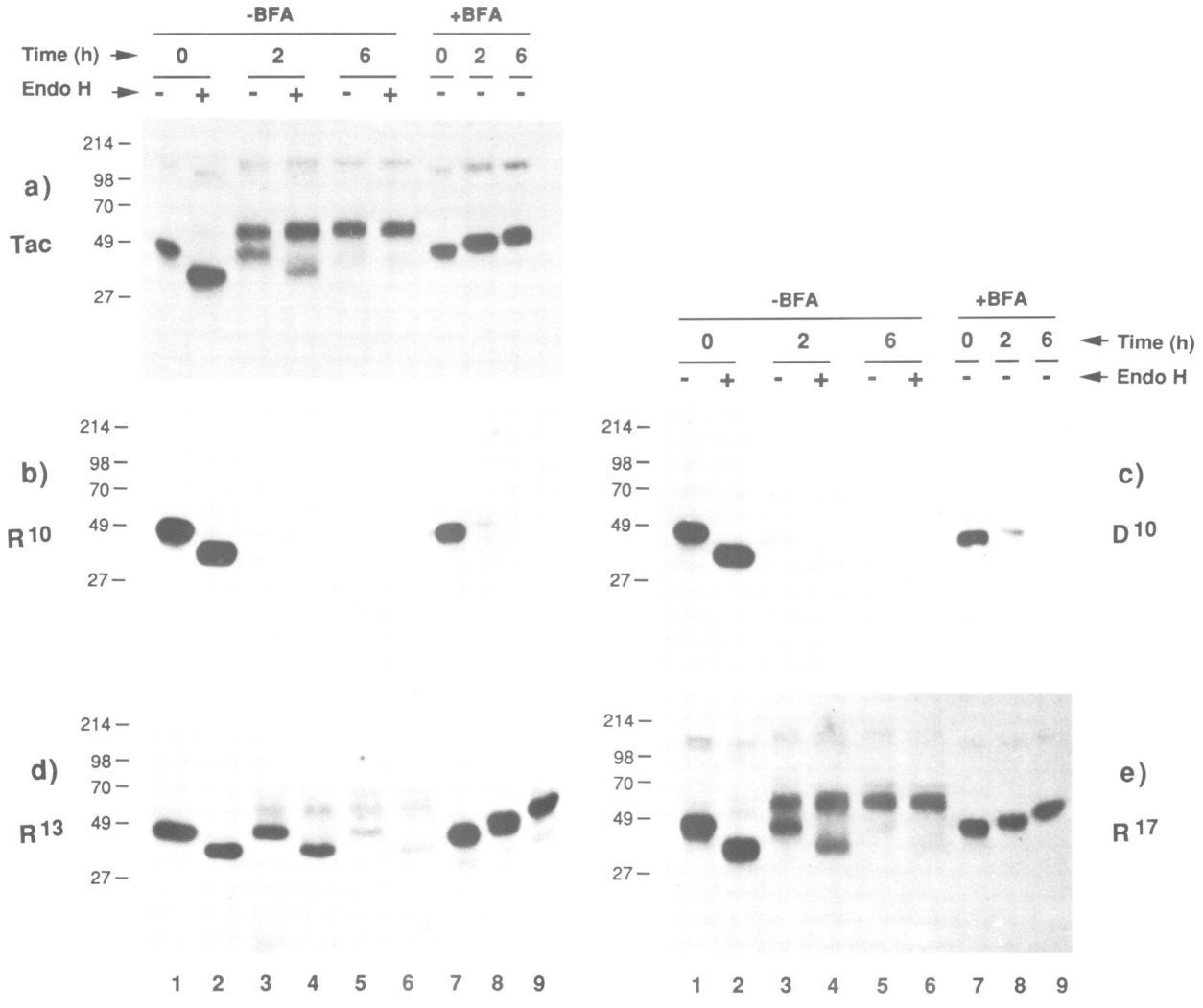

Fig. 1. Fate of Tac mutants containing single R or D residues in their transmembrane domains. Single R or D residues were placed in the predicted transmembrane domain of the human Tac antigen by oligonucleotide-directed mutagenesis. Analysis of the sequence of the human Tac cDNA using the algorithm of Kyte and Doolittle (1982) predicts a single membrane-spanning domain of 19 amino acids (residues 220–238 of the mature protein, Leonard *et al.*, 1984). For simplicity, positions within this sequence were numbered from the lumenal to the cytoplasmic end of the transmembrane domain (see Figure 6a). COS-1 cells were transfected with expression plasmids encoding normal Tac (**a**), or the Tac mutants R^{10} (**b**), D^{10} (**c**), R^{13} (**d**) or R^{17} (**e**) as indicated in the figure. Lanes 1–6 , (–BFA): The transfected cells were metabolically labeled with [^{35}S]-methionine for 30 min 37°C, after which they were placed in regular culture medium at 37°C for 0, 2, or 6 h, as indicated. Labeled Tac proteins were isolated by immunoprecipitation using a monoclonal antibody directed against a lumenal epitope of the Tac antigen (7G7, Rubin *et al.*, 1985a). Immunoprecipitates were either not treated (–) or treated with Endo H (+) prior to analysis by SDS–PAGE on 10% acrylamide gels. Lanes 7–9, (+BFA): The transfected cells were analysed as described for lanes 1–6, save that the experiment was performed in the continuous presence of 5 μg/ml brefeldin A (BFA) in the culture media. Immunoprecipitates from cells analysed in the presence of brefeldin A were not treated with Endo H (–). The positions of mol. wt markers are indicated. Relative mol. wts (M_r) are expressed as $10^{-3} \times M_r$.

