17. C. Quilliet, M. Kléman, M. Benillouche, F. Kalb, *C. R. Acad. Sci. Ser. II* **319**, 1469 (1994); C. Quilliet, C. Blanc, M. Kléman, *Phys. Rev. Lett.* **77**, 522 (1996).
18. J. Daicic *et al*., *J. Phys. II France* **5**, 199 (1995).
19. A. Maldonado, W. Urbach, R. Ober, D. Langevin, *Phys. Rev. E* **54**, 1774 (1996).
20. K. M. McGrath, *Langmuir* 13, 1987 (1997).
21. A. Firouzi *et al*., *Science* **267**, 1138 (1995).
22. R. A. Laudise and D. W. Johnson Jr., *J. Non-Cryst. Solids* **79**, 155 (1986).
23. R. Vacher, T. Woignier, J. Pelous, E. Courtens, *Phys. Rev. B* **37**, 10503 (1988).
24. J. Phalippou, T. Woignier, M. Prassas, *J. Mater. Sci.* **25**, 3111 (1990).
25. D. W. Schaefer *et al*., *J. Non-Cryst. Solids* **145**, 105 (1992).
26. A. Emmerling and J. Fricke, *ibid*., p. 113; P. Scheuerpflug, M. Hauck, J. Fricke, *ibid*., p. 196.
27. D. Posselt, J. S. Pederson, K. Mortensen, *ibid*., p. 128.
28. S. Torquato, unpublished results.
29. M. W. Tate, E. F. Eikenberry, S. M. Gruner, *Rev. Sci. Instrum.* **68**, 47 (1997).
30. Supported by the U.S. Army Research Office (grant DAAH04-95-1-0102), the U.S. Department of Energy (grant DE-FG02-87ER60522), and the Materials Research Science and Engineering Center program of NSF (grant DMR-940032).

27 January 1997; accepted 4 June 1997

# A Di-Acidic Signal Required for Selective Export from the Endoplasmic Reticulum

Noriyuki Nishimura and William E. Balch*

Transport of membrane proteins between intracellular compartments requires specific sequences in the protein cytoplasmic domain to direct packaging into vesicle shuttles. A sequence that mediates export from the endoplasmic reticulum (ER) has proved elusive. A di-acidic signal (Asp-X-Glu, where X represents any amino acid) on the cytoplasmic tail of vesicular stomatitis virus glycoprotein (VSV-G) and other cargo molecules was required for efficient recruitment to vesicles mediating export from the ER in baby hamster kidney cells. The existence of such a signal provides evidence that export from the ER occurs through a selective mechanism.

Vesicular transport of proteins between intracellular compartments is coordinated by the activity of coat complexes that direct the selection of cargo through cytoplasmic signals and budding from membranes (*1*, *2*). These coat complexes include clathrin, which mediates sorting of receptors containing Tyr-based motifs from the trans-Golgi network and the cell surface (*3*); COPI, which binds di-lysine motifs and functions to direct retrograde transport of proteins from pre-Golgi and Golgi compartments (*4*, *5*); and COPII, which participates in protein export from the ER (*1*). A signal that directs recruitment of proteins to COPII-coated vesicles remains to be defined.

VSV-G is a type 1 transmembrane protein that forms a homotrimer (*6*). It has a 29–amino acid oligomer (29-mer) cytoplasmic tail at its COOH-terminus that may be important for ER export (*7*). Consistent with this possibility, we found that addition of a 29-mer cytoplasmic tail peptide to an in vitro ER to Golgi transport assay in baby hamster kidney (BHK) cells inhibited VSV-G transport in a dose-dependent manner (Fig. 1A). We also analyzed the transport of truncated VSV-G mutants in vivo with a vaccinia virus transient expression system. A mutant lacking the entire 29–amino acid cytoplasmic tail of VSV-G is defective in folding and oligomerization (*7*) and exits the ER very slowly (Fig. 1B). In contrast, a mutant lacking 26 amino acids of the cytoplasmic tail is not defective in folding or oligomerization (*7*), yet is also exported at a reduced rate (Fig. 1B).

