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The CD3-γ and CD3-δ subunits of the T cell antigen receptor can be expressed within distinct functional TCR/CD3 complexes

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The T cell receptor for antigen (TCR) consists of two glycoproteins containing variable regions (TCR-α/β or TCR-γ/δ) which are expressed on the cell surface in association with at least four invariant proteins (CD3-γ, -δ, -ε and -ζ). CD3-γ and CD3-δ chains are highly homologous, especially in the cytoplasmic domain. The similarity observed in their genomic organization and their proximity in the chromosome indicate that both genes arose from duplication of a single gene. Here, we provide several lines of evidence which indicate that in human and murine T cells which expressed both the CD3-γ and CD3-δ chains on their surface, the TCR/CD3 complex consisted of a mixture of αβγεζ and αβδεζ complexes rather than a single αβγδεζ complex. First, a CD3-γ specific antibody failed to co-immunoprecipitate CD3-δ and conversely, several CD3-δ specific antibodies did not coprecipitate CD3-γ. Secondly, analysis of a panel of human and murine T cell lines demonstrated that CD3-γ and CD3-δ were expressed at highly variable ratios on their surface. This suggested that these chains were not expressed as a single complex. Thirdly, CD3-γ and CD3-δ competed for binding to CD3-ε in transfected COS cells, suggesting that CD3-γ and CD3-δ formed mutually exclusive complexes. The existence of these two forms of TCR/CD3 complexes could have important implications in the understanding of T cell receptor function and its role in T cell development.

Key words: CD3/stoichiometry/T cell receptor

Introduction

T cells recognize antigens through the use of a receptor which is composed of at least six different polypeptide chains. Two of the chains (α and β), which constitute the T cell receptor (TCR), contain regions of variable sequence which interact with processed nominal antigen in conjunction with components of the major histocompatibility complex (see Clevers *et al.*, 1988 for review). In 0.5–10% of human peripheral blood lymphocytes, the TCR is formed by two different membrane glycoproteins, namely TCR-γ and TCR-δ (see Brenner *et al.*, 1988; Strominger, 1989, for review). Four invariant chains, are physically associated with the TCR: CD3-γ, -δ, -ε and -ζ (Clevers *et al.*, 1988). A fifth chain, CD3-η, has been detected in murine and human T cells as disulfide linked dimers with CD3-ζ (Baniyash *et al.*, 1988; Orloff *et al.*, 1989). However, the majority of CD3-ζ forms a 32 kd homodimer. A murine CD3-η cDNA has been cloned and shown to be probably derived by alternative splicing from the CD3-ζ gene (Jin *et al.*, 1990). Whereas human CD3-γ (28 kd) and CD3-δ (20 kd) are glycosylated proteins, the CD3-ε (20 kd) and CD3-ζ (16 kd) chains do not have any attached oligosaccharide side chains (Clevers *et al.*, 1988). Human CD3-γ, -δ and -ε are located within a 50 kd region on chromosome 11 (Tunnacliffe *et al.*, 1988; Evans *et al.*, 1988). The genes for CD3-γ and CD3-δ have a high degree of sequence homology and are organized in opposite orientations on the chromosome and are separated by a 1.4 kb long non-coding region (Tunnacliffe *et al.*, 1987; Saito *et al.*, 1987). CD3-ζ shows little homology with CD3-γ, -δ or -ε and maps to chromosome 1 (Weissman *et al.*, 1988).

One function of the CD3 proteins is to regulate the transport of the TCR chains to the cell surface. Studies of T cell mutants have demonstrated that a complete CD3 complex is required for efficient assembly and expression of the TCR heterodimer (Sussman *et al.*, 1987; Alarcon *et al.*, 1988a; Sancho *et al.*, 1989; DeWaal *et al.*, 1990; Ashwell and Klausner, 1990, for review). High level expression of the CD3 complex also requires the TCR chains as demonstrated in mutant T cells which lack either the TCR-α or TCR-β chains (Weiss and Stobo, 1984). However, some murine (Ley *et al.*, 1989) and human (Carrell *et al.*, 1987) T cell lines express low levels of CD3 proteins in the absence of the TCR chains. Thus, the mechanisms involved in retaining incomplete TCR–CD3 complexes may not be completely efficient. A second proposed function for the CD3 complex is in signal transduction following antigen–MHC interaction. Studies of T cell mutants which either lack expression of CD3-ζ (Sussman *et al.*, 1988; Weissman *et al.*, 1989) or have an impaired association of CD3-ζ with the other chains of the complex (Alarcon *et al.*, 1988a; Sancho *et al.*, 1989), have also provided supporting evidence for a role of the CD3 complex in signal transduction. In these mutants, low amounts of CD3-ζ$^-$ complexes are expressed on the surface which are unable to release lymphokines, mobilize calcium or generate inositol phosphates upon stimulation with anti-TCR/CD3 antibodies (Sussman *et al.*, 1988; Sancho *et al.*, 1989).

