addition of reagent during a 72-hour time course. After rhPF4 treatment, cell proliferation could be restored by the addition of fresh medium containing growth factors and heparin, indicating that rhPF4 suppressed the HUVE cell response to growth factor stimulation without cytotoxicity. Growth factor–stimulated human dermal fibroblast or keratinocyte proliferation were not inhibited by rhPF4 at concentrations that completely suppress HUVE cell growth. The observation that rhPF4 did not prevent the growth of these cell lines after they were stimulated with basic fibroblast growth factor (bFGF) indicated that rhPF4 inhibition of HUVE cell proliferation was not due to binding and sequestering of soluble bFGF. In experiments we conducted, rhPF4 also failed to inhibit proliferation of several human and murine tumor cell lines even at concentrations two to five times those that inhibited endothelial cell growth.

The inhibitory effect of recombinant PF4 on HUVE cells is correlated with the inhibition of DNA synthesis (Fig. 2B). The concentration of rhPF4 found to be highly inhibitory in both assays (~25 μg/ml), although far above that normally found in human plasma (10 ng/ml), is theoretically attainable at sites of platelet aggregation and α-granule release (*19*). The C-41 peptide extensively inhibited cell proliferation, although substantially higher concentrations of this peptide were required to produce inhibition comparable to that produced by full-length rhPF4 (Fig. 2C). The amino-terminal peptide (N-29) was not inhibitory even at very high doses.

The suppression of growth of vascular endothelial cells without inhibition of other cell types closely associated with capillaries suggests a possible physiological role for PF4 in the regulation of vessel development. In contrast, a highly specific platelet-derived endothelial cell mitogen has recently been cloned and characterized (*20*), suggesting that a balance of platelet proteins may be important for the control of angiogenesis. Regardless of its physiological function, the inhibitory specificity of rhPF4 suggests that it might be useful for the treatment of pathological vascularization. Although angiostatic in vitro and in vivo, protamine is not used for the clinical control of neovascularization because of unacceptably high toxicity (*21*). Because rhPF4 is identical in sequence to the natural protein, it would be expected to be less immunogenic and perhaps better tolerated than protamine. Further investigation of the conditions and growth factors that regulate the vascularization process should facilitate the development of agonists and antagonists for treatment of angiogenic diseases.

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A Peptide Sequence Confers Retention and Rapid Degradation in the Endoplasmic Reticulum

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A nonlysosomal pathway exists for the degradation of newly synthesized proteins retained within the endoplasmic reticulum (ER). This pathway is extremely selective: whereas some proteins are rapidly degraded, others survive for long periods in the ER. The question of whether this selectivity is due to the presence within the sensitive proteins of definable peptide sequences that are sufficient to target them for degradation has been addressed. Deletion of a carboxyl-terminal sequence, comprising the transmembrane domain and short cytoplasmic tail of the α chain of the T cell antigen receptor (TCR-α), prevented the rapid degradation of this polypeptide. Fusion of this carboxyl-terminal sequence to the extracelluar domain of the Tac antigen, a protein that is normally transported to the cell surface where it survives long-term, resulted in the retention and rapid degradation of the chimeric protein in the ER. Additional mutagenesis revealed that the transmembrane domain of TCR-α alone was sufficient to cause degradation within the ER. This degradation was not a direct consequence of retention in the ER, as blocking transport of newly synthesized proteins out of the ER with brefeldin A did not lead to degradation of the normal Tac antigen. It is proposed that a 23–amino acid sequence, comprising the transmembrane domain of TCR-α, contains information that determines targeting for degradation within the ER system.

