Proc. Natl. Acad. Sci. USA
Vol. 88, pp. 10619–10623, December 1991
Immunology

Surface expression of alternative forms of the TCR/CD3 complex

(receptor reconstitution/immunomagnetic selection)

DIETMAR J. KAPPES AND SUSUMU TONEGAWA

Howard Hughes Medical Institute at Center for Cancer Research and Department of Biology, Massachusetts Institute of Technology, Cambridge, MA 02139

Contributed by Susumu Tonegawa, August 19, 1991

ABSTRACT **T-cell antigen receptor (TCR) heterodimers of both the $\alpha\beta$ and $\gamma\delta$ types are expressed at the surface of T cells only in association with a complex of invariant chains called CD3. The requirement for individual CD3 components to achieve TCR surface expression was examined by cotransfection of a non-T-cell line with TCR α and β, as well as CD3 δ, ε, γ, and ζ, cDNAs. Both transient and stable transfectants expressing TCR and CD3 epitopes at the cell surface were generated. By transfection of TCR and CD3 components in different combinations, the TCR chains, as well as the CD3 ε and ζ chains, were each shown to be essential for reconstituting surface expression. On the other hand, CD3 δ and γ chains could be used alternatively, providing evidence for two different types of TCR/CD3 complexes.**

The T-cell receptor (TCR)/CD3 complex consists of a clonally distributed TCR heterodimer (either $\alpha\beta$ or $\gamma\delta$), which mediates major histocompatibility complex-restricted antigen-specific recognition, and five clonally invariant CD3 chains, δ, ε, γ, ζ, and η (1–3). The function of CD3 has not been established, although its substantial intracellular domains would suggest that it couples TCR stimulation to signal transduction. The structure and stoichiometry of the CD3 complex remain equally obscure. Available evidence suggests a minimum stoichiometry for the TCR/CD3 complex of $\alpha\beta\gamma\delta\varepsilon_2\zeta_2$ (3–5). Each of the components is also generally thought to be essential for efficient assembly and transport of the complex to the cell surface. This assumption is based on studies of a limited range of T-cell mutant lines lacking expression of one or another TCR/CD3 component (6–11). The alternative direct approach of *de novo* reconstitution of the TCR/CD3 complex at the surface of non-T cells by cotransfection with the genes encoding the putative components is hampered by the complexity of the receptor and the resulting low likelihood of coexpressing all required gene products within the same cell. We have overcome this problem by using the highly sensitive technique of immunomagnetic selection to identify and purify both transient and stable transfectants present at very low frequency (12). We show that TCR/CD3 surface expression can, indeed, be reconstituted in non-T cells—i.e., no T-cell-specific components other than TCR and CD3 subunits are required. By omission of particular chains we demonstrate that TCR α and β chains as well as CD3 ε and ζ chains are essential for surface expression, whereas CD3 δ and γ chains are individually dispensable. Alternative expression of these two types of receptor might result in differences in signal transduction during thymic development or antigen stimulation.

MATERIALS AND METHODS

Plasmids. Full-length cDNAs encoding TCR α and β chains expressed in the cytotoxic T lymphocyte (CTL) line 2C (13), as well as CD3 chains δ, ε, γ, and ζ (14–17), were cloned in the sense orientation into the expression vector CDM8 (18) between the *Hin*dIII or *Sac* I sites at the 5′ end and the *Xba* I site at the 3′ end. In most cases insertion into the vector CDM8 involved the prior attachment of linkers encoding appropriate restriction sites at one or both ends of the cDNAs to generate the required complementary overhangs.

Transfection and Immunomagnetic Selection. Equal amounts of each cDNA of interest, to a total of 20 μg per transfection, were introduced by a modified $CaPO_4$ coprecipitation procedure (19) into HeLa cells grown to 70% confluency on a standard tissue culture plate. For detection of transient surface expression the cells were removed from the plates 60 hr after transfection with 0.3% EDTA and rosetted with magnetic microspheres (Nichols Institute, San Juan Capistrano, CA) coated with the clonotypic monoclonal antibody (mAb) 1B2 (20). For generation of stable transfectants positive cells purified at the transient stage were repeatedly retransfected with all six TCR and CD3 cDNAs and reselected with mAb 1B2 until clones were derived that maintained expression in culture permanently. The use of immunomagnetic selection to purify transfectants expressing exogenous surface products is described in more detail in ref. 12.