Existing models of the TCR–CD3 complex suggest a receptor which has a stoichiometry of $\alpha\beta\gamma\delta\epsilon_2\zeta_2$ (Blumberg *et al.*, 1990). Here we provide evidence that the CD3-γ and CD3-δ subunits could be expressed as separate complexes on the surface of T cells. In addition, studies with antigen

specific T cell clones suggested that both types of complexes, the CD3-γ-containing complex and the CD3-δ-containing complex, are functionally active.

Results

Isolation of TCR/CD3 complexes lacking either CD3-γ or CD3-δ

We have determined the specificity of a panel of anti-CD3 antibodies through the use of COS cells transiently transfected with individual components of the TCR−CD3 complex. Thus, we were able to identify antibodies which specifically recognized the CD3-γ, -δ or -ε chains (Table I). Of particular note, HMT-3.2 immunoprecipitated CD3-γ from COS cells transfected with the CD3-γ cDNA but not from COS cells transfected with either the CD3-ε or CD3-δ cDNAs (Figure 1). Thus, HMT3.2 is the first monoclonal antibody which is specific for the human CD3-γ chain. However, in similar studies HMT3.2 reacted equally well with murine CD3-γ and CD3-δ chains (not shown). Other antibodies, such as APA 1/2 (Figure 1A), appeared to be CD3-δ specific (Table I). Deglycosylation of the proteins with *N*-glycosidase F was performed to assess whether the proteins immunoprecipitated were, in fact, CD3-γ and CD3-δ. As shown in Figure 1B, the proteins immunoprecipitated by HMT3.2 were digested to a polypeptide backbone of 16 kd, strongly suggesting that these proteins are forms of CD3-γ. Similarly, monoclonal antibody APA 1/2 immunoprecipitated a 20 kd protein that was reduced to a polypeptide backbone of 15 kd, suggesting that this protein was in fact CD3-δ. Some antibodies did not immunoprecipitate CD3 proteins from COS cells that were transfected with individual CD3 chains suggesting that they recognized epitopes which were derived from the quaternary structure. For instance, although OKT3 recognized human CD3-ε in murine T cells transfected with this chain (Transy *et al.*, 1989), it did not immunoprecipitate isolated CD3-γ, -δ or -ε chains (Figure 1A). These chain specific antibodies were then used to dissect the interactions of the CD3-γ and CD3-δ chains with the other chains of the TCR−CD3 complex.

A comparison of the immunoprecipitates obtained with an anti-CD3-ε (HMT-3.1) and an anti-CD3-γ antibody (HMT3.2) from [^{35}S]methionine labeled Jurkat cell lysates is shown in Figure 2A. The anti-CD3-γ antibody coprecipitated CD3-ε in addition to CD3-γ but failed to coprecipitate CD3-δ. The inability of the anti-CD3-γ antibody to coprecipitate CD3-δ was also apparent when mild detergent conditions (digitonin) was utilized (Figure 2B). In contrast to the anti-CD3-γ antibody, the anti-CD3-ε antibody HMT3.1 coprecipitated CD3-δ in addition to CD3-γ, CD3-ε and CD3-ω, as well as the 18 kd precursor of CD3-γ (Berkhout *et al.*, 1988) (indicated as γ* in Figure 2A). Similarly, other anti-CD3-ε antibodies, including SP34, Leu4, UCHT1 and APA 1/1 (data not shown), coprecipitated both the CD3-γ and CD3-δ chains. On the other hand, immunoprecipitation with an anti-CD3-δ antibody (J31, Figure 2B) resulted in the co-isolation of CD3-ε but not CD3-γ.

Although the CD3-γ, CD3-δ, and CD3-ε chains can sometimes be separated by SDS−PAGE, the deglycosylation of CD3-γ and CD3-δ usually allows a better identification of the three chains. Deglycosylation experiments with *N*-glycosidase F were, therefore, performed to prove that the anti-CD3-γ antibody, HMT3.2, did not coprecipitate CD3-δ and that, similarly, the anti-CD3-δ antibody J31 did not coprecipitate CD3-γ (Figure 2C). The anti-CD3-ε antibody, SP34, however coprecipitated both CD3-γ and CD3-δ. The absence of CD3-γ in anti-CD3-δ immunoprecipitates was also observed with five additional anti-CD3-δ antibodies (APA 1/2, SP64, SP8, SP78 and SP19; data not shown). Interestingly, the complexes isolated with the CD3-γ, CD3-δ and CD3-ε specific antibodies contained CD3-ζ and the TCRα/β heterodimer as detected by analysis of the immunoprecipitates with two-dimensional electro-

