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Genetic and immunochemical evidence for CD4-dependent association of p56^{lck} with the $\alpha\beta$ T-cell receptor (TCR): regulation of TCR-induced activation

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Recent observations suggest that the tyrosine kinase p56^{lck} is involved in the transduction of transmembrane signals through the antigen specific T cell receptor (TCR) in CD4$^+$ T cells. By means of *in vitro* kinase assays, we have found that p56^{lck} coprecipitated with the TCR from lysates of a murine CD4$^+$ T cell line in the absence of TCR-mediated stimuli. Analysis of CD4$^-$ mutants and CD4-transfected cells shows that p56^{lck}-TCR association occurred only when CD4 was present. The functional importance of CD4:p56^{lck}-TCR association was demonstrated by low activating potential of rare clonotypic antibodies which did not coprecipitate CD4:p56^{lck}, as well as by total or partial loss of anti-TCR or antigen induced stimulation in CD4$^-$ cells, which could be recovered by CD4 transfection. Complementation assays using different anti-TCR antibodies suggest that cross linking of TCR-p56^{lck}:CD4 plus structural changes in the complex are needed for efficient transduction of activating signals through the TCR in these cells.

Key words: CD4/*lck*/T-cell receptor/tyrosine kinases

Introduction

Mature T lymphocytes expressing the coreceptor molecule CD4 or CD8 specifically recognize peptide antigens associated with class II or class I molecules of the major histocompatibility complex (MHC), respectively (Swain, 1983). Coreceptor function is based on the potentiation of T-cell receptor (TCR)-mediated stimuli when these molecules and the TCR are in close proximity (Eichmann *et al.*, 1987; Saizawa *et al.*, 1987; Rojo *et al.*, 1989). Coreceptors bind to MHC molecules expressed in the surface of antigen presenting cells (APCs) (Doyle and Strominger, 1987; Norment *et al.*, 1988), thus favoring their proximity to the TCR during antigen recognition (Janeway *et al.*, 1989b; Salter *et al.*, 1989; Dianzani *et al.*, 1992). Experiments using CD4 and CD8 constructs which do not bind the tyrosine protein kinase p56^{lck} associated with the intracytoplasmic tail of CD4/CD8 (Rudd *et al.*, 1988; Veillette *et al.*, 1988) show that this kinase is necessary for coreceptor function (Miceli *et al.*, 1990; Glaichenhaus *et al.*, 1991).

Protein tyrosine kinases are essential to T cell activation through the TCR. TCR-mediated stimuli are blocked by inhibitors of protein tyrosine kinases (June *et al.*, 1990b; Mustelin *et al.*, 1990) and tyrosine phosphorylation of different polypeptides is the earliest intracellular signal detected after TCR stimulation (June *et al.*, 1990a; Mustelin *et al.*, 1990). The substrates of this phosphorylation include phospholipase C (PLC) γ1 and CD3 ζ chains (Samelson *et al.*, 1986; Park *et al.*, 1991; Weiss *et al.*, 1991). PLC γ1 is activated by TCR ligands to induce breakdown of inositol phospholipids (Weiss *et al.*, 1986), and tyrosine phosphorylation is likely to be responsible for its increased catalytic activity (Nishibe *et al.*, 1990; Cantley *et al.*, 1991). Activation of PLC γ1 gives rise to inositol phosphates and diacylglycerol, which in turn produce increased intracellular Ca^{2+} and translocation of protein kinase C to the plasma membrane necessary to activate clonal expansion [reviewed in Weiss *et al.* (1986); Klausner and Samelson (1991)].

Neither the α-β chains of the TCR, nor the invariant chains of the CD3 complex non-covalently bound to the TCR possess intrinsic tyrosine kinase activity. However, different studies show that the cytoplasmic tail of CD3ϵ and ζ are needed for coupling the TCR to different intracellular signaling pathways (Frank *et al.*, 1990; Irving and Weiss, 1991; Romeo and Seed, 1991; Letourneur and Klausner, 1992; Wegener *et al.*, 1992). The nature of the protein tyrosine kinase(s) that intervene in TCR-mediated activation has not been fully elucidated. Immunochemical evidence from a murine hybridoma (Samelson *et al.*, 1990) or normal rat or human lymphocytes (Beyers *et al.*, 1992; Gassmann *et al.*, 1992) indicates that a lymphocyte specific isoform of p59^{fyn}, a protein tyrosine kinase of the *src* family, coprecipitates or colocalizes with the TCR complex. Enhanced responses to TCR-mediated signals in *fyn* transgenic mice (Cooke *et al.*, 1991), or in cell lines transfected with activated forms of p59^{fyn} (Davidson *et al.*, 1992) suggest a key role for this kinase in signal transduction. Recently, a second protein of 70 kDa with tyrosine kinase activity has been found associated with ζ chains and CD3ϵ upon TCR stimulation (Wange *et al.*, 1992).

The lymphocyte specific tyrosine kinase p56^{lck} has been also implicated in TCR-mediated stimuli. In p56^{lck-} mutants of the human leukemic T cell line Jurkat, TCR-mediated activation and signaling is defective, and p56^{lck} transfection restores the ability to respond to TCR stimulation (Straus and Weiss, 1992). Furthermore, thymocyte development is blocked in transgenic mice lacking p56^{lck} (Molina *et al.*, 1992).

Coprecipitation, colocalization, fluorescence energy transfer and functional evidence indicate that CD4 and CD8 and the TCR-CD3 complex are associated, or can be induced to associate by certain TCR ligands in the absence of APCs (Tite *et al.*, 1986b; Saizawa *et al.*, 1987; Anderson *et al.*, 1988; Kupfer and Singer, 1988; Janeway *et al.*,