THE IDENTITY AND FUNCTIONS OF intracellular organelles in eukaryotic cells are established by the specific targeting of appropriate proteins among these membrane compartments. The idenfification within proteins of signals that determine their fates within the cell has facilitated an understanding of this targeting (*1*). These peptide signals contain the information for import of cytosolic proteins (or nascent proteins) into the endoplasmic reticulum (ER), mitochondria, chloroplasts, peroxisomes, and nuclei. Within the secretory system, proteins have many fates, including retention within specific organelles or transport to the plasma membrane, secretory granules, and lysosomes. Proteins are targeted to the latter two organelles by information that is localized to peptide sequences or structural motifs (*2*). We have described one potential fate of proteins that are inserted into the ER but fail to be transported into the Golgi system. We observed that some of these proteins are rapidly degraded by a nonlysosomal process, which we refer to as ER degradation (*3–5*). One characteristic of this degradative system is its selectivity. This selectivity became apparent in studies of the fate of newly synthesized chains of the multisubunit T cell antigen receptor (TCR). Normally, this seven-chain complex ($\alpha\beta\gamma\text{-}\delta\epsilon\zeta_2$) assembles in the ER soon after synthesis. If these chains do not reach the Golgi system, either because of incomplete assembly or pharmacologic manipulation [for example, treatment of cells with the fungal

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antibiotic brefeldin A (BFA)], three of the chains (α, β, and δ) are rapidly degraded, whereas the others (γ, ε, and ζ) are stable for relatively long periods within the ER (*3–5*). Among the stable chains, we have found that ε and ζ are not targeted for ER degradation, regardless of whether they are free or associated with other TCR chains. In contrast, γ is only stable if it remains associated with ε. Expression of γ in the absence of other TCR subunits results in its rapid degradation by the ER pathway (*5*). Two explanations for this selectivity, each implying a specific model for ER degradation, are: (i) the ER may uniformly contain the responsible proteases and the selectivity may be a reflection of the intrinsic sensitivity of the target protein; and (ii) the site of degradation may be a specific region of the ER or an associated organelle, with selection occurring at the level of protein sorting within the ER. In either case, the identification of a target sequence that selects proteins for ER degradation would be an essential step in understanding the biology of this process.

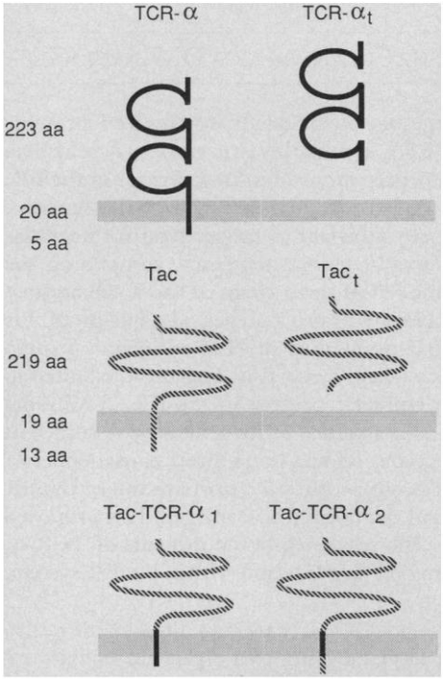

Fig. 1. Schematic representation of the proteins used in this study. The number of amino acids (aa) in the extracellular, transmembrane, and cytoplasmic domains of TCR-α and Tac is shown. DNAs encoding TCR-α_t, Tac$_t$, Tac–TCR-α_1, and Tac–TCR-α_2 were constructed by oligonucleotide-directed mutagenesis (*20*). TCR-α and TCR-α_t DNAs were cloned into the expression plasmid pSVL (Pharmacia). Tac, Tac$_t$, Tac–TCR-α_1, and Tac–TCR-α_2 were cloned into the expression plasmid pCDM8 (*21*). Plasmids were transfected into COS cells (*22*) and metabolic labeling studies were carried out 60 to 84 hours after transfection (*23*). The amino acid sequence of the shorter peptide of the TCR-α chain (amino acids 221 to 243 of the mature protein) that was able to confer retention and degradation in the ER to the Tac antigen is NLS**VMGLRILLLKVAGFNLLMTL**, with bold residues indicating the presumed transmembrane domain contained within this sequence (*24*).