Table I. Specificity of antibodies used in this paper

Antibody	Origin	Reactivity[b] with human CD3	Reactivity with murine CD3	Surface[c] staining
HMT3.2	hMab[a]	CD3-γ	CD3-γ, CD3-δ	−
SP8	mMab	CD3-δ	ND	−
SP19	mMab	CD3-δ	ND	−
SP64	mMab	CD3-δ	CD3-δ	−
SP78	mMab	CD3-δ	ND	−
APA 1/2	mMab	CD3-δ	ND	−
J31	RS	CD3-δ	−	−
SP34	mMab	CD3-ε	−	+
HMT-3.1	hMab	CD3-ε	CD3-ε	−
APA 1/1	mMab	CD3-ε	ND	−
Leu4	mMab	CD3-ε + γ or δ	−	+
OKT3	mMab	CD3-ε + γ or δ	−	+
145-2C11	hMab	−	CD3-ε	+

[a]mMab: mouse monoclonal antibody. hMab: hamster monoclonal antibody. RS: rabbit antiserum. ND: not determined.
[b]Reactivity with human and murine CD3 was evaluated by immunoprecipitation from COS cells individually transfected with each chain.
[c]Surface staining was assessed by indirect immunofluorescence of human and murine T cell lines and hybridomas.

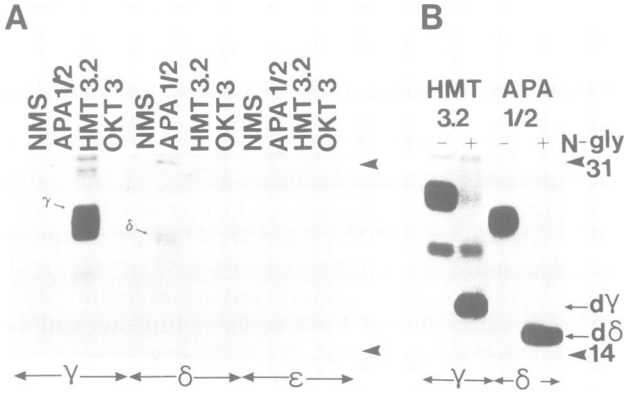

Fig. 1. Characterization of anti-CD3 antibodies. **A** COS cells were transfected with 2 μg of plasmids pSR-γ, MNC8-δ or pSR-ε and labeled 48 h later with a [^{35}S]methionine and [^{35}S]cysteine mixture during 1 h. After labeling, cells were lysed in NP40-containing buffer and immunoprecipitated with the antibodies indicated on the top. NMS is non-immune mouse serum. Arrowheads indicate the relative mobility of carbonic anhydrase (31 kd) and lysozyme (14.3 kd) run in parallel. **B** (+) *N*-glycosidase F (N-gly) treatment of HMT 3.2 and APA 1/2 immunoprecipitates from COS cells transfected with CD3-γ and CD3-δ respectively. Half of the immunoprecipitates were left undigested (−). The positions of the deglycosylated forms of CD3-γ (dγ) and CD3-δ (dδ) are indicated with arrows.

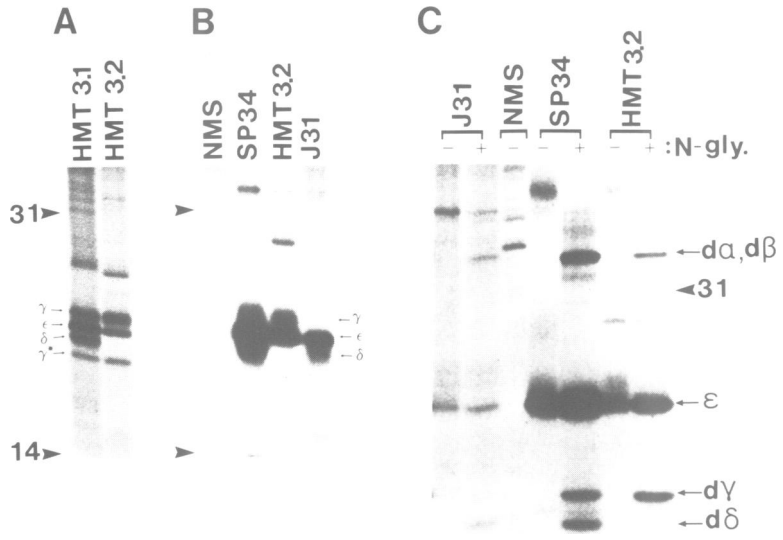

Fig. 2. Immunoprecipitation of the TCR–CD3 complex with antibodies specific for CD3-γ, -δ and -ε. **A** Jurkat cells labeled for 3 h with [^{35}S]methionine plus [^{35}S]cysteine were lysed in 1% NP40 and immunoprecipitated with either an anti-CD3-ε antibody (HMT-3.1) or an anti-CD3-γ antibody (HMT-3.2). After immunoprecipitation, the samples were subjected to electrophoresis on 12.5% polyacrylamide gels. The position of the CD3 subunits detected is indicated with arrows. γ* symbolizes a partially glycosylated form of CD3-γ. **B** Jurkat cells were labeled overnight as indicated in panel **A**, lysed in digitonin–Triton X-100 buffer and immunoprecipitated with: non-immune mouse serum as a control (NMS), anti-CD3-ε (SP34), anti-CD3-γ (HMT-3.2) and anti-CD3-δ (J31) antibodies. **C** (+) *N*-glycosidase F treatment (*N*-gly) of anti-CD3 immunoprecipitates obtained from digitonin–Triton X-100 lysates of Jurkat cells labeled for 10 min with [^{35}S]methionine and [^{35}S]cysteine and chased for 24 h in complete RPMI 1640 medium. Half of the immunoprecipitates were left unincubated with *N*-glycosidase F (−). The deglycosylated forms of TCR-α, TCR-β, CD3-γ, and CD3-δ are indicated with arrows.