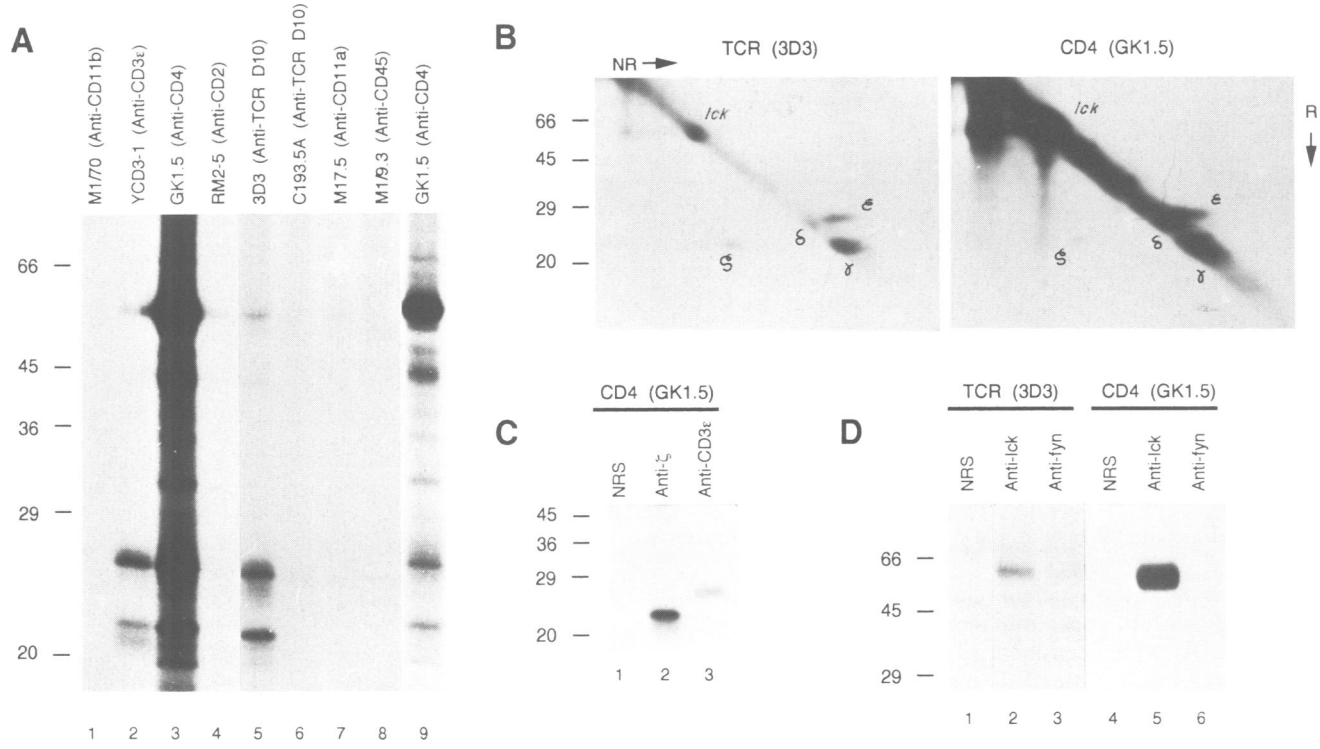

Fig. 1. Analysis of molecular associations by *in vitro* immune complex kinase assays after immunoprecipitation from SR.D10 cell lysates. **(A)** Immune complex kinase assays after immunoprecipitation with monoclonal antibodies to the molecules indicated. Exposure time was 4 days. Lane 9 is lane 3 exposed for 1 day. **(B)** Two-dimensional analysis of phosphorylated proteins in *in vitro* kinase reactions after immunoprecipitation with clonotypic anti-TCR antibody 3D3 or anti-CD4 antibody GK1.5. Exposure was 5 days. **(C)** Identification of phosphorylated polypeptides from CD4 immunoprecipitates by reprecipitation with anti ζ-η antiserum, YCD3-1 anti-CD3ϵ monoclonal antibodies or normal rabbit serum (NRS). Exposure time was 3 days. **(D)** After immunoprecipitation with antibodies to CD4 (GK1.5) or the TCR (3D3) and kinase reactions, the phosphorylated proteins were eluted and reprecipitated as described in Materials and methods with anti-*fyn*, or anti-*lck* antisera or control rabbit serum (NRS). Exposure time was 10 (1–3) or 3 days (4–6). Molecular masses in kDa are indicated.

1989b; Mittler *et al.*, 1989; Rojo *et al.*, 1989; Dianzani *et al.*, 1990; Burgess *et al.*, 1991; Beyers *et al.*, 1992; Collins *et al.*, 1992; Dianzani *et al.*, 1992; Suzuki *et al.*, 1992). These data suggest that, if the protein tyrosine kinase p56^{lck} bound to CD4 or CD8 is physically associated with the TCR–CD3 complex, this should occur largely in a CD4- and CD8-dependent manner.

Here we describe immunochemical as well as genetic evidence for the stable association between TCR–CD3 and p56^{lck} using a subline (SR.D10) on the CD4$^+$ murine T cell clone D10.G4.1. In the conditions used, p56^{lck} is the only protein tyrosine kinase which can be detected in association with the TCR. Analysis of a CD4$^-$ mutant of SR.D10 (SR.CD4$^-$.F1), and CD4 transfectants of these CD4$^-$ cells show that CD4 is needed for p56^{lck} association to the TCR. The association between TCR–CD3 and p56^{lck}–CD4, together with cross linking of the TCR–CD3, is needed for efficient activation through the TCR, although this association occurs independently of activation. However, TCR cross linking is not enough for optimal stimulation, as only certain TCR ligands induce further changes in the complex that allow efficient signaling for cell proliferation.

Results

Stable association between TCR–CD3 and CD4–lck in D10 lysates

Previous results in D10 cells have shown that anti-D10 TCR antibodies that induce efficient activation induce CD4 association to the TCR (Saizawa *et al.*, 1987; Rojo *et al.*, 1989) as well as tyrosine phosphorylation of ζ chains and other cellular substrates (Dianzani *et al.*, 1992). To analyze the presence of protein tyrosine kinases associated to the TCR, and the role of CD4, we performed *in vitro* kinase assays in immunoprecipitates of anti-TCR antibodies with different activating abilities.

Lysates of SR.D10 cells were immunoprecipitated using antibodies to different D10 TCR epitopes, as well as to CD3ϵ, CD4 or other functionally relevant surface molecules like CD2, CD45 or LFA-1 (CD11a/CD18) (Figure 1A). SR.D10 is a hyper-reactive variant clone of the D10.G4.1 line of murine helper cells (M.Ronda *et al.*, manuscript in preparation; see also Figure 4). This variant was chosen because its ability to grow in the presence of interleukins made it possible to generate and select the CD4$^-$ mutants described below.

In vitro kinase assays revealed a strong kinase activity associated to CD4 immunoprecipitates, as well as a weak kinase activity associated to immunoprecipitates of anti-CD3 antibodies or clonotypic antibodies like 3D3 (Figure 1A). Strikingly, we could not detect significant kinase activity in immunoprecipitates of another clonotypic antibody binding a different epitope in the TCR (C193.5A), indicating the specificity of kinase binding in 3D3 or CD3 immunoprecipitates (Figure 1A). The absence of kinase activity associated to C193.5A precipitates is correlated to important activation defects of this antibody (see Figure 5 below). The specificity of kinase associations was also shown by the

absence of kinase activity in immunoprecipitates of other surface molecules like LFA-1 (Anti-CD11a) or CD45 (Figure 1A). In accordance with results in other systems, a minor but reproducible kinase activity coprecipitated with CD2 (Figure 1A) (Beyers *et al.*, 1992). Reprecipitation of phosphorylated polypeptides with anti-phosphotyrosine antibodies and analysis of labeled phosphoaminoacids indicated the presence of a tyrosine kinase in CD4 and TCR immunoprecipitates (not shown). The labeled polypeptides in TCR−CD3 immunoprecipitates included polypeptides with molecular weights (20−30 kDa) similar to CD3 complex chains, as well as a weaker band at 56 kDa (Figure 1A).

Two-dimensional analysis and reprecipitation with appropriate antibodies allowed the identification of some of these polypeptides. The low molecular weight polypeptides labeled in TCR (3D3) immunoprecipitates behaved as the γ, ζ and ϵ chains of the CD3 complex and ζ-η polypeptides by two-dimensional acrylamide gel electrophoresis (Figure 1B).

Phosphorylated polypeptides in CD4 immunoprecipitates included bands of apparent molecular weights between 20 and 30 kDa, with electrophoretic mobility similar to CD3 polypeptides in one- or two-dimension electrophoresis, as well as a very prominent band of 56 kDa, as expected for *lck* (Figure 1A and B). CD3ϵ and ζ/η chains could be identified by reprecipitation of phosphorylated polypeptides from CD4 immunoprecipitates (Figure 1C) or TCR immunoprecipitates (data not shown) with appropriate antibodies or antisera, directly demonstrating the physical association and coprecipitation of TCR−CD3 and CD4.

Reprecipitation of the labeled polypeptides after *in vitro* kinase assays of TCR (3D3) or CD4 (GK1.5) immunoprecipitates using anti-*lck* antisera confirmed the presence of *lck* as a phosphorylated band of 56 kDa from both TCR and CD4 immunoprecipitates (Figure 1D). In the conditions used, the protein tyrosine kinase *fyn* could not be detected in TCR or CD4 immunoprecipitates (Figure 1D), despite its presence in SR.D10 cells (see below).

Other phosphorylated bands in CD4 immunoprecipitates included one polypeptide of 32 kDa which might correspond to one recently described GTP binding protein associated with CD4 (Telfer and Rudd, 1991). The presence of a number of phosphorylated polypeptides in CD4 immunoprecipitates not appearing in TCR precipitates suggests that, in these cells, CD4−*lck* can form distinct molecular complexes with different sets of proteins, one of them being with the TCR−CD3 complex.