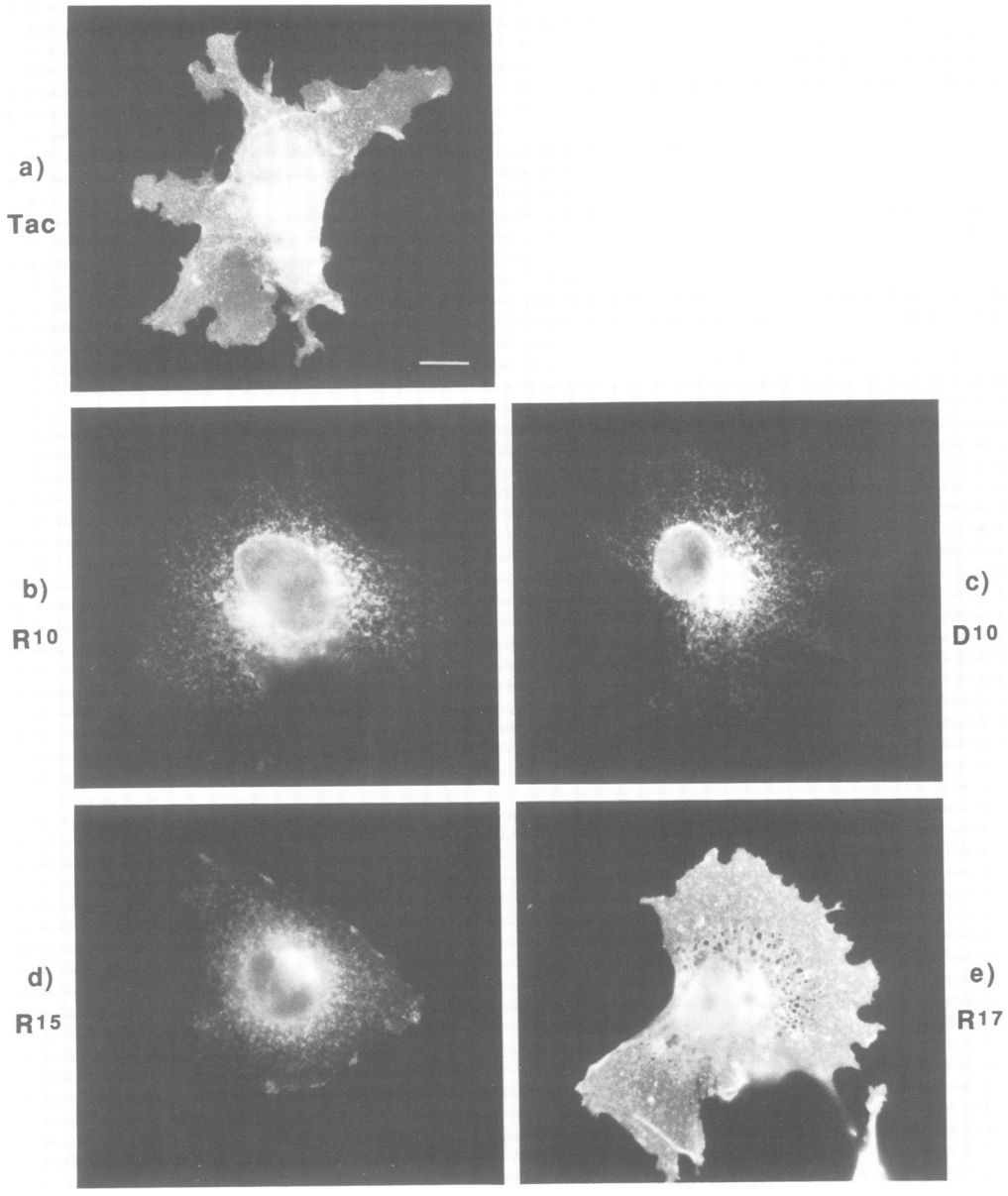

Fig. 2. Immunofluorescence microscopy of cells expressing normal and mutagenized Tac. COS-1 cells expressing normal Tac (a) and the mutants R^{10} (b), D^{10} (c), R^{15} (d) and R^{17} (e) were fixed/permeabilized and stained with a rabbit polyclonal antiserum raised against purified human Tac (R3134, Sharon *et al.*, 1986) and a rhodamine-conjugated second antibody, as described in Materials and methods. Because of the low efficiency of transfection, only 5–15% of cells were found to be positive. Representative cells for each Tac construct are shown. Bar: 10 μm.

consequence of global misfolding of the mutant proteins. Given the limited sensitivity of these assays, however, we cannot rule out the incidence of minor conformational changes or localized regions of unfolding which can trigger a degradative process.

Topology of Tac transmembrane mutants

We next examined whether placement of basic or acidic amino acid residues within the transmembrane domain of Tac still allowed for the integration of the Tac mutants into the ER membrane as transbilayer proteins. The addition of N-linked oligosaccharide chains to the NH_2-terminal ectodomains of the mutant proteins (Figure 1) signified that they were normally translocated into the ER lumen. To determine whether the COOH-terminal tails were exposed on the cytoplasmic face of the ER, isolated rough ER fractions containing metabolically labeled Tac, R^{10}, and D^{10} were incubated for 1 h at 4°C in the absence or presence of 100 μg/ml trypsin. After inhibition of the enzyme, membranes were solubilized and Tac proteins isolated using a monoclonal antibody directed against a lumenal epitope. A similar analysis was performed on an ER fraction containing labeled Tac_t, a form of Tac lacking in transmembrane and cytoplasmic domains (Bonifacino *et al.*, 1990a) and which is therefore completely sequestered within the ER lumen. For the normal Tac antigen, this treatment resulted in a small but appreciable decrease in molecular weight (Figure 4a), an observation consistent with the removal of a portion of its cytoplasmic tail. As expected, the fully translocated Tac_t was protected from the proteolytic enzyme (Figure 4a). Both R^{10} and D^{10} registered decreases in molecular weight comparable to that observed for normal Tac (Figure 4a), thus demonstrating that their COOH-terminal tails were exposed on the cytoplasmic side