To locate the region in the COOH-terminal 26 amino acids responsible for ER export, we generated a series of truncation mutants (Fig. 1C). Deletion of the COOH-terminal five amino acids had no effect on ER to Golgi transport at the 20-min time point, whereas removal of eight amino acids reduced transport by nearly 80% (Fig. 1C). Deletion of residues 1 to 9 or 6 to 13 had no effect, whereas deletion of residues 1 to 13 showed a partial decrease in transport efficiency (Fig. 1C). Deletion of residues 14 to 24 reduced transport to the amount observed for the residue 4 through 29 deletion (del 4-29) truncation (Fig. 1C). These results demonstrate that a spacer region from the membrane is necessary to present a signal critical for ER export that resides within residues 14 to 24.

To identify specific residues required for transport, we substituted amino acid residues 14 to 24 individually with Ala. The Ala scan revealed that neither the Tyr nor Ile residues, which are critical for basolateral sorting (Fig. 1C) (*8*), were required for ER export. Rather, two acidic residues, the Asp at position 21 (D21) and the Glu at position 23 (E23) (Fig. 2A), when mutated individually or in combination, reduced the rate of ER to Golgi transport to that observed for the del 4-29 construct (*9*). The requirement for the DXE sequence was specific because mutation of D21 to E or R, or E23 to D or R, also caused reduced export (Fig. 2A). Examination of transport over a longer time course (Fig. 2B) revealed that the transport defect resulting from mutation of the di-acidic motif predominantly affected the rate of export; the extent of transport was less strongly affected at later time points. The decreased rate of transport correlated with a reduced rate of VSV-G export from the ER as detected by indirect immunofluorescence. Wild-type VSV-G had a prominent Golgi distribution at the 10-min time point, whereas a double mutant in which the DXE signal was mutated to AXA remained localized to the ER (Fig. 2C). ER export of the double mutant appeared to occur through a COPII-mediated pathway (*2*, *10*) because VSV-G transport remained completely sensitive to Sar1 mutants that block the assembly of COPII

**Table 1.** Alignment of the DXE motif found in the cytoplasmic domain of transmembrane proteins. Sequences were obtained from the National Center for Biotechnology Information (NCBI). Asialoglycoprotein receptor, ASGPR; varicella zoster virus glycoprotein I, VZV GPI. The Yxxϕ motifs are underlined. The acidic residues conforming to the di-acidic signal (DXE) involved in ER export are highlighted in bold. Proteins containing a more general (D/E)X(E/D) signal found on the COOH-terminal side of a Yxxϕ motif are listed in (*27*).

| Protein | NCBI identifier | Sequence |
|---|---|---|
| VSV-G* | Virus (138311) | TM-18aa-YT**D**I**E**MNRLGK |
| LAP* | Human (130727) | TM-8aa-YRHVA**D**G**E**DHA |
| CD3δ | Mouse (115986) | TM-21aa-YQPLR**D**R**E**DTQ-14aa |
| CD3γ | Mouse (115994) | TM-21aa-YQPLK**D**R**E**YDQ-12aa |
| ASGPR H1 subunit | Human (126132) | MTKEYQDLQHL**D**N**E**ES-24aa-TM |
| VZV GPI | Virus (138246) | TM-20aa-YAGLPVD**D**F**E**DSESTDTEEEF |
| E-cadherin | Human (399166) | TM-95aa-YDSLLVF**D**Y**E**GSGS-42aa |
| CD3ε (Y177E)* | Mouse (1345709) | TM-35aa-YEPIRKGQR**D**L(Y → **E**/**D**)SGLNQRAV |

*Proteins with known functional di-acidic exit signals.

Departments of Molecular and Cell Biology, The Scripps Research Institute, 10550 North Torrey Pines Road, La Jolla, CA 92037, USA.

*To whom correspondence should be addressed. E-Mail: webalch@scripps.edu

coats (*11*). These results demonstrate that a di-acidic signal immediately flanking the Yxxϕ motif that confers basolateral sorting of VSV-G (Fig. 1C) (*8*) is required for efficient recruitment of VSV-G into COPII vesicles.