Since SR.D10 is a hyper-reactive variant clone of the $CD4^+$ T cell line D10.G4.1, it was important to assess that CD4−*lck* association with the TCR is not a phenomenon particular to this variant. As shown in Figure 2, kinase assays performed using CD3 or CD4 immunoprecipitates from lysates of D10.G4.1 cells or from normal $CD4^+$ T lymphocytes, have similar phosphorylation patterns as immunoprecipitates from SR.D10. Taken together, our results indicate that there is a detectable association between CD4−*lck* and TCR−CD3 even in the absence of TCR mediated stimuli in SR.D10 and other $CD4^+$ T cells.

Generation and characterization of SR.D10 $CD4^-$ mutants

To analyze the role of CD4 in the association of $p56^{lck}$ to the TCR, we obtained a series of $CD4^-$ mutant cell clones

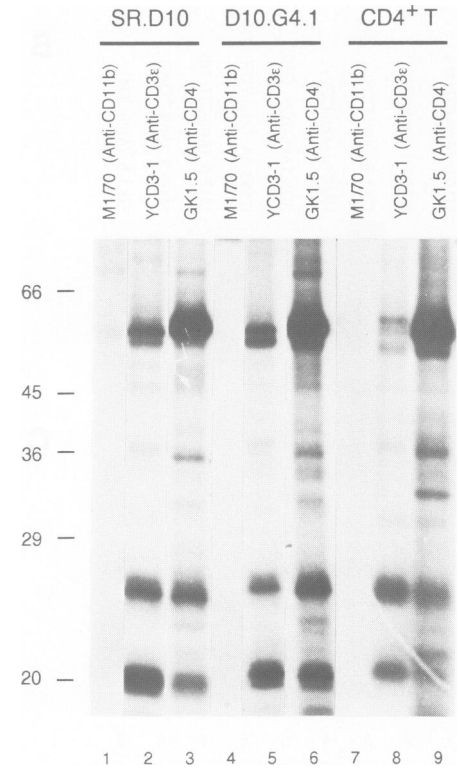

Fig. 2. Kinase assays of CD3, CD4 or CD11b (negative control) immunoprecipitates from lysates of SR.D10 or D10.G4.1 cells or $CD4^+$ T lymphocytes. Exposure time was 3 days. Molecular masses in kDa are indicated.

from SR.D10. Some characteristics of one of these clones ($SR.CD4^-.F1$) are shown in Figures 3, 4 and 5. $SR.CD4^-.F1$ cells lack surface CD4, as determined by flow cytometry with anti-CD4 antibodies (Figure 3A), or CD4 mRNA, as determined by Northern blot (Figure 3B). Yet these cells retain the expression of normal levels of D10 TCRs, as shown by FACS using the clonotypic antibody to D10 TCR 3D3 (Figure 3A), as well as expression of CD2, CD11a/CD18, CD45 and other surface molecules (not shown).

Although $SR.CD4^-.F1$ cells proliferate in the presence of antigen (conalbumin) presented by syngeneic APCs, the response is ~100-fold lower than the response of SR.D10 if the number of APCs needed to achieve a given response is considered (Figure 4). As can be appreciated in this figure, one of the features of the SR.D10 variant is its hyper-reactivity to conalbumin presented by syngeneic APCs, as compared with the original D10.G4.1 clone (Figure 4 and M.Ronda *et al.*, manuscript in preparation). Hyper-reactivity of SR.D10 can be appreciated by comparison of their response to antigen plus graded concentrations of antigen presenting cells (Figure 4), or by comparing the proliferation induced by increasing concentrations of antigen (data not shown). As depicted in this figure, SR.D10 cells show detectable response in the presence of very low amounts of APCs (i.e. $\leq 10^2$ APCs/culture).

The activation of SR.D10 and $SR.CD4^-.F1$ by antibodies directed to the TCR and other surface molecules was also compared. We took advantage of the fact that, like D10.G4.1 cells (Kaye *et al.*, 1983; Tite *et al.*, 1986a; Rojo and Janeway Jr, 1988; Portolés *et al.*, 1989a), SR.D10 proliferation is activated by soluble anti-TCR or anti-CD3

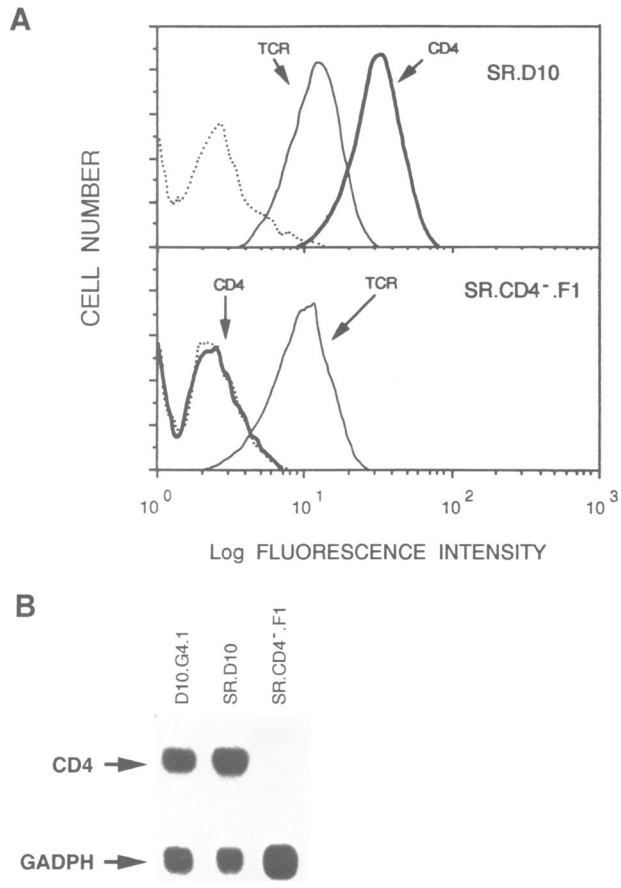

Fig. 3. SR.CD4$^-$.F1 mutant cells lack surface CD4 or CD4 mRNA. (**A**) FACS analysis of cell surface expression of TCR and CD4 in SR.D10 and SR.CD4$^-$.F1 by staining with biotinylated clonotypic antibody 3D3 (thin lines), anti-CD4 antibody GK1.5 (thick lines) or control anti-CD11b antibody M1/70 (dotted lines), followed by fluorescein-conjugated goat anti-biotin antibodies. (**B**) Northern blot analysis of CD4 mRNA in D10.G4.1, SR.D10 or SR.CD4$^-$.F1 cells. Amounts of total RNA were normalized by detection of glyceraldehyde-3-phosphate dehydrogenase (GADPH). Specific probes of CD4 or GADPH are indicated.

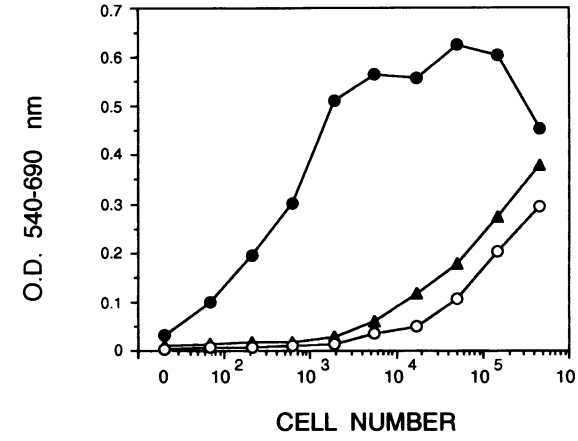

Fig. 4. Response of D10.G4.1, SR.D10, or SR.CD4$^-$.F1, to antigen presented by spleen cells. Cells of the cloned CD4$^+$ T cell line D10.G4.1 (open circles), the hyper-reactive variant clone SR.D10 (closed circles) or CD4$^-$ mutants of SR.D10 (SR.CD4$^-$.F1, closed triangles), were stimulated by antigen (100 μg/ml conalbumin) presented by T-cell- and adherent cell-depleted H-2^k (C3H) spleen cells. Activation was determined by a colorimetric assay as described in Materials and methods.

antibodies added to the cultures (Figure 5A) or fixed to a solid matrix (Figure 5B). Unlike D10.G4.1 (Kaye *et al.*, 1984), SR.D10 cells do not need IL-1 as a costimulator to induce growth factor receptor expression and proliferate in response to anti-TCR−CD3 antibodies (Figure 5). This is likely due to constitutive expression by SR.D10 of IL-4 receptors, as shown by growth in the presence of exogenous IL-4 (Figure 5B and D, see also Figure 7).