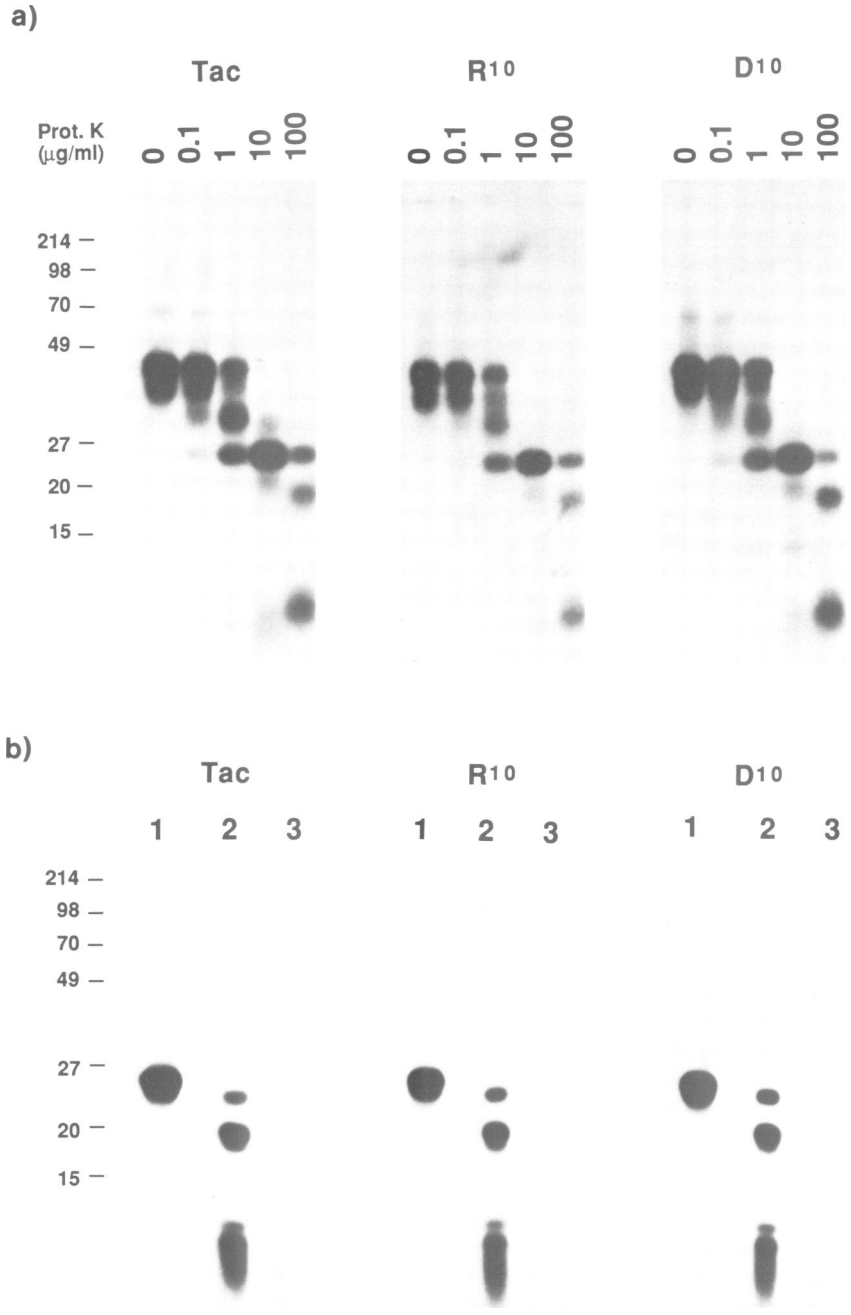

Fig. 3. Limited proteolysis of normal and mutagenized Tac proteins. (**a**) COS-1 cells expressing normal Tac, R^{10} or D^{10} were metabolically labeled for 30 min as described in the Materials and methods section. Tac proteins were isolated by immunoprecipitation with the monoclonal antibody 7G7. The isolated proteins were resuspended in PBS and treated for 15 min at 4°C with varying concentrations of proteinase K, as indicated in the figure. (**b**) Labeled normal Tac, R^{10}, and D^{10} were prepared as described in (a). One half of each sample was kept on ice (lane 1 and 2) and the other half was incubated for 15 min at 95°C in 1% (w/v) SDS and 2% (v/v) β-mercaptoethanol (see Materials and methods) (lane 3). Proteins were then treated with 100 μg/ml proteinase K as described in (a). Samples were analysed by SDS–PAGE on 13% acrylamide gels under either nonreducing (lane 1) or reducing conditions (lanes 2 and 3). The positions of molecular weight markers are indicated. Molecular weights (M_r) are expressed as $10^{-3} \times M_r$.

of ER vesicles. In all cases addition of 1% (w/v) Triton X-100 prior to proteolysis resulted in extensive digestion of the labeled proteins (data not shown). These findings indicate that the newly synthesized R^{10} and D^{10} mutant proteins have a transbilayer topology, indistinguishable from that of normal Tac.

Despite the apparently normal topology of the mutant proteins, the presence of potentially charged residues within their transmembrane domains may alter the nature of their association with the ER membrane. This possibility was examined by treatment of membranes containing normal and mutant forms of Tac with 0.1 M sodium carbonate, pH 11.3, as described by Howell and Palade (1982) and Fujiki *et al.* (1982). This procedure results in the extraction of lumenal and peripheral proteins, while integral membrane proteins remain bound to the membrane. As expected, whereas the normal Tac antigen remained with the membrane fraction, Tac_t was readily extracted upon treatment with the sodium

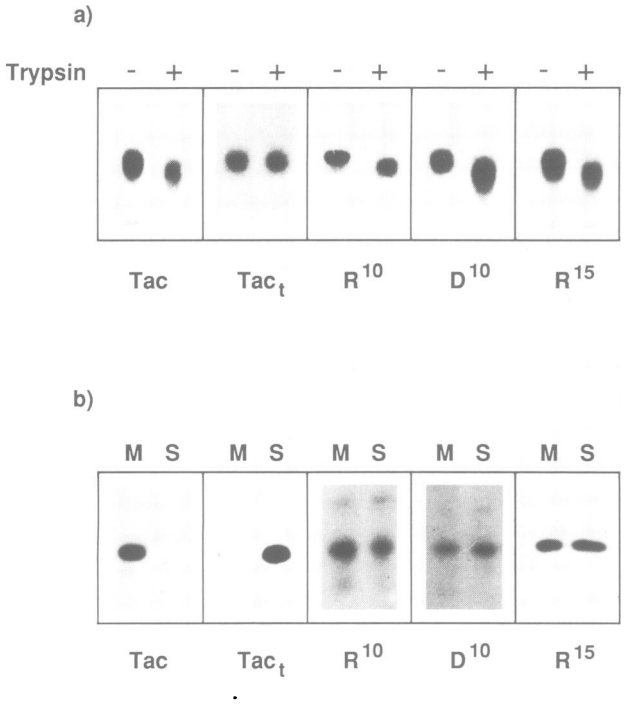

Fig. 4. Topology and membrane association of Tac transmembrane mutants. (**a**) COS-1 cells expressing normal Tac, Tac$_t$, R^{10}, D^{10} or R^{15} were metabolically labeled with [^{35}S]methionine for 30 min. Cells were then disrupted and ER fractions prepared by sedimentation on sucrose gradients. ER fractions derived from the different transfectants were then incubated for 1 h at 4°C in the absence (−) or the presence of 100 μg/ml trypsin (+). As a control for the effectiveness of trypsin digestions, ER fractions were also treated with trypsin in the presence of 0.5% (w/v) Triton X-100 (not shown). After inhibition of the enzyme, Tac species were isolated with a monoclonal antibody reacting with a lumenal epitope (7G7) and resolved by SDS−PAGE. (**b**) Membrane fractions containing labeled Tac, Tac$_t$, R^{10}, D^{10} or R^{15} were prepared as described in Materials and methods and treated with 0.1 M sodium carbonate, pH 11.3. After separation of membrane-bound (M) and soluble (S) proteins, Tac proteins were isolated by immunoprecipitation with the polyclonal antibody R3134 and resolved by SDS−PAGE.