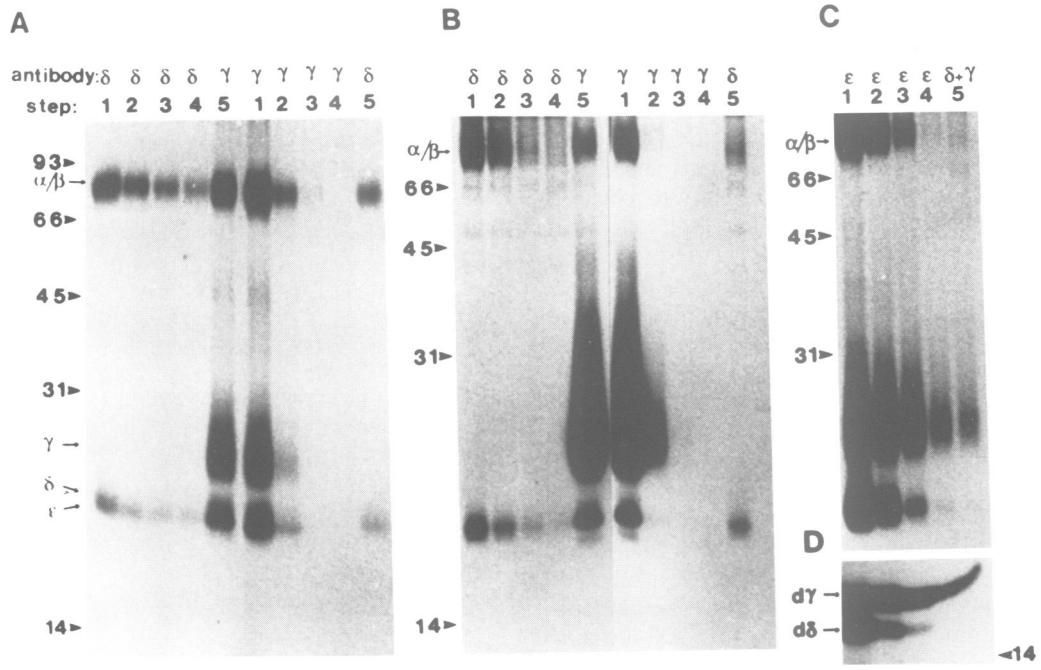

Fig. 3. Immune preclearing of CD3-γ and CD3-δ containing complexes. **A.** HPB-ALL cells were labeled with ^{125}I by the lactoperoxidase method, lysed in digitonin–Triton X-100 buffer and sequentially immunoprecipitated sequentially four times with either an anti-CD3-δ antibody (APA 1/2) or with an anti-CD3-γ antibody (HMT-3.2). After the last preclearing with the anti-CD3-γ antibody, the supernatant was incubated with the anti-CD3-δ antibody. Similarly, after the last preclearing with the anti-CD3-δ antibody the remaining supernatant was immunoprecipitated with the anti-CD3-γ antibody. The immunoprecipitates from each step were analyzed by electrophoresis under non-reducing conditions. **B.** HPB-ALL cells were radioiodinated and lysed in 1% NP40. The lysate was divided into three aliquots, one of which was subjected to sequential preclearing with an anti-CD3-δ antibody (J31) before immunoprecipitation with an anti-CD3-γ antibody (HMT 3.2). Another aliquot was precleared four times with HMT-3.2 before immunoprecipitation with J31. **C.** The third aliquot of the lysate used in panel **B**, was precleared by four sequential immunoprecipitations with an anti-CD3-ε antibody (SP34). After preclearing, the lysate was finally incubated with a mixture of J31 and HMT-3.2. **D.** Half of each immunoprecipitate shown in panel C was digested with *N*-glycosidase F to allow a better separation of the CD3-ε and CD3-δ chains. The positions of the deglycosylated forms of CD3-γ and CD3-δ are indicated (dγ and dδ).

phoresis under non-reducing−reducing conditions (data not shown). Therefore, in contrast to the CD3-ε-specific antibodies, the anti-CD3-γ and anti-CD3-δ antibodies precipitated TCR−CD3 complexes, from T cells, which lacked either CD3-δ or CD3-γ.