Fluorescent Staining. HeLa cells and transfectants were removed from tissue culture plates as described above. All cells were washed and stained with the first mAb in phosphate-buffered saline/0.2% fetal calf serum for 30 min at 0°C, washed, and then reincubated under the same conditions with goat anti-mouse or anti-hamster immunoglobulin. Samples were analyzed on a FACSscan.

Radiolabeling and Immunoprecipitation. Radioiodinations were done by the lactoperoxidase method. Lysis was in either 1% digitonin or 0.5% Triton X-100 (21). Lysates were precleared with normal rabbit serum and *Staphylococcus aureus* (Pansorbin; Calbiochem) for 30 min and immunoprecipitated overnight at 4°C. Gel electrophoresis was done by standard methods.

RESULTS

Initially we examined whether the known TCR/CD3 components are sufficient for cell-surface expression of the receptor complex; HeLa (human epithelial carcinoma) cells were transiently cotransfected with six cDNAs encoding the TCR α and β chains of the murine allospecific (anti-H-2^d) CTL line 2C (13) and the murine CD3 components δ, ε, γ, and ζ (14–17). CD3 η, which is apparently structurally interchangeable with ζ chain, was not considered in these studies (22–24). Surface expression was assayed by rosetting with magnetic microspheres coated with an anticlonotypic mAb, 1B2 (20), followed by magnetic selection of rosetted cells. By this method we could reproducibly detect TCR-positive cells at a frequency of 10^{-4} (Fig. 1*A*). We then systematically

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. §1734 solely to indicate this fact.

Abbreviations: TCR, T-cell receptor; CTL, cytotoxic T lymphocyte; mAb, monoclonal antibody.

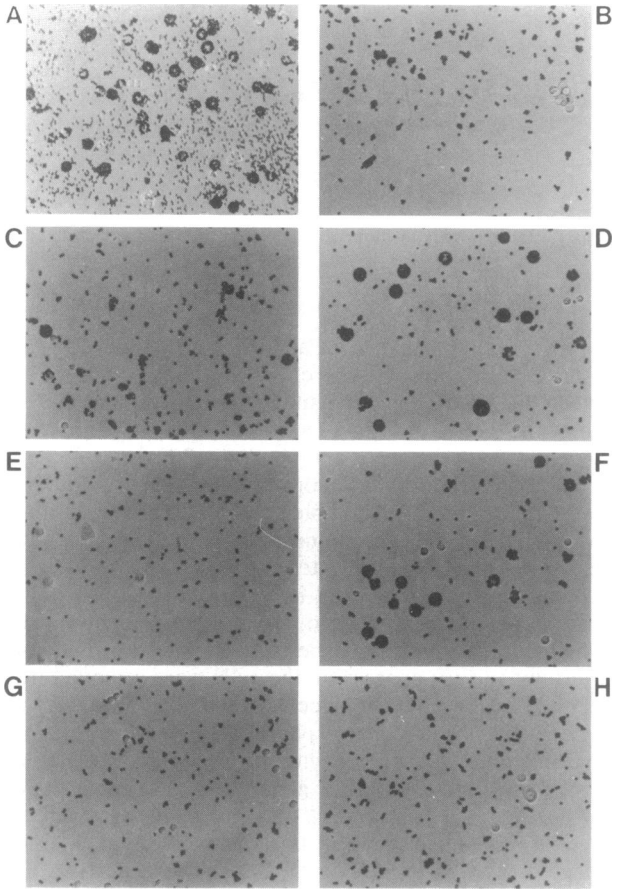

FIG. 1. Surface expression of the TCR/CD3 complex on transiently transfected HeLa cells. In *A* the transfection mixture contained cDNAs encoding TCR α and β chains and CD3 δ, ε, γ, and ζ chains (14–17). The same cDNAs were used with omission of TCR α (*B*), TCR β (*C*), CD3 δ (*D*), CD3 ε (*E*), CD3 γ (*F*), CD3 ζ (*G*), and CD3 δ and CD3 γ (*H*). Surface expression was detected by rosetting with magnetic particles coated with clonotypic mAb 1B2 (20) as described. Cells expressing the requisite epitope are recognizable on micrographs as relatively large spheres studded with dark magnetic particles, as distinct from the smaller unbound magnetic particles scattered around them.