carbonate solution (Figure 4b). Interestingly, both the R^{10} and D^{10} mutant proteins could be partially solubilized by this treatment (Figure 4b), suggesting that they were more loosely integrated into the lipid bilayer. This observation raised the prospect that the presence of potentially charged amino acid residues within transmembrane domains might result in some rate of release of proteins into the ER lumen, which could precede their rapid degradation.

To examine the relationship between degradation and the strength of association with membranes, we compared the fate of Tac-TCR-α, a rapidly degraded transmembrane chimera containing two basic residues in its membrane-spanning domain (Bonifacino *et al.*, 1990a), with that of a truncated form of Tac-TCR-α lacking a cytoplasmic tail (Figure 5a). The absence of a cytoplasmic tail containing basic amino acid residues after a hydrophobic stretch has been shown to significantly enhance the probability that the protein would be completely translocated and extruded into the ER lumen (Kuroiwa *et al.*, 1991). Indeed, removal of the cytoplasmic tail from the Tac-TCR-α chimeric protein resulted in increased extraction with sodium carbonate (from 44% to 70%, Figure 5b). However, pulse−chase analysis of cells expressing these constructs proved that whereas the full length, presumably transmembrane chimeric protein was rapidly degraded, the truncated protein was still retained in the ER but degraded at a much slower rate (Figure 5c). Thus, increasing the probability of release from the membrane does not favor, but rather precludes rapid degradation of the protein. An additional observation arguing against release from the membrane as the event that triggers degradation is the finding that the mutant protein R^{15} is relatively stable in the ER (see below), despite a similar degree of extractability with sodium carbonate as R^{10} and D^{10} (Figure 4b).

Dependence on the position and nature of potentially charged transmembrane residues

The next series of experiments were aimed at establishing whether the effect of potentially charged transmembrane residues on the fate of newly synthesized proteins was dependent on the position and nature of the amino acid side chains. To this end, single R, D, K, E or H residues were placed at various positions within the transmembrane domains of the Tac antigen. Placement of R residues at positions 5 or 8 of the Tac transmembrane domain produced mutant proteins which failed to be transported to the Golgi system and were instead rapidly degraded, similar to the R^{10} mutant (Table I). Substitution of residues at positions 13 and 15 of the Tac transmembrane domain by R residues, however, resulted in mutants displaying a different phenotype (Figure 1d and Table I). The newly synthesized proteins showed impaired transport out of the ER relative to the normal Tac antigen, as evidenced by the reduced amount of Golgi-processed, endo H-resistant forms after 2 and 6 h of chase (Figure 1d and Table I). Despite their lengthy stay in the ER, however, the mutant proteins R^{13} and R^{15} were only slowly or undetectably degraded over a 6 h period, both in the absence and presence of brefeldin A (Figure 1d and Table I). Immunofluorescence microscopy of COS-1 cells expressing R^{15} revealed a relatively heterogeneous pattern, with variable degrees of staining of the ER, Golgi system and plasma membrane (Figure 2d). In contrast to the mutations described above, placement of R residues at positions 17 and 18 of the Tac transmembrane domain, closer to the membrane−cytoplasm interface, had no effect on intracellular transport or stability of the mutant proteins (Figure 1e and Table I). Like normal Tac, these mutant proteins accumulated at the cell surface (Figure 2e). Thus, depending upon the position of the mutation, insertion of R residues within a transmembrane domain resulted in mutant proteins displaying one of three different phenotypes (i) rapid degradation in the ER, (ii) impaired transport out of the ER and slow degradation (even in the presence of brefeldin A), or (iii) normal transport to the cell surface and resistance to degradation in the ER.

Other mutants containing transmembrane D, K, E or H residues exhibited phenotypes similar to the R mutants described above and could be assigned to the same three classes (Table I and Figure 6). Like the D^{10} mutant, proteins having D residues at positions 8, 13 and 15 (D^{8}, D^{13} and D^{15}) were not transported out of the ER and were rapidly degraded (Table I). In contrast, D^{5} was able to partially exit the ER and was not detectably degraded when completely retained within the ER upon addition of brefeldin A (Table I). Mutants D^{2}, D^{17}, D^{18}, and D^{19}, on the other hand, were similar to normal Tac (Table I). Comparison of the fates of the different R and D transmembrane mutants