To determine if mutation of the DXE motif interfered with VSV-G maturation, we examined the kinetics of folding and oligomerization of wild-type and mutant VSV-G. Folding of wild-type VSV-G involves sequential interaction with the chaperones immunoglobulin-binding protein (BiP) and calnexin (*6, 12, 13*). Wild-type VSV-G was released from calnexin with a half-time ($t_{1/2}$) of ~10 min. Release of either the single- or double-Ala substituted forms of VSV-G from calnexin occurred at the same or slightly faster rate than that observed for wild-type VSV-G (Fig. 3A). We also observed similar or accelerated rates of trimerization of the VSV-G tail mutants compared with the wild-type protein (Fig. 3B). The accelerated kinetics of folding and trimerization of VSV-G mutants are analogous to those previously reported for a truncated VSV-G lacking 28 residues of the cytoplasmic tail (*7*). Because mutation of the di-acidic signal did not interfere with VSV-G maturation, folding and oligomerization do not appear to be the steps responsible for the reduced rate of export of VSV-G mutants containing the Ala-substituted DXE motif.

If the DXE motif functions as an exit signal, then these residues should confer export to a protein that is normally retained in the ER. We generated chimeras in which the transmembrane and cytoplasmic domains of the α subunit of the T cell receptor (TCR) were replaced by those of VSV-G (Fig. 4A). TCRα, like VSV-G, is a type 1 transmembrane glycoprotein. Unlike VSV-G, TCRα cannot exit the ER in the absence of other

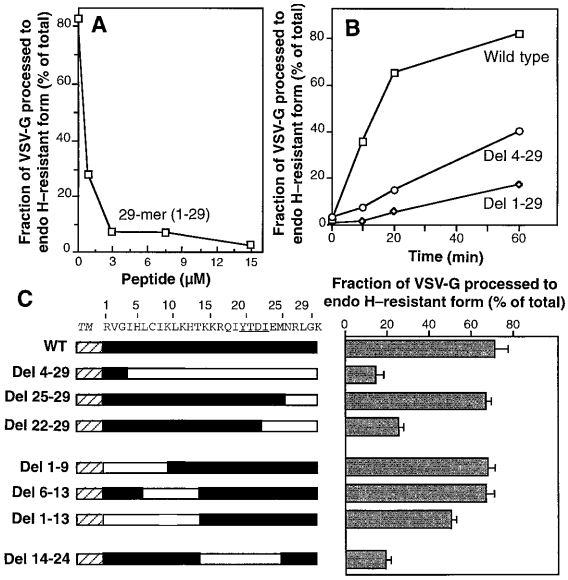


**Fig. 1.** The effect of the cytoplasmic tail of VSV-G on ER to Golgi transport in vitro and in vivo. (**A**) Normal rat kidney cells were infected with tsO45 VSV, a thermoreversible variant retained in the ER at the restrictive temperature (39.5°C) (*32, 33*), labeled for 10 min with [$^{35}$S]Met at 39.5°C, permeabilized, and incubated in vitro at the permissive temperature (32°C) for 60 min with the indicated concentration of 29-mer peptide corresponding to the cytoplasmic tail of VSV-G. Transport to the Golgi was measured by appearance of endo H–resistant forms (*34*). (**B**) BHK cells were transfected with the pAR plasmid encoding wild-type VSV-G or the indicated deletion mutant as described (*35*), labeled for 10 min with [$^{35}$S]Met, and chased with unlabeled Met for the indicated times at 37°C. The fraction of VSV-G processed to endo H–resistant forms was determined as described (*36*). (**C**) Transport of deletion mutants (left panel) was measured at a chase time of 20 min as described above.