investigated the requirement for each component by selectively omitting individual cDNAs from the transfection. When CD3 ε, CD3 ζ, or either of the TCR chains was omitted, expression was either abolished (β and ε chains) or reduced by 50–100 fold (α or ζ chains) (Fig. 1 *B*, *C*, *E*, and *G*). This result agrees with previous reports that surface TCR and CD3 expression were both lost, or vastly reduced, in mutant T-cell lines lacking expression of TCR α, TCR β, or CD3 ζ chains (6–10). The effects of omitting either δ or γ chains were quite different; only a very slight, if any, decrease in numbers of rosettes was evident (Fig. 1 *D* and *F*). Omission of both δ and γ chains caused essentially complete loss of surface expression of the mAb 1B2 epitope (Fig. 1*H*). The experiment was repeated several times, and similar results were obtained. Rosetting was also done with an anti-CD3ε mAb, 2C11 (25) (data not shown). In transfections involving all six chains, mAb 2C11 was found 2- to 4-fold less sensitive in detecting rosettes than mAb 1B2. Omission of CD3 ε chain abolished rosetting with this mAb, as expected, and omission of CD3 ζ chain reduced mAb 2C11 rosetting, although less substantially than was observed with mAb 1B2. Omission of δ chain had little effect, but omission of γ chain reduced rosetting with mAb 2C11 considerably. The apparent contrast between the latter observation and the mAb 1B2 rosetting data (see Fig. 1*F*) may reflect a greater sensitivity of mAb 2C11 in this assay to differences in cell-surface receptor density and/or in epitope conformation resulting from the absence of CD3 γ chain. Although other studies have examined the assembly of TCR/CD3 products in transiently transfected non-T cells, no cell-surface expression has been reported. In one instance, this can be attributed to the omission of the essential ζ chain (26). In another case only intracellular complexes were discussed (27). In any event, surface expression of the low frequency described here would be undetectable by many other assays, including fluorescence-activated cell sorter analysis.

To biochemically analyze the TCR/CD3 complexes produced in our experiments, we proceeded to generate stable transfectants. Cotransfection of TCR/CD3 cDNAs with an antibiotic resistance marker followed by drug selection failed to produce any stable clones expressing TCR/CD3 epitopes, presumably because of the low probability of simultaneous integration of so many exogenous genes. Consequently, we tried another procedure in which the same population of cells was subjected to several consecutive rounds of transfection with all of the cDNAs of interest included at each round. Only those cells immunomagnetically selected for transient surface expression were retained for use in the succeeding round. Stable transfectants were eventually produced by this procedure (12), probably due to the gradual accumulation of stably integrated cDNAs with each successive round of transfection. Thus HeLa cells were repeatedly transfected with all six TCR/CD3 cDNAs and selected with mAb 1B2. Two clones, 4A and 7.85, were generated, and both proved to be also reactive with mAb 2C11 (Fig. 2 *A–D*). The level of expression detected by mAb 2C11 was the same or slightly higher than that of the murine T lymphoma EL4 (Fig. 2*E*). By Northern (RNA) analysis we demonstrated the presence of