Figure 5A also shows clear differences between anti-TCR antibodies in inducing activation of SR.D10. Soluble antibodies like the clonotypic antibody 3D3 induce strong proliferation, whereas antibodies like C193.5A or F23.1, induce low or negligible proliferation. Previous studies have shown that these differences in activation properties between antibodies are linked to the particular epitope recognized in the V region of the TCR, as well as to their ability to induce CD4−TCR association that can be detected by comodulation or cocapping (Saizawa *et al.*, 1987; Rojo and Janeway Jr, 1988; Rojo *et al.*, 1989). All anti-TCR antibodies induce proliferation of SR.D10 when adsorbed to the wells, and their differences in activation efficiency become less apparent (Figure 5B). Induction of SR.D10 proliferation can also be achieved with anti-CD3ϵ antibody (YCD3−1, Figure 5A and B).

Data depicted in Figure 5 also show that antibodies to other surface molecules like CD4, CD11a or CD45 do not activate SR.D10 proliferation, even under conditions (i.e. antibody-coated culture wells) where all anti-TCR antibodies assayed induced clear proliferation (Figure 5B).

Proliferation induced by soluble antibodies to the TCR or CD3ϵ, like 3D3 or YCD3-1, was abolished in CD4$^-$ mutant cells (Figure 5C). Still, anti-TCR or anti-CD3 antibodies adsorbed to the culture wells could activate CD4$^-$ mutant cells (Figure 5D), albeit higher concentrations of antibodies were usually needed. Similar activation defects have been observed in other CD4$^-$ mutant clones of SR.D10 cells (unpublished results).

CD4 is needed for lck association to the TCR and efficient activation

To show that *lck* association to the TCR depends on the presence of CD4, we compared the kinase activity coprecipitated with the TCR in SR.D10 or SR.CD4$^-$.F1 lysates (Figure 6). In the conditions described here, no detectable kinase activity could be found associated with TCR immunoprecipitates from SR.CD4$^-$.F1 cell lysates, suggesting that *lck* was the main, if not the only, protein tyrosine kinase which coprecipitated with the TCR in SR.D10 cells (Figure 6A). Furthermore, data in Figure 6A clearly indicates that *lck* needs CD4 to be associated to the TCR complex, thus confirming TCR−CD3−CD4−*lck* association.

It should be noted that both SR.D10 and SR.CD4$^-$.F1 have readily detectable levels of *lck* and *fyn* protein tyrosine kinases, as determined by autophosphorylation of these molecules in *in vitro* kinase assays of immunoprecipitates (Figure 6B). In fact, results obtained in different assays indicate that, while the activity of *fyn* is consistently higher in SR.CD4$^-$.F1 than in SR.D10 cells, the levels of *lck* can be higher in SR.D10 than in CD4$^-$ mutant cells (Figure 6B) or lower (M.J.Feito *et al.*, in preparation), but the

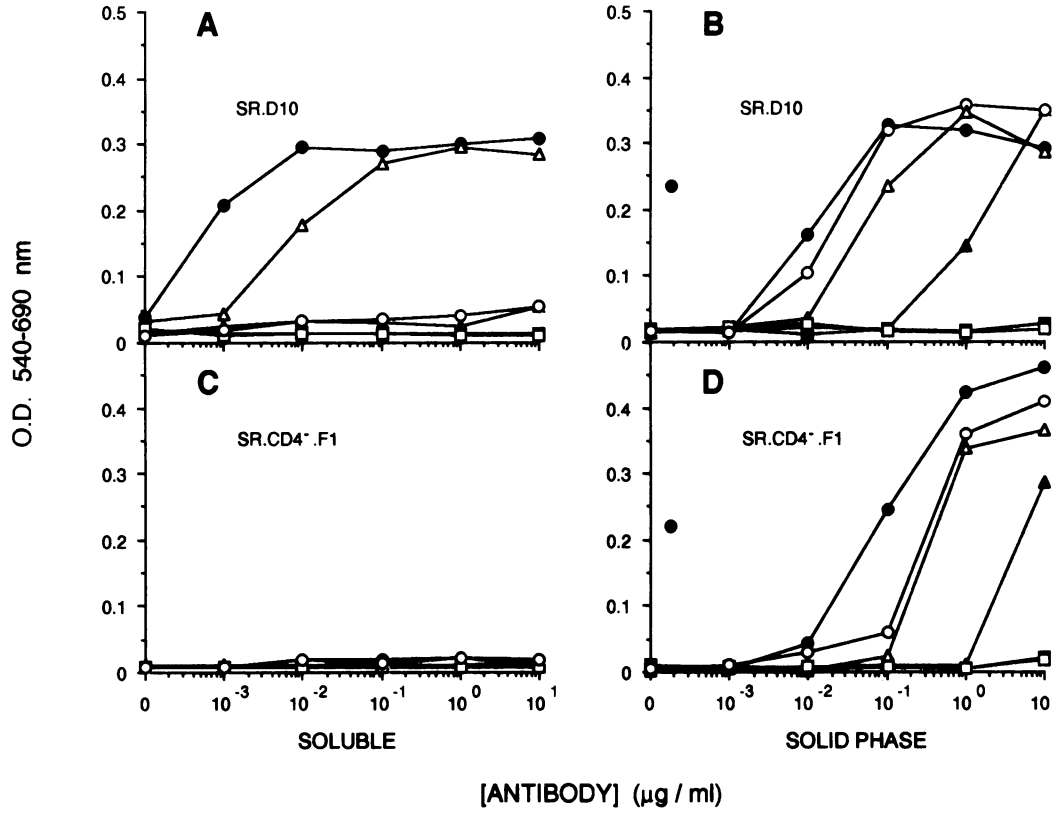

Fig. 5. Activation of SR.D10 and SR.CD4$^-$.F1 mutant cells by antibodies. SR.D10 cells (**A** and **B**) or CD4$^-$ mutant cells SR.CD4$^-$.F1 (**C** and **D**), were cultured in the presence of the indicated concentrations of monoclonal antibodies (A and C), or in culture wells previously coated with antibodies at the concentrations shown in B and D. The assay included the clonotypic antibodies 3D3 (closed circles) and C193.5A (closed triangles), anti-TCR Vβ antibody F23.1 (open circles), antibodies to CD3ϵ (YCD3-1, open triangles), CD4 (GK1.5, closed squares) and CD45 (M1/9.3, open squares). Single closed circles in B and D indicate the response of the corresponding cell line to 0.5 U/ml of murine recombinant IL-4.

response to TCR-mediated stimuli is always lower in the CD4$^-$ mutants.

Further proof of the importance of CD4 in *lck* association with the TCR–CD3 complex as well as in TCR-mediated activation comes from the analysis of CD4 transfectants of SR.CD4$^-$.F1 mutants. As depicted in Figure 6A, transfection of murine CD4 restores not only the kinase activity associated with CD4 immunoprecipitates, but also the kinase coprecipitated by anti-TCR antibodies like 3D3 or F23.1. As clearly seen in this figure, the electrophoretic mobilities of the phosphorylated polypeptides in TCR or CD4 precipitates from SR.D10 or from one representative clone of mouse CD4-transfected cells (mCD4.F3) are indistinguishable (i.e. compare phosphorylated polypeptides in lanes 2, 3 and 6 with lanes 13, 14 and 17 in Figure 6B). Similar results were obtained in all the CD4 transfectants analyzed (not shown). These results demonstrate the importance of CD4 expression for the association of the *lck* kinase with the TCR, which is independent of previous activation through the TCR.