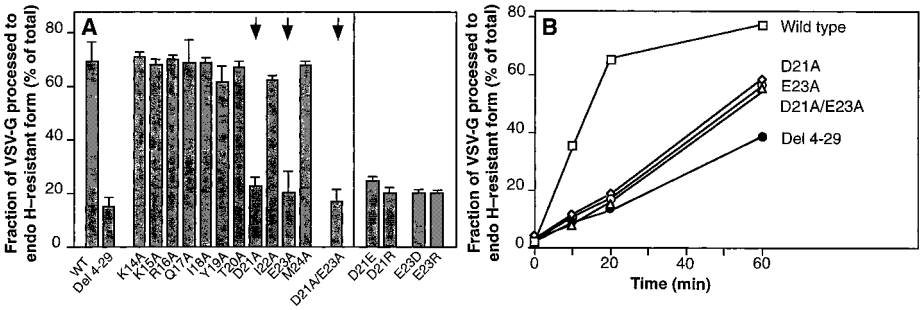


**Fig. 2.** Requirement of two acidic residues in the cytoplasmic tail of VSV-G for efficient export from the ER. (**A** and **B**) Transport of VSV-G mutants to the Golgi in BHK cells was measured at a chase time of 20 min (A) or for the indicated time (B) as described (Fig. 1B). Arrows in (A) indicate the DXE mutants. (**C**) BHK cells were transfected at 39.5°C with the pAR plasmid encoding either tsO45 VSV-G wild-type (a and c) or the D21A/E23A double mutant (aa mutant). After 4 hours of transfection, cells were shifted directly to ice, at which point tsO45 VSV-G was found in the ER (a and b), or to 32°C for 10 min to permit rapid folding and export (c and d). The distribution of tsO45 VSV-G was determined by indirect immunofluorescence as described (*33*). Arrows (c) indicate prominent labeling of Golgi compartments. The arrowhead (d) shows typical retention of the double mutant in the ER. After 60 min of incubation at 32°C, both wild-type and double-mutant proteins were detected on the cell surface (*11*).

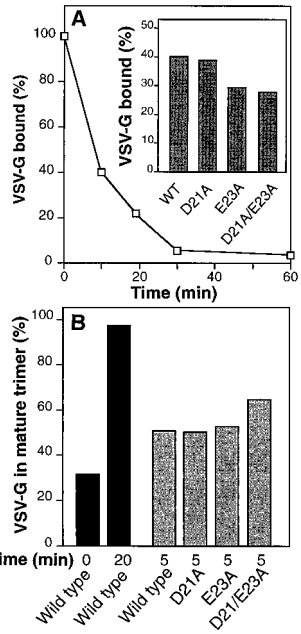


**Fig. 3.** Effect of D21A, E23A, and the D21A/E23A double mutants on release from calnexin and VSV-G trimerization. (**A**) BHK cells were transfected with the pAR plasmid encoding wild-type VSV-G, labeled for 10 min with [$^{35}$S]Met, and chased with unlabeled Met for the indicated times at 37°C. The fraction of VSV-G bound to calnexin was determined as described (*12, 37*). Calnexin-bound VSV-G at each time point is expressed relative to that observed at 0 min (100%). (Inset) BHK cells were transfected with the pAR plasmid encoding the indicated VSV-G construct. WT, wild type. Shown is the amount of each bound to calnexin at the 10-min time point. (**B**) BHK cells were transfected with the pAR plasmid encoding wild-type VSV-G or the indicated tail mutant, labeled for 10 min with [$^{35}$S]Met, and chased with unlabeled Met for the indicated times at 37°C. Trimer formation was determined as described (*7*). The fraction of VSV-G converted from the monomer to trimer is expressed as percentage of total VSV-G at the indicated time points.

TCR subunits and is rapidly degraded after synthesis (*14*). The determinant directing retention and degradation of TCRα in the ER is present in the transmembrane domain (*15*). A chimera containing the cytoplasmic domain of VSV-G linked to the luminal and transmembrane domains of TCRα was rapidly degraded and did not exit the ER, suggesting that the DXE signal cannot override the dominant effects of the transmembrane retention and degradation signal. In contrast, when the extracellular domain of TCRα (TCRαT) was expressed in the absence of the transmembrane region, it was not degraded and was stably retained in the ER (Fig. 4B) (*16*). A chimera containing the extracellular domain of TCRαT joined to the transmembrane and cytoplasmic domains of VSV-G (TVVwt) was efficiently (>30%) exported (Fig. 4B). Export was completely blocked in a chimera in which the di-acidic motif was mutated to Ala residues (TVVaa). The cytoplasmic DXE motif, therefore, is sufficient to direct ER export.

To determine whether the DXE export signal found in the VSV-G cytoplasmic tail functions in other transmembrane proteins, we found that lysosomal acid phosphatase (LAP), a type 1 transmembrane glycoprotein which is targeted to lysosomes through the plasma membrane by a Yxxϕ signal (*17*, *18*), also had a DXE signal on the COOH-terminal side of its Yxxϕ motif (Table 1). In LAP, substitution of this DXE motif with Ala impairs internalization from the cell surface, but does not affect basolateral sorting (*19*). To test whether the di-acidic motif found in LAP is also critical for efficient ER export, we generated a chimera between the cytoplasmic tail of LAP and TCRαT with the transmembrane region of VSV-G as a linker (TVLwt). TVLwt was efficiently exported from the ER (Fig. 4B) and export was largely inhibited when the Asp and Glu residues in the LAP sequence were mutated to Ala residues (TVLaa). Thus, the DXE motif in LAP, like VSV-G, also serves to promote efficient export from the ER.