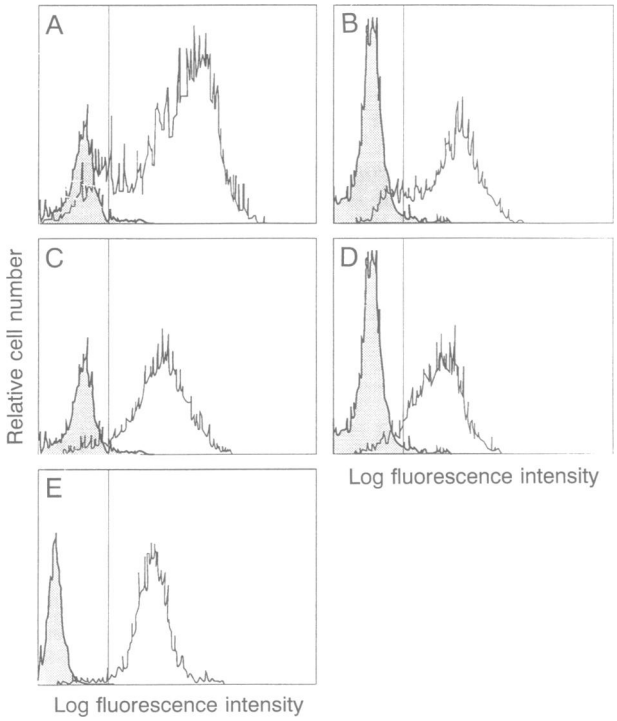

FIG. 2. Surface expression of the TCR/CD3 complex on stable HeLa cell transfectants. Transfectants 4A (*A* and *B*) and 7.85 (*C* and *D*) as well as the murine T lymphoma EL4 (*E*) were stained with mAbs to CD3 ε (25) (*A*, *C*, and *E*) or TCR clonotype (20) (*B* and *D*). Shaded peaks indicate negative controls, either untransfected HeLa cells stained with the same mAbs as the transfectants and fluorescein-conjugated goat anti-hamster antibodies or, in *E*, EL4 cells stained only with fluorescein-conjugated goat anti-hamster antibodies.

CD3 ε and ζ transcripts, as well as TCR α and β transcripts, in clones 4A and 7.85. The two clones differed strikingly, however, in their expression of CD3 δ and γ transcripts: clone 4A expressed γ transcript but lacked detectable δ transcript, whereas 7.85 expressed δ transcript but lacked detectable γ transcript (data not shown). This result conformed with our expectations from the transient transfection experiments described above. 4A and 7.85 transfectant cells were surface-radioiodinated and solubilized. Material immunoprecipitated from the extract by mAb 2C11 was then analyzed under reducing conditions. The immunoprecipitate contained all components predicted from Northern analysis (Fig. 3 *Upper*). Under nonreducing conditions, TCR α/β and CD3 ζ migrated more slowly, which indicates that they are disulfide linked, as in normal T cells (data not shown). To resolve CD3 δ, γ, and ε more effectively, two-dimensional nonreduced/reduced SDS/PAGE was done, which confirmed the absence of CD3 δ chains in 4A cells and CD3 γ chains in 7.85 cells (Fig. 3 *Lower*). The fact that expression of the mAb 2C11 epitope was roughly the same for both stable transfectants contrasts with the transient transfections where the omission of CD3 γ chain, but not of δ chain, considerably reduced the frequency