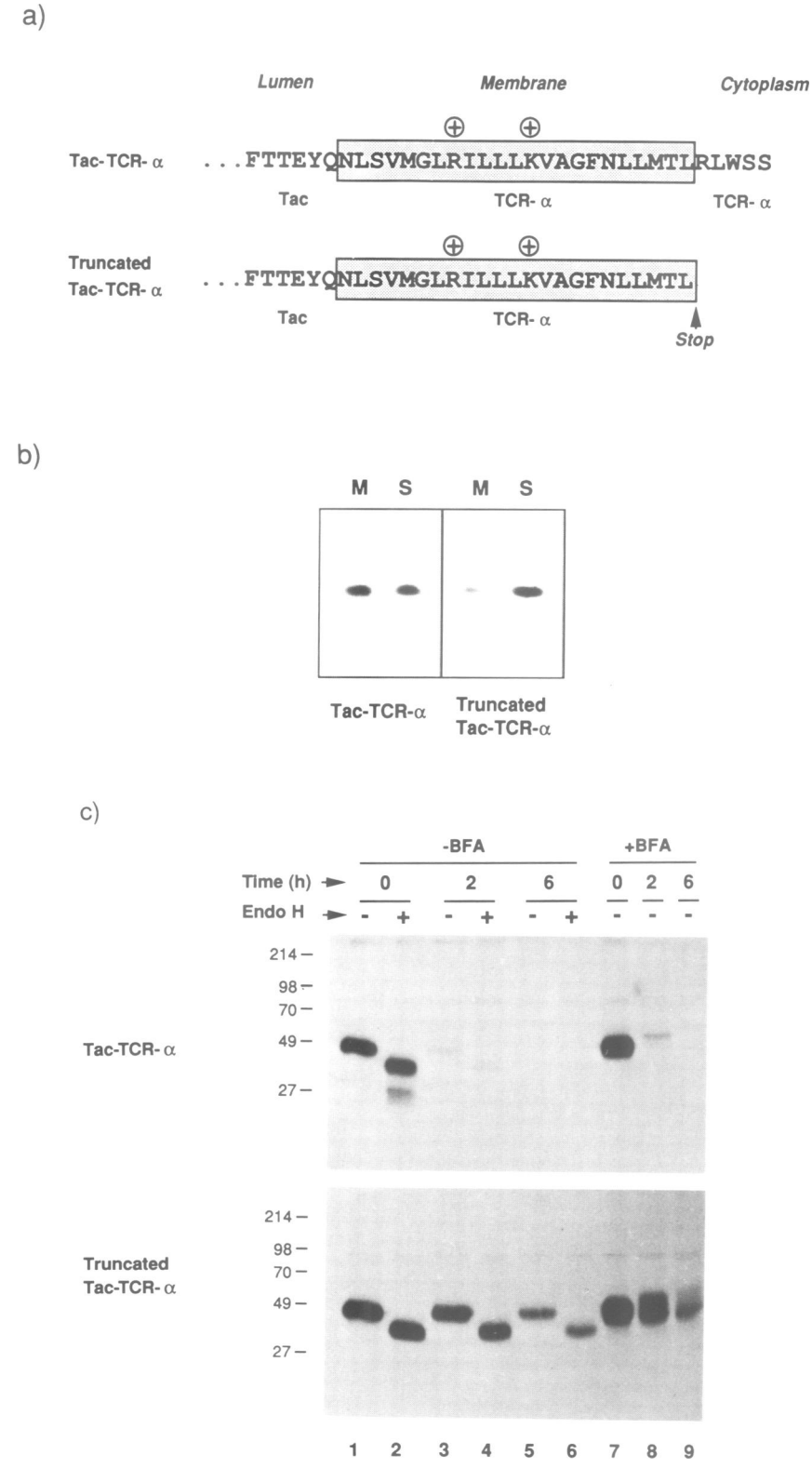

Fig. 5. A transmembrane topology favours rapid degradation in the ER. (**a**) Amino acid sequences of the transmembrane domains and adjacent regions of a Tac−TCR-α chimeric protein (Tac−TCR-α_1, Bonifacino *et al.*, 1990a,b) and a truncated form of this protein. A DNA encoding the truncated Tac−TCR-α was constructed by placing a translation stop codon at the boundary of sequences encoding the transmembrane and cytoplasmic domains of Tac−TCR-α, using oligonucleotide-directed mutagenesis (Kunkel, 1985). The positions of basic amino acid residues within the transmembrane sequence of TCR-α are indicated by the circled plus signs. (**b**) Extraction of membrane fractions containing labeled Tac−TCR-α and truncated Tac−TCR-α with 0.1 M sodium carbonate, pH 11.3. Tac proteins were isolated by immunoprecipitation of membrane-bound (M) and soluble (S) proteins, as described in Materials and methods. (**c**) Fate of Tac−TCR-α and truncated Tac−TCR-α in transfected COS-1 cells. Analysis by pulse−chase metabolic labeling was performed as described in the legend to Figure 1. The positions of molecular weight markers are indicated. Molecular weights (M_r) are expressed as $10^{-3} \times M_r$.

Table I. Characteristics of Tac transmembrane mutants

Construct	% Mature at 2 h	$t_{1/2}$ in BFA (min)
Tac	42	>360
R^2	ND	>360
R^5	<1	40
R^8	<1	30
R^{10}	<1	10
R^{13}	11	340
R^{15}	12	>360
R^{17}	29	>360
R^{18}	33	>360
D^2	40	>360
D^5	8	>360
D^8	<1	40
D^{10}	<1	50
D^{13}	<1	20
D^{15}	<1	30
D^{17}	21	330
D^{18}	43	>360
D^{19}	36	>360
K^5	26	>360
K^8	6	160
K^{10}	10	140
K^{15}	16	>360
E^5	31	>360
E^8	2	140
E^{10}	1	100
E^{13}	<1	220
E^{15}	4	140
H^5	13	>360
H^8	27	240
H^{10}	2	180
H^{13}	14	>360
H^{15}	22	>360

Single R, D, K, E or H residues were placed within the transmembrane domain of Tac at the positions indicated by superscripts. The fate and sensitivity to degradation of various mutant proteins in transfected COS-1 cells were examined in experiments identical to those described in legend to Figure 1. Transport of each mutant protein out of the ER and into the Golgi system was quantified by measuring the percentage of newly synthesized protein at time 0 that was processed into a mature, Endo H-resistant form after a 2 h chase. In order to examine the sensitivity to degradation in the ER of the different proteins, regardless of whether or not they can exit the ER, the rate of degradation of each newly synthesized protein was determined in the presence of brefeldin A. Half lives ($t_{1/2}$ in BFA) were calculated by densitometric scanning of autoradiograms from pulse–chase experiments and regression analysis of the data assuming first-order kinetics. ND: not determined.