We searched for such a sequence by dissecting domains of the α chain of the TCR (TCR-α), a sensitive target of ER degradation. Mature TCR-α found on the antigen-specific 2B4 cell line is a 42- to 44-kD transmembrane glycoprotein with a core molecular mass of 32 kD (*6*). It has an NH_2-terminal extracellular domain of 223 amino acids, a single transmembrane domain of 20 amino acids, and a short cytoplasmic tail of 5 amino acids (*7*) (Fig. 1). The extracellular domain contains four consensus sequences for the addition of N-linked oligosaccharide chains (*7*), of which only three appear to be glycosylated in the full-length protein (*8*). The fourth site (Asn^{221}-Leu^{222}-Ser^{223}) is immediately adjacent to the transmembrane domain and is probably not glycosylated because of steric hindrance. When TCR-α is expressed in fibroblasts by either transient or stable transfection, it never reaches the Golgi system (*3, 5*) (Fig. 2A, lanes 1 and 2). Instead, it is apparently retained in the ER, a fate common to many isolated subunits of multicomponent complexes. However, following a lag after synthesis, TCR-α is degraded (*3, 5*) (Fig. 2B) by a nonlysosomal process (*3–5*).

To determine the fate of the large extracellular domain of TCR-α in the absence of a membrane anchor, we produced a soluble form of TCR-α (TCR-α_t, Fig. 1) by inserting a termination codon into the cDNA after the codon for Met^{225} of the mature protein, near the boundary between the extracellular and transmembrane domains. When TCR-α_t was expressed in COS cells, it remained sensitive to endoglycosaminidase H (endo H) even at long chase times (Fig. 2A, lanes 3 and 4). As resistance to endo H is acquired by processing in the Golgi system, this observation indicates that TCR-α_t was also retained in the ER. The decreased migration upon SDS–polyacrylamide gel electrophoresis (SDS-PAGE) of TCR-α_t with respect to the full-length TCR-α (Fig. 2A, lanes 1 and 3) is probably due to the addition of a fourth N-linked carbohydrate chain to the consensus sequence located at the end of the extracellular domain (*8*). The core polypeptide size of TCR-α_t (Fig. 2A, lane 4) was consistent with the predicted truncation. Triton X-114 phase partitioning (*9*) demonstrated that the truncation indeed resulted in a soluble protein, and sucrose gradient centrifugation analysis in nonionic detergents revealed that neither full-length nor truncated TCR-α were aggregated (*8*). Consistent with the retention of TCR-α_t in the ER, examination of the culture medium showed that no truncated TCR-α was secreted (*8*). Analysis of the fate of TCR-α_t in COS cells revealed that, in contrast to full-length TCR-α, the truncated protein was not degraded over an 8-hour period (Fig. 2B). To assess whether the lack of degradation of TCR-α_t was due to the lack of a membrane anchor or to the additonal N-linked glycosylation, we produced a different truncated TCR-α by the addition of a translation stop codon after the codon for Thr^{207} of the mature protein. This resulted in the deletion of the fourth consensus sequence for N-linked glycosylation. Pulse-chase studies with this truncated protein showed that despite its being retained in the ER, this protein was also not degraded by the nonlysosomal pathway (*8*). These observations suggested that the COOH-terminal