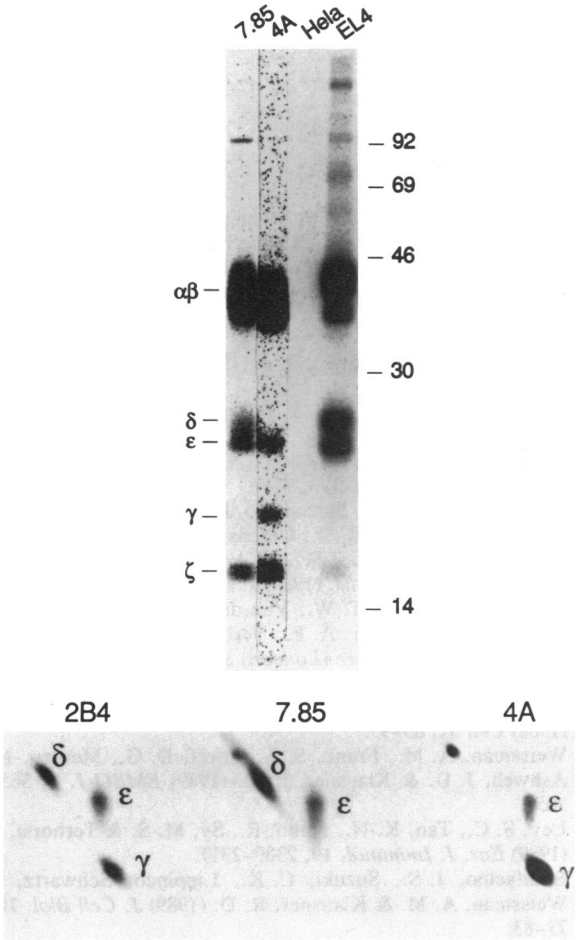

FIG. 3. SDS/PAGE analysis of TCR/CD3 complex expressed on stable HeLa transfectants. Cells were surface-radioiodinated, solubilized in detergent, and immunoprecipitated with anti-CD3ε mAb. Proteins were separated by one-dimensional SDS/PAGE (*Upper*) or two-dimensional unreduced/reduced SDS/PAGE (*Lower*). When overexposed, a band appears in *Upper* in the EL4 lane at the position corresponding to CD3 γ. In *Lower* only the autoradiograph segments below the 30-kDa marker in the reduced dimension that contain CD3 δ, ε, and γ are shown; also note that the small spot present on the diagonal just above the position of CD3 δ in the two HeLa transfectants is nonspecific.

of immunomagnetic rosetting with mAb 2C11 (but not with mAb 1B2; see above). The γ-less transient transfectant population, which remained detectable by mAb 2C11 rosetting as well as mAb 1B2 rosetting, presumably consisted of high expressors that had taken up the greatest amounts of exogenous cDNAs. Apparently this is the population that corresponds to the γ-less stable transfectant, 7.85, which stains brightly with mAb 2C11.

DISCUSSION

Our results have several clear implications for the structure and assembly of the TCR/CD3 complex. No T-cell-specific components other than those tested are required for cell-surface expression. Thus the factor variously designated CD3-Ω or T cell receptor-associated protein (28, 29), which appears to associate transiently with the TCR/CD3 complex inside the cell, either is not required for assembly and transport or is not T-cell-specific. The data further confirm that TCR $\alpha\beta$ and CD3 $\varepsilon\zeta$ are essential for surface expression. The existence of two forms of TCR/CD3 complex, one lacking CD3 δ and the other lacking CD3 γ, calls into question the idea that CD3 δ and γ chains pair in an obligatory fashion with TCR α and β chains, respectively, an hypothesis based on the observations that CD3 γ chain can be covalently cross-linked to TCR β chain and that CD3 δ chain can associate intracellularly with TCR α chain (26, 30, 31). Our data suggest that such pairings are not necessary for efficient assembly and transport of TCR/CD3 complex to the cell surface. Our observations are also difficult to reconcile with a report of a murine T-cell mutant that is defective for TCR/CD3 surface expression, apparently due to the lack of CD3 δ chain (11). However, other murine T-cell lines have recently been described that are partially deficient in CD3 δ or γ expression and yet retain TCR surface expression (32). Perhaps, these discrepancies can be explained by differences in relative levels of synthesis of TCR/CD3 components.

In our transient transfection assay, the number of cells showing reactivity to mAb 1B2 was as high when δ or γ chain was omitted as it was when both were present. This result suggests that the δ-less and γ-less complexes were formed and transported as efficiently as putative complexes containing both δ and γ chains. This result argues against the possibility that δ-less and γ-less complexes reach the cell surface only under exceptional conditions, specifically when very high internal levels of one or more CD3 components are present. If this were the case, then only cells that have taken up introduced genes at a high-copy number will express TCR at the cell surface when either δ or γ chain is omitted from the transfection. Thus, a considerable decrease in positive cells would be expected relative to transfections containing both chains. In view of our data, it must be questioned whether a CD3/TCR complex containing both CD3 δ and γ chains exists at all. Indeed, studies using antibodies thought specific for CD3 δ or γ chains to determine whether these components coprecipitate from the surface of T cells give conflicting results (30, 32, 33). Two alternative models for the structure of the TCR/CD3 complex expressed on our transfectants, as well as a possible complex containing both δ and γ chains, are shown in Fig. 4.