Intriguingly, the anti-TCR antibody F23.1 coprecipitated similar kinase activity from cell lysates of SR.D10 or CD4 transfectants as the activating antibodies 3D3 or YCD3-1 (Figure 6A). Yet F23.1 induced negligible SR.D10 proliferation (Figure 5A). Possible reasons for this behavior will be discussed below.

CD4–lck association with the TCR: consequences for TCR-mediated activation

The functional meaning of CD4–*lck* association with the TCR is set forth by recovery or augmentation of different TCR-mediated stimuli upon CD4 transfection (Figure 7). So, response to antigen presented by low numbers of syngeneic APCs in two representative CD4 transfectant clones (mCD4.F3 and mCD4.B3) closely resemble the original SR.D10 cells (Figure 7A). These transfectants expressed TCR and CD4 levels similar to those expressed by SR.D10 (data not shown).

Restoration of functional properties in CD4 transfectants is also clearly appreciated, in the absence of accessory cells, using anti-TCR antibodies like 3D3 to induce proliferation (Figure 7B). Neither 3D3 nor low amounts of APCs plus antigen induced detectable proliferation of SR.CD4$^-$.F1 mutant cells (Figures 4, 5C, 7A and B) or CD4$^-$ clones, like Neo.D1, transfected with control vectors (Figure 7).

As in SR.D10 cells, proliferation of CD4-transfected clones like mCD4.F3 and mCD4.B3 could not be induced by certain, inefficient soluble anti-TCR antibodies like C193.5A or F23.1 (not shown), even though F23.1 coprecipitated kinase activity from lysates of SR.D10 and CD4 transfected cells (Figure 6A).

Is TCR–CD3–CD4–lck cross linking sufficient for efficient activation?

The experiments presented here suggest that maintaining CD4-mediated association of *lck* with the TCR is necessary to obtain optimal activation through the TCR (Figures 1, 4, 5 and 7). However, one anti-TCR antibody (F23.1) coprecipitated *lck*–CD4 from SR.D10 lysates or lysates of CD4 transfectants (Figure 6A), yet produced negligible proliferation upon direct addition to the cultures (Figures 5A and 8). This antibody is putatively capable of inducing

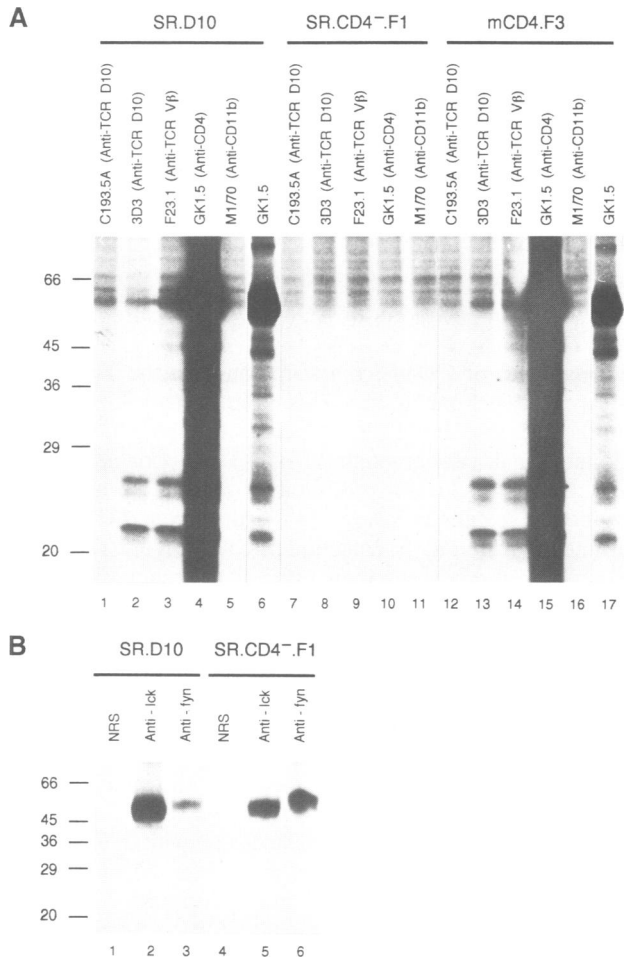

Fig. 6. *In vitro* kinase assays in immunoprecipitates from lysates of SR.D10, SR.CD4$^-$.F1 or CD4-transfected cells (mCD4.F3). (**A**) *In vitro* kinase assays in immunoprecipitates from lysates of SR.D10, SR.CD4$^-$.F1 mutants, or mCD4.F3 mouse CD4 transfectants using monoclonal antibodies to the TCR, CD4 or CD11b (negative control), as indicated. Exposure time was 3 days, except lanes 6 and 17, which are lanes 4 and 15, respectively, exposed for 24 h. (**B**) Kinase activity associated in immunoprecipitates from SR.D10 or SR.CD4$^-$.F1 lysates using NRS, rabbit anti-*lck* antiserum or rabbit anti-*fyn* antiserum. Exposure was 12 h. Molecular masses in kDa are indicated.

cross linking and oligomerization of CD4−*lck* along with the TCR, but this is not enough to induce SR.D10 proliferation, as shown in Figures 5 and 8. It has previously been suggested that only certain TCR ligands of D10 cells (like the clonotypic antibody 3D3) induce conformational changes in the TCR which, along with TCR cross linking, are needed for delivery of an activating signal (Saizawa *et al.*, 1987; Rojo and Janeway Jr, 1988; Janeway *et al.*, 1989a). As in D10.G4.1 cells, proliferation of SR.D10 can be achieved by combinations of F23.1 antibodies and monovalent Fab fragments of the efficient activator antibody 3D3 (Figure 8). These two antibodies bind non-overlapping epitopes in D10 TCR (Rojo and Janeway Jr, 1988; Dianzani *et al.*, 1992), and neither stimulus alone induces proliferation (Figure 8). On the other hand, 3D3 Fab fragments competitively inhibited SR.D10 activation by the homologous antibody 3D3. Taken together, these data suggest that changes in the TCR induced by 3D3 Fab plus cross linking of TCR achieved with F23.1 are needed for the functional triggering of the CD4-associated *lck* tyrosine kinase and initiation of signal transduction to the cells.

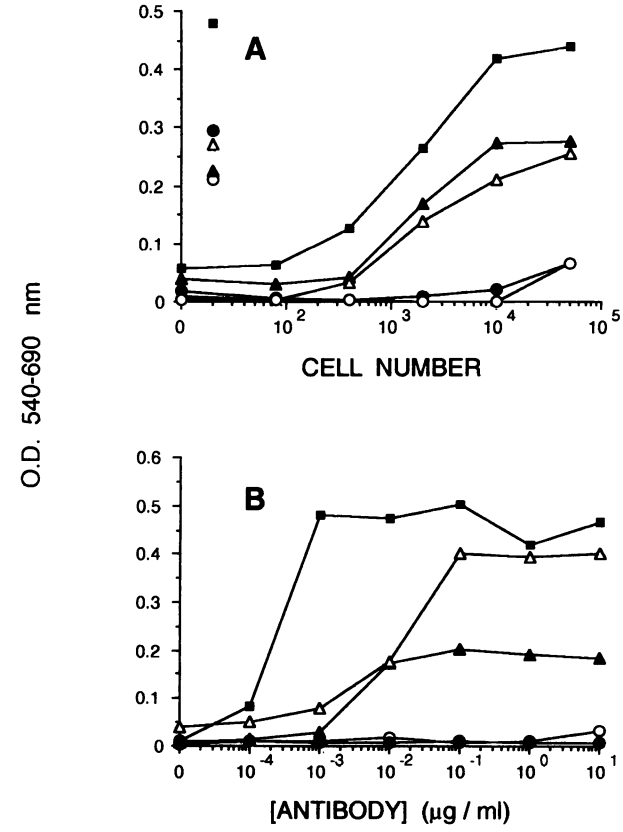

Fig. 7. Influence of CD4 expression on responsiveness to TCR-mediated stimuli. (**A**) Antigen stimulation of SR.D10 (closed squares), SR.CD4$^-$.F1 (open circles), clones of SR.CD4$^-$.F1 cells transfected to express neomycin resistance (Neo.D1, closed circles) or wild type CD4 (mCD4.F3, closed triangles, and mCD4.B3, open triangles). These cells were tested for activation by conalbumin (100 μg/ml) in the presence of the indicated numbers of mitomycin C-treated TAK lymphoma cells as antigen-presenting cells. Proliferation induced in the different cell lines by recombinant mouse IL-4 (5 U/ml) is indicated by single symbols. (**B**) Activation of SR.D10 (closed squares), SR.CD4$^-$.F1 (open circles), Neo.D1 (closed circles), mCD4.F3 (closed triangles) or mCD4.B3 (open triangles) by 3D3 clonotypic antibody added to cultures at the concentrations indicated.