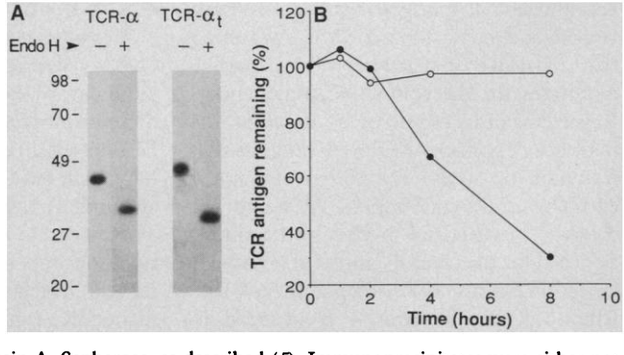

Fig. 2. Fate of TCR-α and TCR-α_t in the ER. (**A**) COS cells expressing TCR-α and TCR-α_t were pulse-labeled for 30 min with [^{35}S]methionine in carrier-free medium, and were then incubated for 3 hours at 37°C in the presence of unlabeled methionine. Cell pellets were extracted with Triton X-100, and TCR-α proteins were isolated from lysates by immunoprecipitation with a monoclonal antibody to 2B4 cell TCR-α bound to protein A–Sepharose, as described (*5*). Immunoprecipitates were either not treated (−) or treated (+) with endo H as described (*4*) before analysis by SDS-PAGE on 13% acrylamide gels. The positions of molecular mass markers (expressed in kilodaltons) are indicated to the left. (**B**) Similarly, COS cells expressing TCR-α and TCR-α_t were pulse-labeled for 30 min with [^{35}S]methionine in carrier-free medium and then incubated for the designated times in methionine-containing medium. Extraction of cell pellets, immunoprecipitation, and analysis by SDS-PAGE were performed as in (A). The relative amount of TCR-α and TCR-α_t with respect to the amount at 0 hours was determined by densitometric scanning of autoradiograms. Similar results were obtained in four independent experiments. TCR-α (●); TCR-α_t (○).

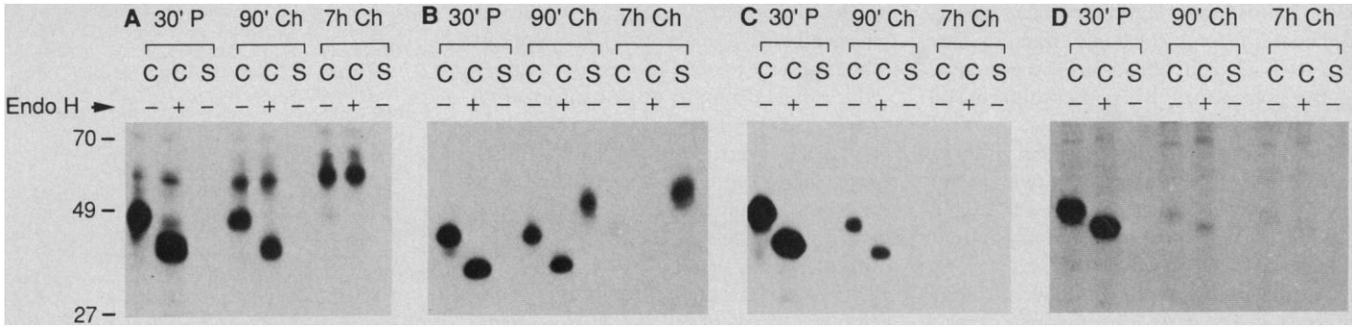

Fig. 3. Effect of transferring peptide sequences of TCR-α to the extracellular domain of Tac on retention and degradation in the ER. COS cells expressing Tac (**A**), Tac_t (**B**), Tac–TCR-α_1 (**C**) or Tac–TCR-α_2 (**D**) were pulse-labeled for 30 min at 37°C with [^{35}S]methionine in carrier-free medium and then were either not incubated (30′ P) or were incubated for 90 min (90′ Ch) or 7 hours (7h Ch) at 37°C in regular culture medium. Cells (C) and culture supernatants (S) were collected. Cell pellets were extracted with Triton X-100, and Tac proteins were isolated from detergent-solubilized cells and culture supernatants by immunoprecipitation with the monoclonal antibody anti-Tac (*13*) as described (*5*). Immunoprecipitates were either not treated (−) or treated (+) with endo H as described (*4*), before analysis by SDS-PAGE on 10% acrylamide gels. The position of molecular mass markers (expressed in kilodaltons) are shown on the left.

region (transmembrane plus cytoplasmic domains) was required for the degradation of TCR-α retained in the ER.