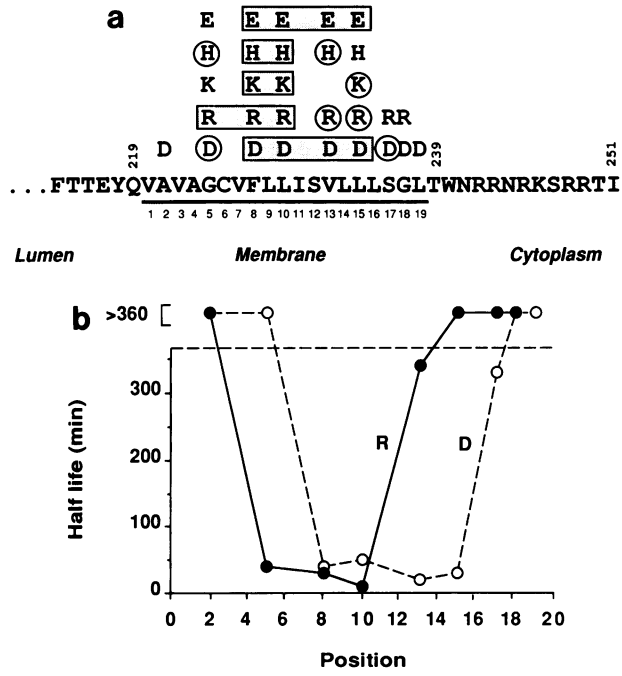

Fig. 6. (**a**) Phenotypes of Tac transmembrane mutants. On the basis of the characteristics shown in Table I, Tac transmembrane mutants were grouped into three different phenotypic classes: (A) proteins that are rapidly degraded in the ER (boxed), (B) proteins whose transport out of the ER is impaired but are only slowly degraded (circled), and (C) proteins that are normally transported to the cell surface (plain letters). Assignment to each phenotypic class was based on the following criteria: class (A) proteins with half lives of 240 min or less in the presence of brefeldin A, class (B) proteins that are poorly processed into endo H-resistant forms (21% or less of the mature form at 2 h of chase) and have a half life of >240 min in the presence of brefeldin A, and class (C) proteins that are efficiently processed into endo H-resistant forms (>21% mature at 2 h of chase) and are resistant to degradation even in the presence of brefeldin A (half lives >360 min). (**b**) Position-dependence of degradation induced by transmembrane R or D residues. The half lives of Tac mutants containing transmembrane R or D residues were plotted against the position of these residues within the transmembrane domain. Positions were numbered from the lumenal to the cytoplasmic side of the membrane as shown in (a).

revealed an interesting pattern of phenotypic effects (Figure 6). Addition of either basic or acidic residues resulted in rapid degradation when centrally placed in the Tac transmembrane domain. Relative to R mutations, however, the region where D mutations caused rapid degradation was somewhat longer and slightly shifted towards the cytoplasmic side of the membrane (Figure 6a and b). This 'degradation zone' appeared to be flanked on both sides by a short segment in which addition of R or D resulted in inefficient transport out of the ER and slow degradation (Figure 6a). Positioning of R or D residues near the boundaries between the transmembrane and the lumenal or cytoplasmic domains, in a region where the charged side chains could potentially interact with the polar lipid head groups or the aqueous environment, had little or no effect on the fate of the mutant proteins (Figure 6a). Placement of K, E or H residues near the middle of the Tac transmembrane domain also induced increased sensitivity to degradation in the ER, although their effects tended to be less pronounced and the regions of degradation more restricted than those observed for R and D mutations (Table I and Figure 6a).

Discussion

The analysis reported here demonstrates that placement of a single potentially charged residue within the transmembrane domain of a typical integral membrane protein can result in its retention and rapid degradation in the ER. Both R and D residues are effective in causing rapid degradation when placed at any of several central positions within the membrane-spanning domain of the Tac antigen. Substitution with K and E residues can have a similar effect,

although the rate of degradation is considerably lessened and the degradation zone somewhat more restricted. In at least two positions near the middle of the transmembrane sequence, H residues can likewise lead to increased sensitivity to degradation. Interestingly, these effects of potentially charged residues appear to correlate with the high levels of free energy required to partition their potentially charged side chains from water into a lipid bilayer. The energy cost associated with this transfer is higher for R or D than for K or E, with H requiring the least free energy of the group (Engelman *et al.*, 1986). If this correlation can be extended to other amino acid residues, then the strongly polar Q and N residues, which have transfer free energies higher than H (Engelman *et al.*, 1986), would also be expected to alter the fate of integral membrane proteins in a similar way when centrally placed within a transmembrane domain.

Thermodynamic considerations have led to the view that charged residues cannot reside within the hydrophobic interior of the membrane (reviewed by Engelman *et al.*, 1986; Singer, 1990). Interaction with the membrane in a discharged form is a more favourable alternative, although still costly in terms of transfer free energy (Engelman and Steitz, 1981; Honig and Hubbel, 1984). In either case, we were concerned that interrupting a hydrophobic transmembrane sequence by insertion of a potentially charged amino acid residue could result in complete translocation into the ER lumen by interfering with the stop-transfer function of the sequence. However, this does not appear to be the case for at least two of the Tac mutants exhibiting the most extreme phenotypic changes (R^{10} and D^{10}). Despite a weaker interaction with the lipid bilayer suggested by the partial extraction with sodium carbonate (Figure 4b), at any given point in time a majority of the R^{10} and D^{10} mutant proteins have a bitopic configuration (Figure 4a). The ability of these sequences to function as membrane-spanning domains may be explained by the fact that the decrease in free energy achieved by partitioning their predominantly hydrophobic helices into a lipid bilayer still exceeds the increase due to the presence of a strongly polar residue (Engelman *et al.*, 1986). Although the exact position of the potentially charged groups relative to the lipid bilayer and their physical state (i.e. bound water or ions, oligomerization, etc) have not been defined, the placement of such groups in a thermodynamically unfavourable situation has dramatic effects on the fate of the mutant proteins.

How might these potentially charged residues act in promoting retention and degradation in the ER? Given that these residues are positioned within the transmembrane domain, one possibility is that they act directly, for example by forming charge pairs and/or hydrogen bonds with polar groups present within the transmembrane domain of a putative recognition protein that is part of the ER retention/degradation apparatus. Indeed, we have recently demonstrated that a single potentially charged residue, when placed within a transmembrane domain, can solely mediate stable interaction with a protein containing an appropriately placed residue capable of assuming an opposite charge (Cosson *et al.*, 1991). Such charge-pair interactions within the transmembrane domain can completely explain the assembly of certain subunits of the TCR with one another (Manolios *et al.*, 1990). One characteristic of these charge pair interactions is their marked dependence on the relative positions of the charges within their transmembrane domains (Cosson *et al.*, 1991), which might explain the sharp change in the effect of these residues on ER degradation as a function of position, as demonstrated in Figure 6b. Alternatively, the effect of such highly polar residues within transmembrane domains on the stability of the proteins may be mediated not via intramembrane interactions but rather via indirect effects on the local structure of the transmembrane helices or adjacent regions of the molecules, or by perturbation of the lipid bilayer.