CD3-γ and CD3-δ appear in two distinct TCR/CD3 complexes on the surface of human T cells

Immunodepletion experiments were performed to determine whether the anti-CD3-γ and anti-CD3-δ antibodies recognized two distinct complexes, or alternatively dissociated CD3-δ or CD3-γ from a single complex. ^{125}I-labeled HPB-ALL lysates were incubated four times with aliquots of the anti-CD3-γ monoclonal antibody HMT 3.2. These sequential immunoprecipitations with the anti-CD3-γ antibody completely removed the CD3-γ containing complexes (Figure 3A, lanes γ1 to γ4) from the radiolabeled cell lysate. A subsequent immunoprecipitation of the CD3-γ depleted lysate with the anti-CD3-δ antibody APA 1/2, was performed to isolate CD3-δ containing complexes (Figure 3A, lane δ5). The amount of the CD3-δ containing complex isolated, was comparable to the amount precipitated with the anti-CD3-δ antibody from a non-depleted radiolabeled lysate (compare lanes δ5 and δ1 in Figure 3A). The presence of CD3-ε in each immunoprecipitate was confirmed by deglycosylation with *N*-glycosidase F to resolve CD3-γ from CD3-δ (data not shown).

In reciprocal experiments, sequential immunodepletions with anti-CD3-δ antibody, APA 1/2, almost entirely removed the CD3-δ containing complexes (Figure 3A, lanes δ1 to δ4). Subsequent immunoprecipitation of the CD3-δ depleted lysate with an anti-CD3-γ antibody (HMT3.2) revealed a quantity of a CD3-γ containing complex which was comparable to that detected from a non-depleted lysate (compare lanes γ5 and γ1 in Figure 3A). The CD3-γ chain was associated with the TCR−α/β heterodimer and CD3-ε rather than as an isolated chain. Similar results were obtained when a different anti-CD3-δ antibody (J31) was used (Figure 3B).

These experiments demonstrated, therefore, that the anti-CD3-γ and the anti-CD3-δ antibodies used did not dissociate CD3-δ or CD3-γ, respectively, from the remaining TCR−CD3 complex. Rather, the results suggested that the CD3-γ and -δ subunits formed two different complexes.

The preceding experiments predicted that preclearing the lysates with an antibody specific for a subunit common to the CD3-γ and CD3-δ containing complexes would result in the depletion of both complexes. Therefore, the following experiment was performed. After preclearing with the anti-CD3-ε antibody SP34 (Figure 3C, lanes ε1 to ε4), subsequent immunoprecipitation with a mixture of anti-CD3-γ and anti-CD3-δ antibodies produced less CD3-γ and CD3-δ chains than the last anti-CD3-ε immunoprecipitation (compare lane γ + δ5 with lane ε4 Figure 3C). This indicated that the anti-CD3-ε antibody efficiently removed both CD3-γ and -δ containing complexes. To better distinguish CD3-γ from CD3-δ in this experiment, aliquots of the immunoprecipitates shown in Figure 3C were subjected to digestion with *N*-glycosidase F which resulted in the 16 kd and 15 kd deglycosylated CD3-γ and CD3-δ polypeptides, respectively (Figure 3D). The total amounts of CD3-γ plus CD3-δ diminished after each immunoprecipitation with the anti-CD3-ε antibody. Of note, whereas the amounts of CD3-γ and CD3-δ coprecipitated after the first incubation with SP34 were equal (lane ε1), the amounts of CD3-δ were more diminished in comparison to CD3-γ after the last anti-CD3-ε incubations (see Figure 3D, lanes ε3 and 4). Since the SP34 antibody does not react with either the CD3-γ or CD3-δ chains, these results suggested that SP34 had a higher affinity for the CD3-δ containing complex than for the CD3-γ containing complex. A steric effect of the larger CD3-γ chain on the binding of SP34 to CD3-ε may be a possible explanation for this phenomenon.

In conclusion, these results demonstrated that CD3-γ and CD3-δ formed different complexes, on the surface of a human T cell line, which could be isolated by CD3-γ and CD3-δ specific antibodies.

CD3-γ and CD3-δ compete for binding to the CD3-ε subunit

We have previously shown that the interactions between the CD3-γ, CD3-δ and CD3-ε chains take place very early in the assembly process (Alarcon *et al.*, 1988b). By transfecting COS cells with either CD3-ε and CD3-γ or CD3-ε and CD3-δ, we have shown that CD3-ε was able to associate with either chain independently (Berkhout *et al.*, 1988). In this section, experiments were performed to investigate whether CD3-γ and -δ subunits bound to overlapping or identical sites on CD3-ε. To investigate this point, a competition experiment was designed in which COS cells were cotransfected with varying amounts of the CD3-γ and CD3-δ cDNAs. The total amount of plasmid DNA used for each transfection was maintained constant by the addition of the appropriate amount of the pSRαneo plasmid without an insert. After immunoprecipitation of the metabolically labeled lysates with an anti-CD3-ε antibody, the amounts of coprecipitated CD3-γ and CD3-δ chains were determined by deglycosylation with *N*-glycosidase F. As shown in Figure 4, CD3-γ and CD3-δ competed with each other for the binding to CD3-ε, as increasing concentrations of one chain displaced the alternate chain. This experiment suggested that