To assess whether the transmembrane domain plus cytoplasmic tail of TCR-α could transfer the information for ER degradation to an otherwise stable protein, we fused this sequence to a truncated form of the α chain of the human interleukin-2 (IL-2) receptor (also known as Tac antigen). Mature Tac is a 55-kD transmembrane glycoprotein with a core molecular mass of 33 kD (*10*). It has an extracellular domain of 219 amino acids, a single membrane-spanning domain of 19 amino acids, and a 13–amino acid COOH-terminal cytoplasmic tail (*10*) (Fig. 1). The extracellular domain of Tac contains two consensus sequences for N-linked glycosylation and several sites for the addition of O-linked carbohydrates. When expressed in COS cells, the Tac antigen is rapidly transported out of the ER to the Golgi system, where its carbohydrate chains are characteristically modified (Fig. 3A). From the Golgi system, Tac is transported to the cell surface, where it can be readily detected by immunofluorescent staining of the cells. A truncated form of Tac (Tac_t) was produced by insertion of a translation stop codon into the Tac cDNA after the codon for Gln^{219} of the mature protein, at the predicted external limit of the transmembrane domain (Fig. 1). When this protein was expressed in COS cells, it was rapidly lost from the cells (half-life of 1 hour) (Fig. 3B). However, all of the lost protein could be quantitatively recovered from the culture medium, and the secreted protein had been processed by Golgi enzymes (Fig. 3B). Thus, unlike the truncated TCR-α, truncated Tac is readily transported out of the ER and into the Golgi system, after which it is rapidly secreted as a soluble protein. A chimeric protein (Tac–TCR-α_1) (Fig. 1) consisting of the 219–amino acid NH_2-terminal domain of Tac linked to the 28–amino acid COOH-terminal transmembrane and cytoplasmic domains of TCR-α was constructed. When transiently expressed in COS cells, Tac–TCR-α_1 was rapidly lost after synthesis with no evidence of Golgi processing (Fig. 3C). To more precisely delineate the TCR-α sequences that transferred the phenotype of degradation in the ER to the Tac antigen, we constructed another chimeric protein (Tac–TCR-α_2) by replacing the transmembrane domain of mature Tac (amino acids 220 to 241) by the transmembrane domain of TCR-α (amino acids 221 to 243) (Fig. 1). This second chimera also failed to acquire resistance to endo H and was rapidly degraded in COS cells (Fig. 3D). In contrast, a third chimeric protein, in which the cytoplasmic tail of the Tac antigen (amino acids 245 to 251) was replaced by the cytoplasmic tail of TCR-α (amino acids 244 to 248), was processed normally in the Golgi system and transported to the cell surface (*11*). A more detailed study of the kinetics of degradation of Tac–TCR-α_1 showed that the half-life of this chimeric protein was ~10 min (Fig. 4). None of the lost protein could be accounted for by secretion (Fig. 3C). Solubilization of the cells with 0.2% SDS demonstrated that the loss of the chimeric protein was not due to the formation of aggregates insoluble in Triton X-100 (*11*), as was found for influenza hemagglutinin (*12*). The chimeric protein Tac–TCR-α_1 was immunoprecipitated by three different antibody reagents specific for the external domain of Tac, including two conformationally specific monoclonal antibodies that recognize different epitopes [anti-Tac (*13*) and 7G7 (*14*)] and a polyclonal antiserum [R3134 (*15*)]. Thus, the external domain of Tac was not grossly abnormal. The rate of disappearance of Tac–TCR-α_1 was identical, regardless of the antibody used (*11*). As would be predicted for the nonlysosomal ER degradation pathway, the disappearance of the Tac–TCR-α_1 was not inhibited by 50 m*M* NH_4Cl, an agent that blocks lysosomal degradation of proteins (Fig. 4).

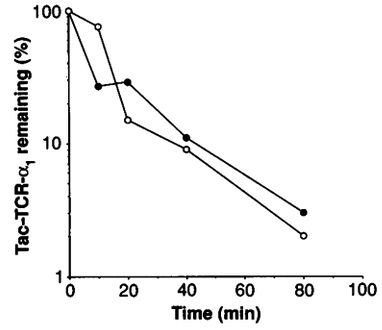

Fig. 4. Kinetics of Tac–TCR-α_1 degradation. COS cells expressing Tac–TCR-α_1 were incubated in the absence (●) or presence (○) of 50 m*M* NH_4Cl for 1 hour at 37°C before pulse-labeling for 15 min at 37°C with [^{35}S]methionine in carrier-free medium. Cells were then incubated for the designated times at 37°C in methionine-containing medium, in the continued absence or presence of 50 m*M* NH_4Cl. Cell pellets were extracted with Triton X-100, and the chimeric protein was analyzed by immunoprecipitation and SDS-PAGE on 10% acrylamide gels. The percentage of Tac–TCR-α_1 remaining with respect to the amount at 0 min was determined by densitometric scanning of autoradiograms.