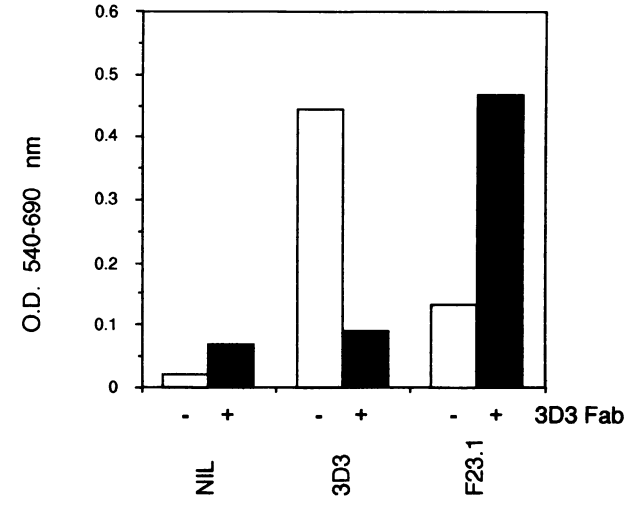

Fig. 8. Monovalent Fab fragments of the clonotypic antibody 3D3 potentiates activation of SR.D10 by F23.1. Cells were cultured in the presence of clonotypic antibody 3D3 (1 ng/ml); anti-TCR Vβ 8 antibody F23.1 (1 μg/ml), or medium (NIL), with or without 3D3 Fab (10 μg/ml), as indicated.

Discussion

CD4–lck association to the TCR

We have obtained immunochemical as well as genetic evidence that CD4–*lck* is associated with the TCR complex in lysates of unstimulated cells from the murine $CD4^+$ T cell clone SR.D10. Phosphorylated proteins from *in vitro* kinase assays of CD4 immunoprecipitates included, apart from $p56^{lck}$, several polypeptides of the CD3 complex (Figure 1B, C and D). The protein tyrosine kinase $p56^{lck}$ could be also recovered from TCR immunoprecipitates (Figure 1D) or CD3 immunoprecipitates (data not shown). Comparison of cells expressing CD4 (SR. D10, mCD4. F3) or not ($CD4^-$ F1) shows that $p56^{lck}$ needs CD4 to coprecipitate with the TCR (Figure 6A). TCR–CD3–CD4–*lck* association in the absence of TCR stimuli could also be observed in lysates of normal $CD4^+$ T lymphocytes isolated from murine spleen or from lysates of D10.G4.1 cells, indicating that it is a common feature, not restricted to a particular $CD4^+$ T cell clone (Figure 2). The weak, non-covalent association between TCR–CD3 and CD4–*lck* can only be observed using mild detergents like CHAPs to lyse the cells, and, in our hands, is destroyed by other detergents like NP-40 or Triton X-100 (data not shown).

In the system here described, $p56^{lck}$ was the only protein tyrosine kinase found in association with the TCR–CD3 complex (Figures 1 and 5). This observation is surprising in view of the immunochemical evidence indicating that $p59^{fyn}$ is the main or only protein tyrosine kinase detected in TCR or CD3 immunoprecipitates (Samelson *et al.*, 1990; Beyers *et al.*, 1992), as well as functional data indicating an important role of *fyn* in TCR-mediated activation mechanisms (Cooke *et al.*, 1991; Davidson *et al.*, 1992). We have obtained preliminary data in other experimental systems indicating that the absence of detectable *fyn* in our reprecipitation experiments is not due to inefficiency of the antiserum used (J.M.Rojo, unpublished results). One reason for these differences might be the relative levels of *lck* and *fyn* kinases in each particular system. In our hands, both SR.D10 and the $SR.CD4^-$.F1 mutants had abundant *lck* and *fyn* tyrosine kinases, although *lck* activity was usually higher than *fyn* (Figure 6B). In contrast, the 2B4 hybridoma used by Samelson *et al.* to detect *fyn* association with the TCR might express very low levels of *lck* (Samelson *et al.*, 1990). In addition, differences between species, cell lines or subsets in *fyn*–TCR and *lck*–CD4–TCR association strength, or the sensitivity of these interactions to dissociation by different detergents could account for the differences observed. For instance, while we have observed CD3–CD4 association in normal mouse $CD4^+$ T lymphocytes lysed with CHAPS (Figure 2), it was undetectable in the same cells using the conditions described by Beyers *et al.* for rat T lymphocytes lysed with Brij 96 (Beyers *et al.*, 1992) (data not shown).

Functional and immunochemical data suggest that CD4 coupling to the TCR takes place in a precise and unique orientation, close to the binding sites of the anti-TCR C193.5A and anti-CD3ε antibodies. The clonotypic antibody C193.5A binds an epitope in the TCR close to CD3ε, and D10.G4.1 activation by C193.5A as well as by anti-CD3ε can be sterically inhibited by anti-CD4 (Rojo and Janeway Jr, 1988; Portolés *et al.*, 1989a; Dianzani *et al.*, 1992). Furthermore, the negligible amount of $p56^{lck}$ coprecipitated by C193.5A (Figures 1A and 6A) suggests that the binding of this antibody disrupts pre-existing TCR–CD4 complexes.

Fluorescence resonance energy transfer analysis in a different system has shown that activating signals like anti-CD3 antibodies induce *lck*-dependent association of CD4 with the TCR (Mittler *et al.*, 1989; Collins *et al.*, 1992). Conversely, our results show that *lck* binding to the TCR greatly depends on the presence of CD4 (Figure 6A). This might indicate that CD4 and *lck* mutually need each other to be associated with the TCR.

Functional implications of CD4–lck association with the TCR

Maintenance of CD4–*lck* association with the TCR is of great functional importance. This is clearly appreciated first, by the fact that anti-TCR antibodies which do not coprecipitate significant amounts of $p56^{lck}$ are poor activators, and second, by loss of proliferation induced by anti-TCR antibodies in cells lacking CD4 (Figure 5A and C), which can be recovered by transfection of CD4 (Figure 7B). CD4 expression also means a more efficient activation by antigen (~100-fold, Figures 4, 7A and our unpublished data). These results are in full agreement with previous data in tumor systems showing the importance of *lck* association to CD4 and CD8 for the coreceptor function of these molecules (Miceli *et al.*, 1990; Chalupny *et al.*, 1991; Glaichenhaus *et al.*, 1991), and reinforce the idea that $p56^{lck}$ needs the expression of coreceptor molecules to be efficiently coupled to the TCR and the activation pathways of T cells. A correlation between coreceptor association with the TCR and functional parameters has been also observed by Carrera *et al.* in thymocytes from mice expressing unique, transgenic, MHC class I-restricted TCRs (Carrera *et al.*, 1992). *In vitro* kinase assays demonstrated the association of TCR chains with CD8 immunoprecipitates from thymocytes, which was dependent on positive selection as it could be detected neither in CD4 precipitates from the same cells, nor in CD8 precipitates from thymocytes of mice expressing a non-restricting MHC. An important difference between these data and ours is that Carrera *et al.* could not find detectable TCR–CD8 or TCR–CD4 association in normal T lymphocytes or T cell lines (Carrera *et al.*, 1992).