The capacity of the CD3 δ and γ chains to substitute for one another seems quite logical, as they are relatively more homologous to each other than to other components of the receptor complex (34). The situation is somewhat paralleled by the relationship between the CD3 ζ and η components (35). The latter chains can form disulfide-linked dimers both among themselves (ζ_2, η_2), with each other (ζ/η), or even with the γ chain of the Fc receptor (ζ/Fc γ, η/Fc γ) (22–24). Thus, there appears to be considerable scope for heterogeneity in composition of the CD3 complex. It is tempting to

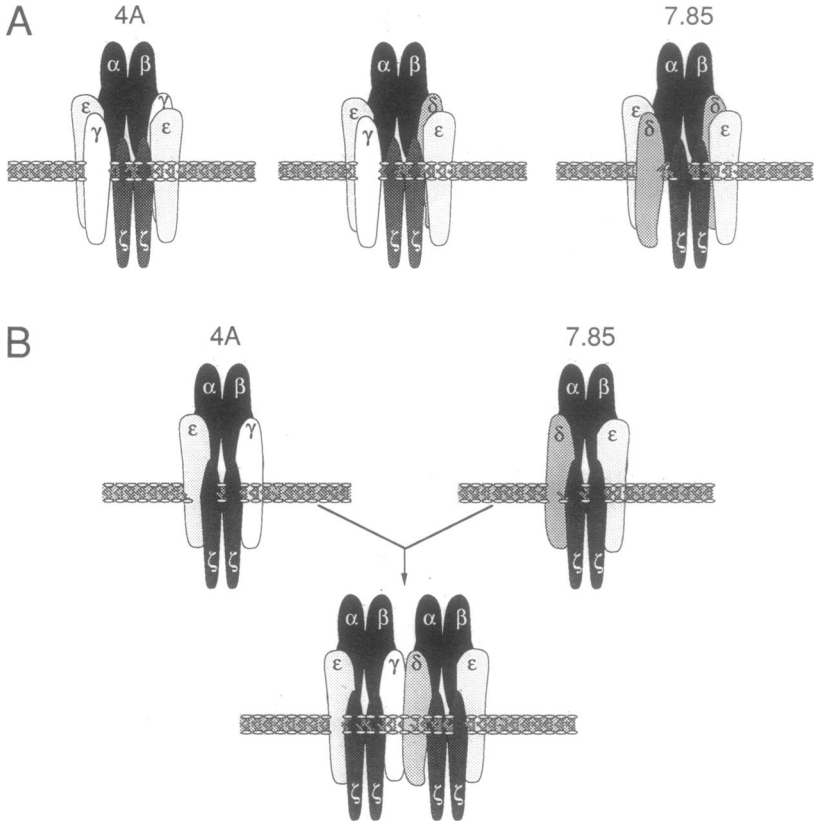

FIG. 4. Two hypothetical models for composition of the TCR/CD3 complex in transfectants 4A and 7.85 and possible mixed complexes containing both CD3 δ and γ. Both models accommodate two CD3 ζ and two ε chains per complex and either two chains of CD3 γ, two chains of δ, or one chain of each. (*A*) Model for the minimal possible stoichiometry based on these prerequisites. (*B*) Model that differs in that it incorporates two TCR α/β heterodimers, rather than one, permitting neutralization of all the negatively charged transmembrane amino acids of the CD3 components by the positively charged TCR transmembrane amino acids.

speculate that the expression of alternative forms of the TCR-associated CD3 complex at different developmental stages might result in different responses to TCR stimulation (e.g., negative selection versus positive selection in the thymus). Although both ζ_2- and $\zeta\eta$-containing complexes transduce signal (24), there is evidence for differences in their response (36, 37). In our case, functional consequences are difficult to assess because neither of our transfectants appears capable of signal transduction, as assayed by CD3 ζ phosphorylation and release of intracellular Ca^{2+} upon receptor cross-linking (data not shown). A plausible explanation for this lack of function (aside from the possibility that both δ and γ chains are required) lies in the absence of other T-cell molecules—e.g., CD8, CD45, and $pp56^{lck}$ (38–40). The transfection protocol used here makes it possible to add further exogenous surface proteins to our transfected cell lines, such as the molecules mentioned above. As far as we are aware, the TCR/CD3 assembly represents the most complex multisubunit receptor reconstituted by transfection so far.