The observations reported here provide an explanation for the role of basic amino acid residues within the transmembrane domains of the TCR-α and -β chains as determinants for rapid degradation within the ER. Similarly, the α subunit of the Fcγ receptor IIIA has a D residue in its transmembrane domain, which has been shown to mediate retention and degradation of the unassembled subunit within the ER (Kurosaki *et al.*, 1991). The ability to alter the fate of the Tac antigen by merely placing a single potentially charged amino acid within its transmembrane domain suggests that the effect of these residues is not restricted to the native TCR or Fcγ membrane-spanning sequences, and that their targeting potential can be manifested in the context of an unrelated hydrophobic sequence. A similar structural determinant might explain the fate of other proteins within the ER. For example, the accelerated degradation of the resident ER enzyme 3-hydroxy-3-methylglutaryl Coenzyme A (HMG CoA) reductase induced by sterol is entirely mediated by the membrane-bound domain of the protein (Gil *et al.*, 1985; Jingami *et al.*, 1987; Skalnik *et al.*, 1988). This domain is composed of multiple membrane-spanning sequences, several of which contain potentially charged amino acid residues (Liscum *et al.*, 1985).

As with cytosolic degradation, there is reason to suspect that ER degradation may function not only in quality control over abnormal proteins, but in regulating the fate of metabolically controlled proteins. An interesting example of this is the secretory protein apolipoprotein B. Davis and colleagues (1990) have shown that apolipoprotein B exists as two distinct pools in the ER: one located within the ER lumen and the other associated with the ER membrane, such that the protein is partially exposed on the cytoplasmic face. Whereas the soluble, lumenal form is secreted from the cell, the membrane-bound form is effectively degraded by a process resembling the aforementioned ER degradation pathway. Analysis of the amino acid sequence of apolipoprotein B reveals the presence of hydrophobic stretches too short to span a membrane as typical transmembrane helices (Davies *et al.*, 1990). However, if one accepts the premise that, under certain conditions, potentially charged or strongly polar amino acid residues can be accommodated within a transmembrane sequence, then the resulting membrane-spanning domains may fit the pattern defined in our studies as a targeting determinant for ER degradation. This supposition is substantiated by the recent studies of Chuck *et al.* (1990) documenting that translocation intermediates of apolipoprotein B are indeed transiently arrested as membrane-spanning proteins.

Observations indicating that certain soluble proteins retained within the ER have relatively short half lives (Sitia *et al.*, 1987; Stoller and Shields, 1989; Lau and Neufeld, 1989; Weiss and Bogen, 1991) clearly suggest that transmembrane motifs are not the only determinants of rapid

degradation within this organelle. Even for transmembrane proteins having potentially charged residues within their membrane-spanning domains, it is important to question whether such residues are both necessary and sufficient for degradation. We have shown that the assembly of a chimeric protein containing the TCR-α transmembrane domain with the CD3-δ chain via the formation of a presumed charge pair largely prevents the ER degradation of the chimera, despite the fact that the destabilizing residues are still within the transmembrane domains of the complex (Bonifacino *et al.*, 1990b). This finding can be the result of either the direct masking of the potentially charged residues such that they go unrecognized by the degradative machinery or by interfering with any indirect effects of the isolated charged residues. In either case, the ability of potentially charged residues to be involved in both targeting the ER degradation and subunit assembly provides a mechanism whereby quality control is achieved by the mutually exclusive use of these residues for either assembly or destruction (Bonifacino *et al.*, 1990b). Thus, potentially charged residues can exist within transmembrane domains but nonetheless be unavailable for signaling degradation.

There are additional examples of proteins that have potentially charged residues within their transmembrane domains but yet are not rapidly degraded in the ER. The HIV-1 envelope glycoprotein precursor, gp160 (Willey *et al.*, 1988), the product of the *neu* oncogene (Bargmann and Weinberg, 1988) and mutagenized forms of the EGF receptor (Kashles *et al.*, 1988), the Semliki forest virus E2 protein (Cutler and Garoff, 1986) and the Rous sarcoma virus envelope glycoprotein (Davis and Hunter, 1987), all have potentially charged transmembrane residues but seem able to exit the ER. A mutant of the vesicular stomatitis virus G protein containing an R residue in its membrane-spanning domain has been shown to remain as an endo H-sensitive form, although by immunofluorescence microscopy, the protein was predominantly localized to a perinuclear structure resembling the Golgi system (Adams and Rose, 1985). Although in some of these cases the localization and fate of the newly synthesized proteins were not examined in detail, the available evidence nonetheless suggests that the effects of potentially charged residues are not entirely independent of their structural context. Factors such as the length and amino acid composition of the transmembrane or perimembrane sequences, interactions with other side chains or the protein backbone, homo- and hetero-oligomerization and intrinsic resistance to proteolysis may all influence the ability of potentially charged residues to cause rapid degradation in the ER.

The selective degradation of proteins whether in the cytosol or in the ER requires the cell to recognize specific structural features of the targeted molecules. Some of these features appear to be surprisingly simple, such as the identity of the NH_2-terminal amino acid for ubiquitin-mediated cytosolic degradation (Bachmair *et al.*, 1986). Similarly, the specific placement of potentially charged residues at particular sites within the transmembrane domains of proteins may provide a simple motif for ER degradation. More recent work on ubiquitin-mediated degradation has pointed to the fact that the NH_2-terminal amino acid may in some cases be necessary but not sufficient for targeted degradation. The separate function of other structural determinants (i.e. an internal lysine residue or a specific degradation sequence) is essential for degradation (Chau *et al.*, 1989; Johnson *et al.*, 1990; Glotzer *et al.*, 1991). Targeting for ER degradation may involve analogous complexity, as appropriately placed charged transmembrane residues may by themselves not be sufficient for ER degradation. An important corollary of this study is that the existence of potentially charged residues within transmembrane domains of type I integral membrane proteins is not necessarily forbidden. However, their placement with hydrophobic transmembrane sequences can have dramatic consequences on the biological properties of proteins and may provide the basis for determining the structural maturation, fate and function of certain membrane-bound proteins.