A possible explanation for the fate of the chimeric proteins was that they were made incompetent for transport from the ER to the Golgi system, a relatively common phenotype of mutated membrane proteins. Accordingly, ER degradation might simply be a consequence of retention in the ER. To test this, we treated cells expressing either the full-length or truncated Tac with BFA. This drug completely blocks movement of proteins from the ER to the Golgi system

(*16, 17*) but has no inhibitory effect on ER degradation (*5*). Accordingly, when the fate of Tac–TCR-α_1 in fibroblasts was examined in the presence of BFA, the chimera still underwent rapid degradation in the ER (Fig. 5). Consistent with previous findings, treatment with BFA resulted in retention of both full-length and truncated Tac within the ER, as determined by immunofluorescence microscopy (*11*). The Tac and Tac_t retained within the ER underwent partial processing of their carbohydrate chains (Fig. 5) by Golgi enzymes that were redistributed to the ER in the presence of BFA (*17*). Because of its effect on transport from the ER to the Golgi system, BFA effectively blocked secretion of Tac_t (Fig. 5). In contrast to Tac–TCR-α_1, neither Tac nor Tac_t were degraded in the presence of BFA, despite being retained in the ER (Fig. 5). Thus, retention in the ER per se does not result in the rapid degradation of these proteins. Information contained within the transmembrane domain of TCR-α appears to be necessary for this process to occur.

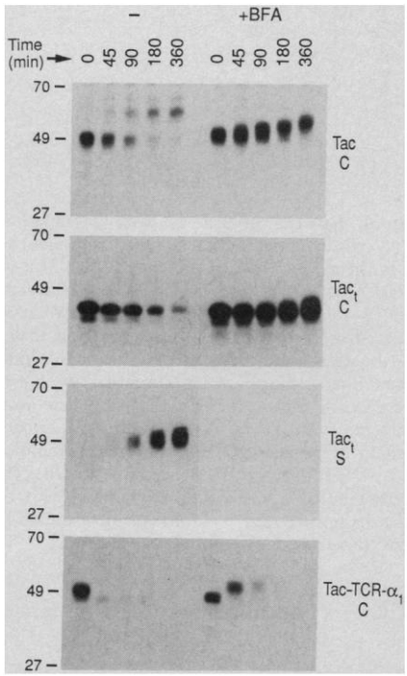

Fig. 5. Effect of retention in the ER on the degradation of Tac and Tac_t. COS cells expressing Tac, Tac_t, and Tac–TCR-α_1 were incubated for 1 hour at 37°C in regular culture medium in the absence (−) or the presence of brefeldin A (5 μg/ml) (+BFA). Cells were pulse-labeled for 30 min at 37°C with [^{35}S]methionine in carrier-free medium and then incubated in regular culture medium for the periods indicated, in the continued absence or presence of BFA. Cells were extracted with Triton X-100, and Tac proteins were isolated from detergent-solubilized cells (C) or culture supernatants (S) with the monoclonal antibody anti-Tac (*13*). No secreted Tac proteins were found in supernatants of cells expressing full-length Tac or Tac–TCR-α_1. The positions of molecular mass markers (expressed in kilodaltons) are shown on the left of each panel.