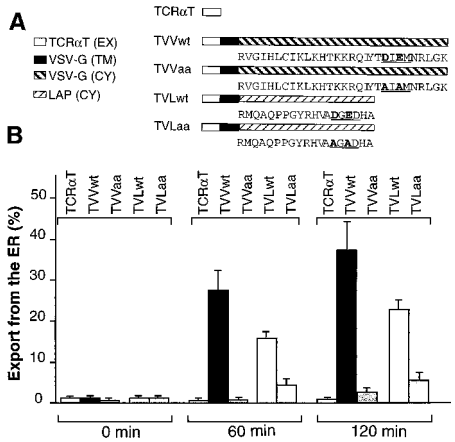


**Fig. 4.** Effect of the di-acidic motif present in VSV-G and LAP on the transport of TCRα. (**A**) The TCRα chimeras that were used are illustrated (*38*). EX, extracellular domain; TM, transmembrane domain; CY, cytoplasmic domain. (**B**) BHK cells were transfected with the pT7 plasmid encoding the indicated TCRα chimera, labeled for 30 min with [$^{35}$S]Met, and chased with unlabeled Met for the indicated times at 37°C. TCRα chimeras were immunoprecipitated with an antibody to TCRα (A2B4) (*39*), and their transport to the Golgi was measured by the appearance of endo H–resistant forms.

Our results provide evidence for a signal that directs the export of cargo from the ER. A diphenylalanine motif (FF) was recently suggested to promote export of proteins from the ER (*20*). However, the processing of a reporter glycoprotein by *cis*-Golgi enzymes continued following mutation of the FF motif to Ala residues (*20*). Whether the FF motif functions in concert with a dilysine motif to direct recycling through interaction with COPI coats or augments export from the ER remains to be determined (*21*–*23*). Whereas the di-acidic signal appears to be critical for controlling the efficiency of export of VSV-G, mutation of the signal does not limit the extent of transport. This accounts for the inability of previous studies to detect the role of the DXE signal in ER export (*8*, *19*, *24*–*26*).

A di-acidic motif located at variable distance on the COOH-terminal side of the Yxxϕ motif can be found in many, but not all, transmembrane cargo molecules exiting the ER (Table 1) (*27*). The juxtaposition of these two signals suggests that such regions in the cytoplasmic domain of cargo proteins may direct sequential interaction with multiple coat machineries. In one of these proteins, the CD3ε subunit of TCR, substitution of Tyr$^{177}$ with an acidic residue such as Glu or Asp, but not with Ala, led to loss of normal retention in the ER by creating a new di-acidic motif on the COOH-terminal side of the Yxxϕ motif (Table 1) (*28*). Our finding that a di-acidic signal on one group of proteins mediates efficient recruitment by COPII coats indicates that cargo recruitment from the ER occurs by a selective mechanism as we previously proposed (*2*, *29*). The presentation of DXE or other signals may be the ultimate determinant in dictating the variable rate of export of cargo from the ER.