We thank members of our laboratory for helpful suggestions and D. Gerber and P. Mombaerts, M.D., for countless hours of entertainment. We gratefully acknowledge Charley Steinberg for reviewing the manuscript, C. Browne for technical assistance, E. Basel for typing the manuscript, and F. Hochstenbach and M. Brenner for advice on protein analysis. D.J.K. is a Fellow of the Damon Runyon–Walter Winchell Cancer Research Fund. This work was supported by grants from Howard Hughes Medical Institute and the National Institutes of Health.

1. Clevers, H. C., Alarcon, B., Wileman, T. E. & Terhorst, C. (1988) *Annu. Rev. Immunol.* **6**, 629–662.
2. Davis, M. M. & Bjorkman, P. J. (1988) *Nature (London)* **334**, 395–402.
3. Ashwell, J. D. & Klausner, R. D. (1990) *Annu. Rev. Immunol.* **8**, 139–167.
4. de la Hera, A., Müller, U., Olsson, C., Isaaz, S. & Tunnacliffe, A. (1991) *J. Exp. Med.* **173**, 7–17.
5. Blumberg, R. S., Ley, S., Sancho, J., Lonberg, N., Lacy, E., McDermott, F., Schad, V., Greenstein, J. L. & Terhorst, C. (1990) *Proc. Natl. Acad. Sci. USA* **87**, 7220–7224.
6. Weiss, A. & Stobo, J. D. (1984) *J. Exp. Med.* **160**, 1284–1299.
7. Ohashi, P. S., Mak, T. W., Van den Elsen, P., Yanagi, Y., Yoshikai, Y., Calman, A. F., Terhorst, C., Stobo, J. D. & Weiss, A. (1985) *Nature (London)* **316**, 606–609.
8. Sussman, J. J., Bonifacino, J. S., Lippincott-Schwartz, J., Weissman, A. M., Saito, T., Klausner, R. D. & Ashwell, J. D. (1988) *Cell* **52**, 85–95.
9. Weissman, A. M., Frank, S. J., Orloff, D. G., Mercep, M., Ashwell, J. D. & Klausner, R. D. (1989) *EMBO J.* **8**, 3651–3656.
10. Ley, S. C., Tan, K.-N., Kubo, R., Sy, M.-S. & Terhorst, C. (1989) *Eur. J. Immunol.* **19**, 2309–2317.
11. Bonifacino, J. S., Suzuki, C. K., Lippincott-Schwartz, J., Weissman, A. M. & Klausner, R. D. (1989) *J. Cell Biol.* **109**, 73–83.
12. Kappes, D. J., Strominger, J. S. & Monos, D., *Gene*, in press.
13. Kranz, D. M., Sherman, D. H., Sitkowsky, M. V., Pasternack, M. S. & Eisen, H. N. (1984) *Proc. Natl. Acad. Sci. USA* **81**, 573–577.
14. van den Elsen, P., Shepley, B. A., Cho, M. & Terhorst, C. (1985) *Nature (London)* **314**, 542–544.
15. Gold, D. P., Clevers, H., Alarcon, B., Dunlap, S., Novotny, J., Williams, A. F. & Terhorst, C. (1987) *Proc. Natl. Acad. Sci. USA* **84**, 7649–7653.
16. Haser, W., Saito, H., Koyama, T. & Tonegawa, S. (1987) *J. Exp. Med.* **166**, 1186–1191.
17. Weissman, A. M., Baniyash, M., Hou, D., Samelson, L. E.,