Materials and methods

DNA recombinant procedures and transfections

Single amino acid substitutions within the transmembrane domain of the Tac antigen were performed by oligonucleotide-directed mutagenesis of a human Tac cDNA (Leonard *et al.*, 1984), according to the method of Kunkel (1985). Construction of DNAs encoding a Tac-TCR-α chimeric protein (Tac-TCR-α) and a truncated, soluble form of the Tac antigen (Tac_t) was described previously (Bonifacino *et al.*, 1990a). DNAs encoding the normal and mutant Tac proteins were cloned into a modified version of the expression plasmid pCDM8 (Seed, 1987), as described (Bonifacino *et al.*, 1990a). COS-1 cells (American Type Culture Collection, Rockville, MD) plated on 150 mm culture dishes were transfected by the calcium phosphate precipitation method (Graham and van der Eb, 1973) using 20 μg of DNA, as previously described (Bonifacino *et al.*, 1990a). At 16 h after transfection, cultures of transfected cells were trypsinized, plated on 35 mm dishes and grown for an additional 24 h.

Metabolic labeling and immunoprecipitation

Transfected cells were labeled with 1 ml of 0.25 mCi/ml [^{35}S]methionine (Tran ^{35}S-label, ICN Radiochemicals, Irvine, CA) in methionine-free Dulbecco's modified Eagle's medium (DMEM) containing 5% dialyzed fetal bovine serum and 0.15 mg/ml gentamicin for 30 min at 37°C. For pulse–chase experiments, labeled cells were incubated in regular culture medium (DMEM containing unlabeled methionine, 10% fetal bovine serum, 0.15 mg/ml gentamicin) for various time periods at 37°C. At each time point, cells were removed from plates, collected by centrifugation and frozen at −70°C before analysis by immunoprecipitation of detergent-solubilized proteins, as previously described (Bonifacino *et al.*, 1990b). Digestions with Endo H (Boehringer) were performed for 16 h at 37°C, as described by Chen *et al.* (1988). Immunoprecipitates were resolved by SDS–PAGE. When indicated, brefeldin A (Epicentre Technologies, Madison, Wisconsin) was added to the culture and labeling media at 5 μg/ml from a 1 mg/ml stock solution in ethanol.

Limited proteolysis of Tac proteins

COS-1 cells expressing normal Tac or Tac mutants were metabolically labeled for 30 min and proteins isolated by immunoprecipitation with the monoclonal antibody 7G7 (a generous gift of David Nelson, NIH, Bethesda, MD). The isolated labeled proteins were resuspended in PBS and treated for 15 min at 4°C with different concentrations of proteinase K (0.1 to 100 μg/ml). In some experiments, immunoprecipitated proteins were denatured by incubation for 15 min at 95°C in 1% (w/v) SDS and 2% (v/v) β-mercaptoethanol followed by the addition of 60 mg/ml iodoacetamide. After 5-fold dilution with 1% (w/v) Triton X-100, denatured proteins were treated with proteinase K. Proteolysis was stopped by the addition of 0.5 mM phenylmethylsulfonyl fluoride and samples were analysed by SDS–PAGE on 13% acrylamide gels under either reducing or non-reducing conditions.

Analysis of the topology of Tac proteins

Transfected COS-1 cells were metabolically labeled for 30 min as described above, washed twice with 0.25 M sucrose/5 mM HEPES buffer, pH 6.8, resuspended in the same buffer and disrupted using a Dounce homogenizer fitted with a tight pestle (30 strokes). A postnuclear supernatant was fractionated by sedimentation on discontinuous sucrose gradients, as described by Bole *et al.* (1986). Membranes banding at the 1.3/2.0 M sucrose interface were collected, diluted 4-fold with PBS and incubated for 1 h at 4°C in the absence or presence of 100 μg/ml trypsin (Sigma Chemical Co., St Louis, MI), with or without 0.5% (w/v) Triton X-100. The reaction was stopped

by the addition of 0.5 mg/ml soybean trypsin inhibitor (Sigma Chemical Co., St Louis, MO). Membranes were solubilized and Tac proteins isolated by immunoprecipitation using the monoclonal antibody 7G7. Samples were analysed by SDS–PAGE on 10% acrylamide gels.

Sodium carbonate extraction of membranes

The association of newly synthesized Tac and Tac mutants with membranes was studied by extraction with a sodium carbonate solution (Howell and Palade, 1982; Fujiki *et al.*, 1982). Metabolically labeled COS-1 cells expressing various Tac constructs were disrupted as described in the previous section and homogenates centrifuged for 10 min at 2000 r.p.m. to remove intact cells and nuclei. Postnuclear supernatants were spun for 15 min at 230 000 *g* in a Beckman TL-100 centrifuge. Membrane pellets were resuspended in 0.5 ml of 0.1 M sodium carbonate, pH 11.3 using a Dounce homogenizer (10 strokes, tight pestle) and incubated for 30 min on ice. The extracted proteins were separated from membranes by an additional centrifugation for 15 min at 230 000 *g*. Membranes were again resuspended in 0.5 ml 0.1 M sodium carbonate, pH 11.3. After neutralization to pH 7 by the addition of 1 N HCl, samples were treated with 0.5% Triton X-100 and solubilized Tac proteins isolated by immunoprecipitation with the polyclonal antibody R3134 (Sharon *et al.*, 1986, a generous gift of Warren Leonard, NIH, Bethesda, MD).

Immunofluorescence microscopy

At 40 h after transfection, cells grown on 12 mm round coverglasses were fixed for 15 min at 25°C with 2% (v/v) formaldehyde in PBS. After rinsing twice with PBS, cells were permeabilized by incubation for 15 min at 25°C with 0.1% (w/v) saponin in PBS. Cells were then incubated for 1 h at 25°C with 50 μl of a 1:500 dilution of the rabbit polyclonal antiserum R3134 (Sharon *et al.*, 1986) in 0.1% (w/v) saponin/0.1% (w/v) bovine serum albumin/PBS (SBP buffer). After washing the excess, unbound antibody with PBS, cells were incubated for 30 min at 25°C with a 1:500 dilution of a rhodamine-conjugated goat anti-rabbit IgG (Cappel) in SBP buffer. The unbound antibody was removed by washing in PBS and coverglasses were mounted on microscope slides using Fluoromount G (Southern Biotechnology Associates, Birmingham, AL).

Acknowledgements

We thank Jennifer Lippincott-Schwartz, Carolyn Suzuki and James Basilion for helpful discussions and critical review of the manuscript. P.Cosson is the holder of a fellowship from EMBO. N.Shah was supported by the NIH Research Fellowship Program as a medical student from the University of Pennsylvania.

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Received on April 11, 1991; revised on June 7, 1991