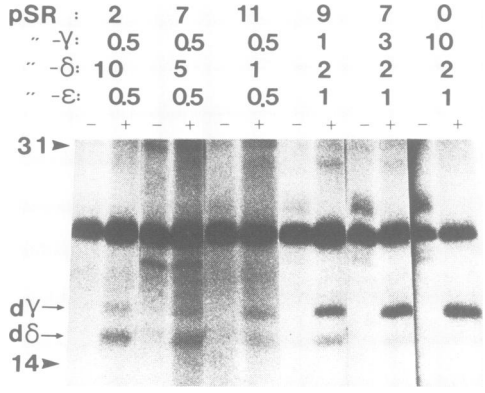

Fig. 4. Competition of CD3-γ and CD3-δ for binding to CD3-ε. COS cells were cotransfected with the amounts (in μg) of CD3 encoding plasmids indicated on the top of the figure. Variable quantities of PSRα vector without insert were added to maintain constant the total amount of DNA in each set of plates. 72 h after transfection the transfected cells were labeled with [^{35}S]methionine and [^{35}S]cysteine for 1 h, lysed in NP40 containing buffer and immunoprecipitated with an anti-CD3-ε antibody (SP34). To evaluate the amounts of CD3-ε and CD3-δ that were coprecipitated in each case, half of each immunoprecipitate was digested with *N*-glycosidase F.

CD3-γ and CD3-δ had a common binding site on CD3-ε and that CD3-ε must form alternate complexes with either CD3-γ or CD3-δ.

In addition, immunoprecipitation with CD3-γ and CD3-δ specific antibodies showed that free CD3-γ and CD3-δ were present in each COS cell lysate (data not shown).

A panel of human T cell lines expressed different ratios of CD3-γ and CD3-δ on their cell surface

In this section, the ratio of CD3-γ and -δ containing complexes on different human T cell lines was determined. To do this, ^{125}I-surface labeled cells were lysed and immunoprecipitated with an anti-CD3-ε antibody (SP34) and the immunoprecipitates were digested with *N*-glycosidase F to resolve the CD3-γ, -δ and -ε chains. As shown in Figure 5, CD3-γ and CD3-δ were expressed at variable ratios ranging from 2.2 in PEER cells to 0.5 in HUT-78 cells. Moreover, the ratios of CD3-γ to CD3-δ protein expression on the surface of some Jurkat cell variants reflected the levels of mRNA which encode for CD3-γ and CD3-δ chains (data not shown). Since each cell line consistently showed the same pattern, this excluded the possibility that these results were biased by differences in radio-iodination. However, it is important to note that the antibody used, SP34, may preferentially immunoprecipitate CD3-δ containing complexes (Figure 3D). Therefore, the data given in Figure 5 are useful to compare different cell lines but not to evaluate the actual amounts of CD3-γ and CD3-δ in each T cell line. The expression of CD3-γ and CD3-δ chains in variable quantities further suggested that the two chains were expressed in distinct complexes.

Expression of CD3-γ and CD3-δ in a panel of murine T cell clones

We have analyzed a series of murine T cell lines with the aim of finding examples of cells which express only the CD3-γ or the CD3-δ type complexes. The murine T−T hybridoma 41A11 expressed equivalent amounts of CD3-γ and CD3-δ as assessed by immunoprecipitation with an anti-CD3-ε antibody after iodination with ^{125}I (145-2C11, Figure 6A). Immunoprecipitation with the HMT-3.2 antibody, that recognizes murine CD3-γ and CD3-δ, isolated both CD3-γ and CD3-δ from 41A11 surface labeled proteins.

Three murine T cell clones (E10, 2G5 and CDC25) expressed a CD3-δ containing complex almost exclusively, since very little or no CD3-γ was detected by immunoprecipitation with either the anti-CD3-ε or the anti-CD3-(γ,δ) antibodies (Figure 6A and B). In contrast, another T cell clone, DN.1.1, and a T cell hybridoma, BY, had very low surface expression of CD3-δ although CD3-γ and CD3-ε were expressed in high levels. Longer exposures of the gels shown in Figure 6 demonstrated the presence of CD3-ζ in all cases (not shown).

Indirect immunofluorescence staining with an anti-CD3-ε antibody (145-2C11) was performed to determine the relative levels of expression of the TCR−CD3 complexes in the T cell lines. As shown in Figure 6C, the T cell lines analyzed stained brightly with the anti-CD3-ε antibody regardless of whether they expressed CD3-γ or -δ containing TCR−CD3 complexes on their surface.