Although the data obtained in D10 cell activation by different anti-TCR antibodies, as well as in other experimental systems, suggested that one function of activating TCR ligands was recruiting CD4 in the proximity of the TCR (Saizawa *et al.*, 1987; Janeway *et al.*, 1988, 1989a, 1989b, 1992; Mittler *et al.*, 1989; Rojo *et al.*, 1989; Collins *et al.*, 1992), the functional meaning of CD4–TCR proximity has not been fully ascertained, and might be different in different cell subsets. Results of co-capping experiments show that memory $CD4^+$ T cells and T_h1 cell lines have high levels of TCR–CD4 association, whereas this association is low in $CD4^+$ lymphocytes of naive phenotype of T_h2 cell lines (Dianzani *et al.*, 1990). If cross linking of TCR–CD4–*lck* complexes was *per se* a prime factor of TCR-mediated activation, one would expect that binding of anti-CD4 antibodies should lead to cross linking of the accompanying TCR and T cell activation. This phenomenon has been observed neither in D10 cells (Figure 5) nor in normal $CD4^+$ T lymphocytes, even those of memory phenotype showing high levels of CD4-associated TCR (Eichmann *et al.*, 1987; Emmrich *et al.*, 1987; Dianzani *et al.*, 1990; Luqman *et al.*, 1992; Suzuki *et al.*, 1992; our unpublished

data). The same conclusion can be drawn from data presented here as well as from previous data showing that anti-TCR antibodies like F23.1 do not efficiently induce D10 cell proliferation (Figure 5A) or tyrosine kinase phosphorylation of cell substrates (Dianzani *et al.*, 1992), while they do maintain the existing CD4–*lck* association with the TCR (Figure 6A). One explanation for the absence of cell proliferation when TCR cross linking takes place in the presence of high amounts of accompanying CD4 (i.e. when using anti-CD4 antibodies) is the existence of negative signals, different from steric inhibition, that ensue under certain circumstances upon CD4 cross linking (reviewed in Janeway, 1992). Some of the polypeptides which coprecipitate with CD4, but not with the TCR (see Figures 1A, B and 6A) might be responsible for the differential response of the cells to TCR or CD4 cross linking.

Experiments using the T_h2 cell line D10.G4.1 and different anti-TCR antibodies suggest another explanation. The activation efficiency of these antibodies correlates with induction of TCR–CD4 proximity and tyrosine phosphorylation of cellular substrates (Saizawa *et al.*, 1987; Janeway *et al.*, 1988, 1989b, 1992; Rojo *et al.*, 1989; Dianzani *et al.*, 1992). However, certain antibodies (i.e. F23.1) which neither activate SR.D10 cells, nor destroy CD4–*lck* association with the TCR–CD3 complex (Figures 5, 6 and 8) induce SR.D10 proliferation when combined with monovalent, non-activating Fab fragments of an efficient anti-TCR antibody like 3D3 (Figure 8). We would argue that these Fab fragments change the TCR complex to allow cross linking by F23.1 to function as an effective signal for cell activation, as previously suggested (Rojo and Janeway Jr, 1988; Janeway *et al.*, 1989a). This is in agreement with the view that the involvement of *lck* in TCR-mediated signals is under different physical constraints depending on the TCR ligand (Haughn *et al.*, 1992). Whether ligand induced changes allow recruiting of CD4–*lck* into the proximity of the TCR complex, activation of *lck*, *fyn* or other TCR-associated kinases, or both, needs further analysis. In addition, this mechanism closely resembles receptor systems possessing intracytoplasmic domains with protein tyrosine kinase activity. In these systems, the binding of specific ligands induces oligomerization as well as conformational changes that activate the receptor associated tyrosine protein kinase to initiate cell signaling (Fantl *et al.*, 1989; Greenfield *et al.*, 1989).

Interestingly, although CD4$^-$ mutants of SR.D10 could not be activated by soluble anti-TCR, they could still be stimulated, albeit with lower efficiency, by anti-TCR or anti-CD3 monoclonal antibodies fixed to a solid matrix (Figure 5D) as well as by antigen (Figure 4 and unpublished results). Whether the activation mechanisms (i.e. tyrosine kinases and substrates) implicated in the activation of CD4$^-$ mutant cells by antigen or solid-phase anti-TCR antibodies are the same as in the original SR.D10 cells should be determined.

The relative implication of different tyrosine protein kinases in the two signal transduction pathways which have been described in the TCR complex, one through ζ chains (Frank *et al.*, 1990; Irving and Weiss, 1991; Romeo and Seed, 1991), the second through CD3ϵ chains (Letourneur and Klausner, 1992; Wegener *et al.*, 1992), should be also assessed, in view of recent data showing that phosphorylation of ζ immunoprecipitates depends on the expression of functional *lck* (Straus and Weiss, 1992).

Materials and methods

Antibodies, plasmids and other reagents

The following monoclonal antibodies were used: 3D3 (anti-D10 TCR, mouse IgG1) (Kaye *et al.*, 1983); C193.5A (anti-D10 TCR, mouse IgG1) (Tite *et al.*, 1986a); F23.1 (anti-Vβ8.1, mouse IgG2a) (Staerz *et al.*, 1985); YCD3-1 (anti-mouse CD3ϵ, rat IgG2c) (Portolés *et al.*, 1989a); GK1.5 (anti-mouse CD4, rat IgG2b) (Dialynas *et al.*, 1983); YT4.2 (anti-mouse CD4, rat IgG2a) (Janeway Jr *et al.*, 1987); M17/5 (anti-CD11a, rat IgG2a) (Sánchez-Madrid *et al.*, 1982); M1/70 (anti-CD11b, rat IgG2b) (Springer *et al.*, 1979); M1/9.3 (pan-CD45, rat IgG2a) (Coffman, 1982); and Y-19 (anti-Thy-1, rat IgG) (Jones and Janeway, 1981) were obtained from Dr Charles A.Janeway Jr, Yale University School of Medicine, New Haven, CT. All antibodies were purified from hybridoma supernatants by affinity chromatography over protein A–Sepharose columns (Pharmacia). Anti-mouse CD2 antibody RM2-5 (rat IgG2b) was from Pharmingen (San Diego, CA). 3D3 Fab fragments were obtained as previously described (Portolés *et al.*, 1989b). Rabbit antipeptide antiserum #387 to murine ζ-η chains was a generous gift of Dr L.Samelson (NICHHD, NIH, Bethesda, ML). Rabbit antisera to human *fyn* (residues 35–51) and human *lck* (residues 22–51), cross reactive with murine *fyn* and *lck*, respectively, were purchased from Upstate Biotechnology, Inc. (Lake Placid, NY). Rabbit antibodies recognizing mouse and rat Ig (RαMRIg) were obtained by passage of rabbit anti-mouse Ig antisera over rat IgG–Sepharose columns. For immunoprecipitation assays, affinity purified RαMRIg antibodies were coupled to CNBr activated Sepharose (1 mg antibody/ml of packed beads). Other antibodies and FITC coupled reagents were from Sigma.

Mouse recombinant IL-1α was a gift of Dr S.Gillis, Immunex Corp., Seattle, WA; human recombinant IL-2 was a gift of Dr M.Gately, Hoffmann-La Roche Inc., Nutley, NJ. Mouse recombinant IL-4 was from Genzyme.

Plasmids pNeoSRα and pSRαmCD4 were kindly provided by Dr Soon-Cheol Hong and Dr Charles A.Janeway, Jr, Yale University School of Medicine. 3-[(3-cholamidopropyl)-dimethylammonio]-1-propanesulfonate (CHAPS) was from Pierce Europe (Oud-Beijerland, Holland). All other reagents were from Sigma.