## REFERENCES AND NOTES


1. R. Schekman and L. Orci, *Science* **271**, 1526 (1996).
2. M. Aridor and W. E. Balch, *Trends Cell Biol.* **6**, 315 (1996).
3. S. L. Schmid, *Annu. Rev. Biochem.*, in press.
4. T. E. Kreis and R. Pepperkok, *Curr. Opin. Cell Biol.* **4**, 533 (1994).
5. H. R. B. Pelham, *Cell* **79**, 1125 (1994).
6. R. W. Doms, D. S. Keller, A. Helenius, W. E. Balch, *J. Cell Biol.* **105**, 1957 (1987).
7. R. W. Doms *et al.*, *ibid.* **107**, 89 (1988).
8. D. C. Thomas and M. G. Roth, *J. Biol. Chem.* **269**, 15732 (1994).
9. Single-letter abbreviations for the amino acid residues are as follows: 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. X indicates any amino acid.
10. R. Schekman *et al.*, *Cold Spring Harbor Symp. Quant. Biol.* **60**, 11 (1996).
11. N. Nishimura and W. E. Balch, unpublished data.
12. A. M. De Silva, W. E. Balch, A. Helenius, *J. Cell Biol.* **111**, 857 (1990).
13. C. Hammond and A. Helenius, *Science* **266**, 456 (1994).
14. R. D. Klausner, *New Biol.* **1**, 3 (1989).
15. J. S. Bonifacino, P. Cosson, R. D. Klausner, *Cell* **63**, 503 (1990).
16. J. S. Bonifacino, C. K. Suzuki, R. D. Klausner, *Science* **247**, 79 (1990).
17. C. Peters *et al.*, *EMBO J.* **9**, 3497 (1990).
18. M. Bruan, A. Waheed, K. von Figura, *ibid.* **8**, 3633 (1989).
19. V. Prill, L. Lehmann, K. von Figura, C. Peters, *ibid.* **12**, 2181(1993).
20. K. Fiedler, M. Veit, M. A. Stamnes, J. E. Rothman, *Science* **273**, 1396 (1996).
21. C. Itin, R. Schindler, H.-P. Hauri, *J. Cell Biol.* **131**, 57 (1995).
22. E. Gaynor and S. D. Emr, *ibid.* **136**, 789 (1997).
23. F. Letourneur *et al.*, *Cell* **79**, 1199 (1994).
24. S. W. Pimplikar, E. Ikonen, K. Simons, *J. Cell Biol.* **125**, 1025 (1994).
25. A. Musch, H. Xu, D. Shields, E. Rodriguez-Boulan, *ibid.* **133**, 543 (1996).
26. K. Matter, E. M. Yamamoto, I. Mellman, *ibid.* **126**, 991 (1994).
27. Other proteins in which the DXE signal can be detected on the COOH-terminal side of the Yxxϕ motif are cystic fibrosis transmembrane regulator, glucose transporter 4, low-density lipoprotein receptor, furin-like protease (PC6B/PC5B), cation independent– and dependent–mannose 6 phosphate receptor, epidermal growth factor receptor, Kex1 protease (yeast), Fc receptor (FcγRII2/FcRII-B2), transferrin receptor, nerve growth factor receptor, vacuolar protein sorting receptor (yeast), and insulin-related receptor.
28. A. Mallabiabarrena, J. Angeles, M. Rico, B. Alarcon, *EMBO J.* **14**, 2257 (1995).
29. W. E. Balch, J. M. McCaffery, H. Plutner, M. G. Farquhar, *Cell* **76**, 841 (1994).
30. O. Kuge *et al.*, *J. Cell Biol.* **125**, 51 (1994).
31. R. Higuchi, B. Krummel, R. K. Saiki, *Nucleic Acids Res.* **16**, 7351 (1988).
32. F. Lafay, *J. Virol.* **14**, 1220 (1974).
33. H. Plutner, H. W. Davidson, J. Saraste, W. E. Balch, *J. Cell Biol.* **119**, 1097 (1992).
34. H. W. Davidson and W. E. Balch, *J. Biol. Chem.* **268**, 4216 (1993). Appearance of endoglycosidase H (endo H)–resistant forms corresponds to the processing of VSV-G oligosaccharides by *cis*- or medial Golgi–associated α-1,2-mannosidases and glycosyltransferases.
35. All mutant constructs were made with polymerase chain reaction (PCR) as described (*30, 31*) and were verified by DNA sequencing.
36. E. J. Tisdale, J. R. Bourne, R. Khosravi-Far, C. J. Der, W. E. Balch, *J. Cell Biol.* **119**, 749 (1992).
37. W.-J. Ou, P. H. Cameron, D. Y. Thomas, J. J. M. Bergeron, *Nature* **364**, 771 (1993).
38. TCRα/VSV-G and TCRα/VSV-G/LAP chimeras were constructed with PCR as described (*30, 31*).
39. L. E. Samelson, R. N. Germain, R. H. Schwartz, *Proc. Natl. Acad. Sci. U.S.A.* **80**, 6972 (1983).


40. Plasmids encoding TCRα and antibody to TCRα (A2B4) were provided by J. S. Bonifacino (NIH, Bethesda, MD). Supported by a grant from NIH (GM 42336).

28 March 1997; accepted 2 June 1997