All of the murine T cell clones analyzed had functional TCR−CD3 complexes, since the stimulation of these clones with antigen or anti-CD3 antibodies induced them to secrete lymphokines, to proliferate or to kill target cells. Table II summarizes the response of the T cell clones to anti-CD3 antibody stimulation as measured by proliferation and IFN-γ secretion. Previous data have shown that the E10, 41A11 and BY murine cell lines could be activated to secrete other lymphokines like IL-2 or IL-4 via stimulation of the TCR−CD3 complex (Ley *et al.*, 1989; Sleckman *et al.*, 1987). Although a possible effect of the residual levels of

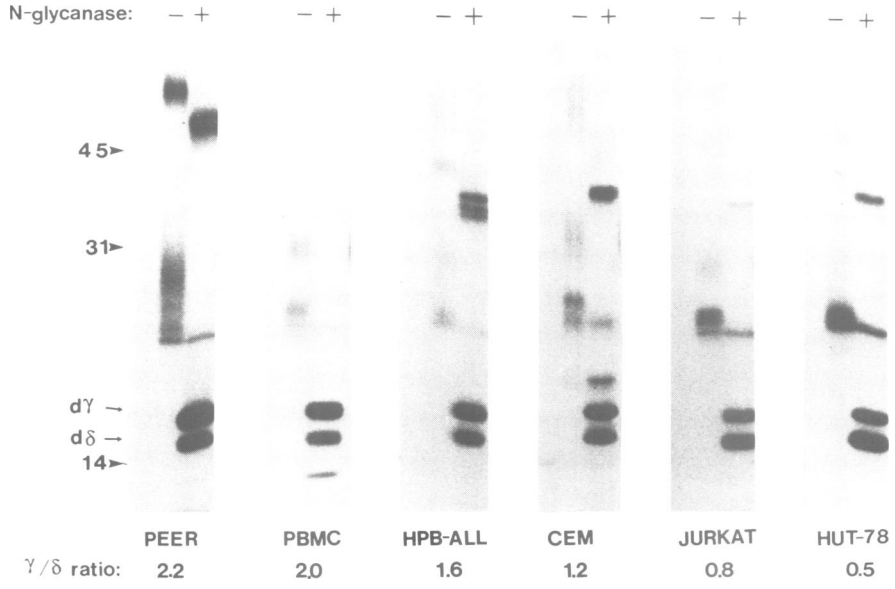

Fig. 5. CD3-γ/CD3-δ ratios in a panel of human T cell lines. 2 × 10^7 cells of the indicated T cell lines and peripheral blood mononuclear cells (PBMNC) were labeled with ^{125}I by the lactoperoxidase method, lysed in 1% NP40 and immunoprecipitated with an anti-CD3-ε antibody (SP34). Half of each immunoprecipitate was digested with *N*-glycosidase F (*N*-glycanase) for a better separation of the coprecipitated CD3-γ and CD3-δ chains. The samples were subjected to electrophoresis on polyacrylamide gels. The positions of the deglycosylated CD3-γ and CD3-δ (dγ and dδ) were located by autoradiography, the protein bands were sliced and counted in a gamma counter. The ratio of CD3-γ to CD3-δ is indicated under the name of each T cell line. The ε/γ + δ ratio was 0.09 to 0.13 in all the cell lines.

CD3-δ detected in BY and DN.1.1 cannot be ruled out, the data presented in Table II suggested that both CD3-γ containing and CD3-δ containing complexes were functionally active.

We have frequently found a CD3-γ negative or low phenotype in murine T cell clones (Figure 6). To determine whether this phenotype reflects a common phenotype found in murine tissues, we analyzed the expression of CD3-γ and CD3-δ in lymphocytes extracted from thymus, lymph nodes and spleen. CD3-γ was barely detectable by immunoprecipitation with the anti-CD3-ε antibody 145-2C11 from thymocytes labeled with ^{125}I by the lactoperoxidase catalysed method (Figure 7). Similar results were obtained by immunoprecipitation with the anti-CD3-(γ,δ) antibody HMT3.2 and by immunoprecipitation from lymph node and spleen lymphocytes (not shown). By contrast, a high level expression of CD3-γ and low CD3-δ were detected in DO11.10 cells labeled by the lactoperoxidase method