Cells and cell lines

SR.D10 is a spontaneous variant cloned from cultures of the murine CD4$^+$ T cell line D10.G4.1 (M.Ronda *et al.*, manuscript in preparation). It was maintained by stimulation every 2 weeks at 5×10^4 cells/ml with mitomycin-C treated H-2^k (C3H) spleen cells (5×10^4 cells/ml) plus antigen (100 μg/ml conalbumin) in Click's medium supplemented with 10% inactivated fetal calf serum (culture medium). D10.G4.1 (Kaye *et al.*, 1983) was maintained as described for SR.D10 except that 10^5 responder cells/ml and 10^6 spleen cells/ml were used. The I-A^k transfected TAK lymphoma (Buerstedde *et al.*, 1988) was obtained from Dr Charles A.Janeway, Jr.

CD4$^+$ T lymphocytes were obtained from spleen cell suspensions of C3H mice. After removal of erythrocytes by hypotonic shock they were resuspended in culture medium in the presence of anti-CD8 and anti-CD11b antibodies and incubated for 30 min at 4°C. After extensive washing, the cells were resuspended in culture medium, and run over a mouse immunoglobulin (Ig)/anti-mouse Ig column as previously described (Wigzell, 1976). The cells collected in the effluent were routinely >95% viable, >95% CD3$^+$ and >90% CD4$^+$.

Generation of CD4$^-$ mutants

SR.D10 cells (10^7 in 10 ml of culture medium) were irradiated (500 Rad, 10 min), and grown for 10 days in culture medium containing 5 U/ml IL-2; 10 U/ml IL-4 and 25 pg/ml IL-1α (interleukin medium). The cells were then selected by panning over GK1.5-coated plates, and unbound cells grown for 4 days in interleukin medium. Panning was repeated, and unbound cells (70% CD4$^-$, 100% TCR$^+$, as determined by flow cytometry) were cloned by limiting dilution in interleukin medium. The resulting clones were checked for CD4 expression by flow cytometry and one CD4$^-$ clone (SR.CD4$^-$.F1) was selected for further use.

Generation of CD4 transfectants

Plasmids pNeoSRα and pSRαmCD4 were obtained from strain DH5α of *Escherichia coli* by the ethidium bromide–cesium chloride method (Sambrook *et al.*, 1989). Plasmids were linearized with *Sca*I and cotransfected (at 3 μg/ml and 10 μg/ml, respectively) into SR.CD4$^-$.F1 (5×10^6 cells in 0.8 ml of PBS) by electroporation at 960 μF and 280 V using a Gene Pulser (Bio-Rad). Electroporated cells were distributed in a flat-bottom 96 well culture plate and cultured overnight in interleukin medium and Geneticin (800 μg/ml) was added to the surviving cells. Neomycin-resistant cells were cloned and clones expressing adequate levels of TCR and CD4 were selected.

Flow cytometry

Cells (5×10^5) were incubated for 30 min at 4°C with saturating amounts of biotin-labeled antibody in 100 μl of PBS, 0.1% NaN_3, 5% FCS (staining buffer). Then, the cells were washed and incubated for 30 min with FITC-conjugated goat anti-biotin (Sigma) in staining buffer. After washing with PBS, the cells were fixed in 1% paraformaldehyde and analyzed on a FACScan (CoulterElectronics).

Immunoprecipitation and in vitro kinase assays

For each immunoprecipitation, 10^7 cells were lysed for 15 min on ice with 0.15 ml of 10 mM CHAPS in 50 mM Tris pH 7.5, 300 mM NaCl, 1 mM PMSF and 10 μg/ml aprotinin. Postnuclear supernatants were precleared twice by rotation with 20 μl of RαMRIg–Sepharose CL-4B beads previously incubated with 5 μg of normal mouse Ig for 1 h at 4°C and washed. Precleared supernatants were added to 20 μl of packed RαMRIg–Sepharose previously coated with 5 μg of appropriate monoclonal antibodies in lysis buffer (1 h, 4°C) and washed. After rotation for 1 h at 4°C, the precipitates were washed five times with 2 mM CHAPS in 50 mM Tris pH 7.5, 300 mM NaCl, 1 mM PMSF and 10 μg/ml aprotinin (washing buffer).

Kinase assays were performed by addition to washed immunoprecipitates of 50 μl of kinase assay buffer (2 mM CHAPS in 25 mM HEPES pH 7.2, 10 mM $MgCl_2$, 3 mM $MnCl_2$) and 10 μCi [γ-^{32}P]ATP (30 Ci/mmol; 1 Ci = 37 GBq) and incubation for 20 min at 25°C with frequent mixing. Reactions were stopped with 60 μl of double strength SDS–PAGE sample buffer and the samples boiled for 5 min. Eluted products were separated on 12% SDS–PAGE gels and visualized by autoradiography. Immunoprecipitation and kinase assays using anti-*fyn* and anti-*lck* antisera were performed as described, except that cell lysates were pre-cleared with protein A–Sepharose (Pharmacia) preincubated with 5 μl of normal rabbit serum and immunoprecipitates were performed with 20 μl of packed protein A–Sepharose preincubated with 5 μl of anti-*fyn* or anti-*lck* antisera. For reprecipitation of phosphorylated proteins, the kinase reaction was stopped by addition of 1 ml of washing buffer and centrifugation. The washed immunoprecipitates were treated to disrupt weak non-covalent bonds between phosphorylated polypeptides by incubation for 30 min on ice with 0.5 ml of 0.1% SDS, 0.2% sodium deoxycholate and 0.1% Triton X-100 in 50 mM Tris pH 7.6, 300 mM NaCl. After centrifugation, aliquots of the supernatant were added to 20 μl of packed protein A–Sepharose previously incubated with antiserum (5 μl/sample) or monoclonal antibody (5 μg/sample) and washed. After rotation for 16 h at 4°C, the beads were washed twice with washing buffer and analyzed by SDS–PAGE electrophoresis as described above.

Proliferation assays

D10 cells, CD4$^-$ mutants and mouse CD4 transfectants were cultured in 100 μl of culture medium in flat-bottomed wells (10^4 cells/well) in the presence of APCs, affinity purified antibodies or interleukins, at the concentration indicated. Where appropriate, antibodies were adsorbed to culture wells by incubation of affinity purified antibodies at the concentration indicated (2 h at 37°C, 50 μl/well in PBS); unbound antibodies were extensively washed before addition of the cells. Activation by antibodies was performed in the absence of IL-1. Antigen presenting cells were either cells of the murine B lymphoma TAK, or spleen cells from C3H mice (I-A^k) depleted of T cells by treatment with anti-Thy antibody plus rabbit complement and then passed through Sephadex-G10 to remove adherent cells (Mishell *et al.*, 1980). All APCs were treated with mitomycin C and washed before use.

After 3 days, cell proliferation was determined by a colorimetric assay using 3-(4,5-dimethylthiazole-2-yl)-2,5 diphenyltetrazolium bromide (MTT) as described (Mossmann, 1983). Initial experiments showed equivalent results using MTT or [^{3}H]thymidine incorporation in this system.

Northern blot

Total cellular RNA was prepared from frozen cell pellets by the guanidine hydrochloride method (Winter *et al.*, 1985). For Northern blot analysis, 10 μg of RNA was resolved on agarose–formaldehyde gels (Sambrook *et al.*, 1989) and transferred to nitrocellulose membrane by vacuum blotting. The membrane was prehybridized at 42°C in a solution containing 50% formamide, 5 × SSC (1 × SSC is: 150 mM NaCl, 15 mM sodium citrate), 5 × Denhardt's solution, 0.1% SDS and 100 μg/ml salmon sperm DNA. Hybridization with specific probes was carried out in the same solution. Mouse CD4 or glyceraldehyde-3-phosphate dehydrogenase specific probes were labeled with ^{32}P by random hexamer extension, as described in Feinberg and Vogelstein (1983). To remove the labeled probes, between hybridizations, the filters were heated (1 h, 65°C) in 50 mM Tris pH 8.0 containing 0.5% SDS, 20 mM EDTA and 0.5 mM sodium pyrophosphate.

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