Our results demonstrate that information for selective degradation by the nonlysosomal ER degradative pathway can be transferred from a protein targeted for destruction to a normally stable protein. It is reasonable to assume that the extracellular domain of Tac is similar, if not identical, whether it is attached to its native transmembrane domain or the TCR-α transmembrane domain. This assumption is supported by conformation-specific antibody reactivity. It is therefore unlikely that the insensitivity of either truncated or full-length Tac to ER degradation (even when retained in the ER) is due to the intrinsic resistance of the extracellular domain to ER proteases. Another example of probable ER degradation is found in the rapid turnover of hydroxymethylglutaryl coenzyme A reductase (*18*). Degradation of this resident ER enzyme is accelerated by sterols. When the membrane-bound domain of this protein is deleted, it is degraded at a much lower rate and degradation is no longer controlled by sterols. Whether the targeting information transferred by the TCR-α sequence is a biochemical or spatial phenomenon is not clear. If the former is the case, then this sequence is the recognition site for a biochemical process that can initiate the degradation of a protein to which it is attached, analogous to signals for cytosolic degradation (*19*). If the latter is true, then this sequence is responsible for the sorting of the TCR-α and Tac extracellular domains to a degradative compartment.

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20. Oligonucleotide-directed mutagenesis was performed as described [T. A. Kunkel, *Proc. Natl. Acad. Sci. U.S.A.* **82**, 488 (1985); ———, J. D. Roberts, R. A. Zakour, *Methods Enzymol.* **154**, 367 (1987)]. cDNAs encoding TCR-α and Tac were inserted into the double-stranded replicative form of M13mp19 DNA. Oligonucleotides 40 or 30 residues long and with one or three mismatches, respectively, were used to introduce translation stop codons. For construction of the Tac–TCR-α_1 hybrid gene, a 910-bp Eco RI–Nac I fragment of the Tac cDNA and a 540-bp Hind II–Xba I fragment of the TCR-α cDNA were cloned in tandem into pUC18 that had been digested with Eco RI and Xba I. The hybrid gene was excised from pUC18, and cloned into M13mp19. Fusion of the two reading frames at the desired point in the sequence was achieved by deletion of 55 bp with the use of a 48-residue mutagenic oligonucleotide encoding amino acids 212 to 219 of Tac fused to amino acids 221 to 228 of TCR-α. We constructed the Tac–TCR-α_2 hybrid gene by mutagenesis using the polymerase chain reaction as described [S. J. Scharf, G. T. Horn, H. A. Erlich, *Science* **233**, 1076 (1986)]. The Tac–TCR-α_1 gene was used as a template. The 5′ amplification primer consisted of a 36-residue oligonucleotide containing an Eco RI site fused to a sequence of the 5′ untranslated region of the Tac cDNA. The 3′ amplification primer consisted of a 66-residue oligonucleotide that was complementary to a sequence comprising codons for amino acids 235 to 243 of TCR-α, codons for amino acids 242 to 251 of Tac, a stop codon (TAG), and an Xba I site. The mutagenized DNAs were completely sequenced and found to contain no other mutations.
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22. COS-1 or COS-7 cells were maintained in culture in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum and gentamicin (0.15 mg/ml). Cells were transfected by the calcium phosphate precipitation method, as described (*5*). Cells transfected with pSVL plasmid constructs were treated for 16 hours at 37°C with 3 m*M* sodium butyrate to increase expression of the DNAs. pCDM8 constructs routinely gave high levels of expression without sodium butyrate treatment. Similar results were obtained with COS-1 and COS-7 cells.
23. For metabolic labeling studies, cells plated on 150-mm culture dishes were washed twice and resuspended in methionine-free medium [methionine-free DMEM containing 5% fetal bovine serum and gentamicin (0.15 mg/ml)]. After preincubation for 15 min at 37°C, cells were labeled with 4 ml of [^{35}S]methionine (0.25 mCi/ml) (Trans ^{35}S-label, ICN Radiochemicals) per plate.
24. Abbreviations for the amino acid residues are: A, Ala; C, Cys; D, Asp; E, Glu; F, Phe; G, Gly; H, His; I, Ile; K, Lys; L, Leu; M, Met; N, Asn; P, Pro; Q, Gln; R, Arg; S, Ser; T, Thr; V, Val; W, Trp; and Y, Tyr.

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