Burgess, W. H. & Klausner, R. D. (1988) *Science* **239,** 1018–1021.
18. Seed, B. & Aruffo, A. (1987) *Proc. Natl. Acad. Sci. USA* **84,** 3365–3369.
19. Chen, C. & Okayama, H. (1987) *Mol. Cell. Biol.* **7,** 2745–2752.
20. Kranz, D. M., Tonegawa, S. & Eisen, H. N. (1984) *Proc. Natl. Acad. Sci. USA* **81,** 7922–7926.
21. Samelson, L. E., Harford, J. B. & Klausner, R. D. (1985) *Cell* **43,** 223–231.
22. Baniyash, M., Garcia-Morales, P., Bonifacino, J. S., Samelson, L. E. & Klausner, R. D. (1988) *J. Biol. Chem.* **263,** 9874–9878.
23. Orloff, D. G., Ra, C., Frank, S. J., Klausner, R. D. & Kinet, J.-P. (1990) *Nature (London)* **347,** 189–191.
24. Clayton, L. K., Bauer, A., Jin, Y.-J., d'Adamio, L., Koyasu, S. & Reinherz, E. (1990) *J. Exp. Med.* **172,** 1243–1253.
25. Leo, O., Foo, M., Sachs, D. H., Samelson, L. E. & Bluestone, J. A. (1987) *Proc. Natl. Acad. Sci. USA* **84,** 1374–1378.
26. Berkhout, B., Alarcon, B. & Terhorst, C. (1988) *J. Biol. Chem.* **263,** 8528–8538.
27. Manolios, N., Bonifacino, J. S. & Klausner, R. D. (1990) *Science* **249,** 274–277.
28. Alarcon, B., Berkhout, B., Breitmeyer, J. & Terhorst, C. (1988) *J. Biol. Chem.* **263,** 2953–2961.
29. Bonifacino, J. S., Lippincott-Schwartz, J., Chen, C., Antwish, D., Samelson, L. E. & Klausner, R. D. (1988) *J. Biol. Chem.* **263,** 8965–8971.
30. Koning, F., Maloy, W. L. & Coligan, J. E. (1990) *Eur. J. Immunol.* **20,** 299–305.
31. Brenner, M. B., Trowbridge, I. S. & Strominger, J. L. (1985) *Cell* **40,** 183–190.
32. Alarcon, B., Ley, S. C., Sanchez-Madrid, F., Blumberg, R. S., Ju, S. T., Fresno, M. & Terhorst, C. (1991) *EMBO J.* **10,** 903–912.
33. Samelson, L. E., Weissman, A. M., Robey, F. A., Berkower, I. & Klausner, R. D. (1986) *J. Immunol.* **137,** 3254–3258.
34. Krissansen, G. W., Owen, M. J., Verbi, W. & Crumpton, M. J. (1988) *EMBO J.* **5,** 1799–1808.
35. Jin, Y.-J., Clayton, L. K., Howard, F. D., Koyasu, S., Sieh, M., Steinbrich, R., Tarr, G. E. & Reinherz, E. L. (1990) *Proc. Natl. Acad. Sci. USA* **87,** 3319–3323.
36. Mercep, M., Bonifacino, J. S., Garcia-Morales, P., Samelson, L. E., Klausner, R. D. & Ashwell, J. D. (1988) *Science* **242,** 571–574.
37. Sussman, J. J., Mercep, M., Saito, T., Germain, R. N., Bonvini, E. & Ashwell, J. D. (1988) *Nature (London)* **334,** 625–628.
38. Marth, J. D., Lewis, D. B., Wilson, C. B., Gearn, M. E., Krebs, E. G. & Perlmutter, R. M. (1985) *EMBO J.* **6,** 2727–2734.
39. Ostergaard, H. L., Shackelford, D. A., Hurley, T. R., Johnson, P., Hyman, R., Sefton, B. M. & Trowbridge, I. S. (1989) *Proc. Natl. Acad. Sci. USA* **86,** 8959–8963.
40. Gabert, J., Langlet, C., Zamoyska, R., Parnes, J. R., Schmitt-Verhulst, A.-M. & Malissen, B. (1987) *Cell* **50,** 545–554.