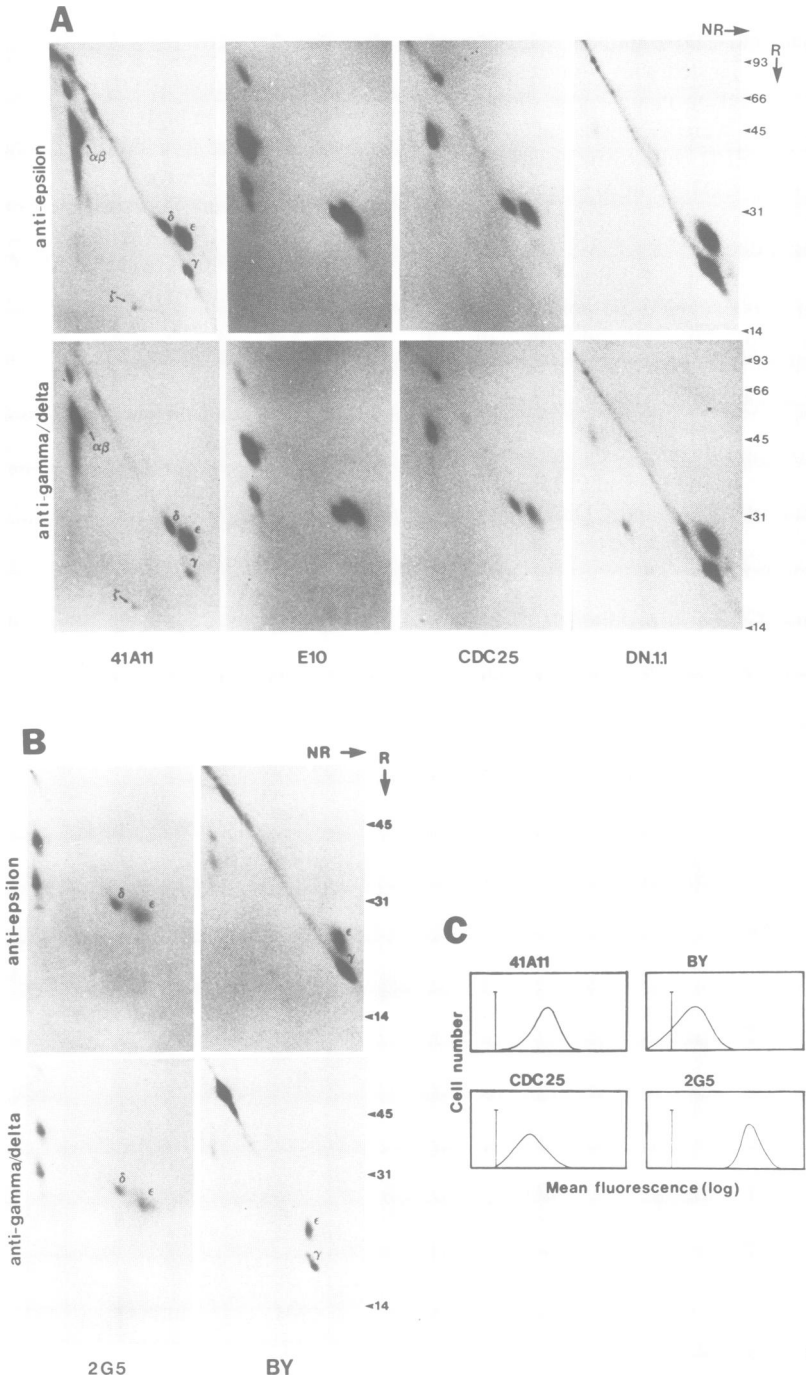

Fig. 6. Analysis of the TCR−CD3 complex in murine T cell clones. **A**,**B**. 10−15 × 10^6 cells of the murine T cell clones indicated in the bottom of the figure were radioiodinated, lysed in a buffered solution of digitonin and Triton X-100 and immunoprecipitated with either 145-2C11 (anti-CD3-ε) or HMT3.2 (anti-CD3-γ and -δ) antibodies. The immunoprecipitates were subjected to two-dimensional electrophoresis under non-reducing/reducing conditions. **C** Intact cells from the murine T cell clones and hybridomas 41A11, BY, CDC25 and 2G5 were analyzed by indirect immunofluorescence staining using 145-2C11 (anti-CD3-ε) as first antibody followed by incubation with a FITC-labeled rabbit anti-hamster IgG antibody. The vertical bar represents the modal position for a non-immune serum control staining.

(Figure 7). Two other labeling methods were used to determine the levels of expression of CD3-γ and CD3-δ in thymocytes and DO11.10 cells: a modified version of the Bolton–Hunter method which labels amino-groups of surface proteins (Thompson *et al.*, 1987) and metabolic labeling with [^{35}S]methionine followed by immunoprecipitation exclusively of surface proteins. As shown in Figure 7, murine thymocytes had low expression of CD3-γ relative to that of CD3-δ by any of the three different labeling methods used. The difference was more accused when a comparison was made with a T-cell hybridoma, DO11.10. The absolute level of expression of CD3-γ and CD3-δ cannot be exactly calculated by these labeling methods but it can be concluded that the ratios of CD3-γ to CD3-δ in murine thymocytes and in DO11.10 cells were very different.

Table II. Activity of murine T cell clones upon stimulation

Clone (antigen+H-2)	Antigen-dependent proliferation[1] (stimulation index)	CD3-mediated induction of IFN-γ[2] (U/ml)
2G5 (Mls^a)	124	4000
E10 (GLT+I-E^d)	73	9000
CDC25 (RGG+I-A^k)	52	ND
DN.1.1 (unknown)	ND	350

[1]Proliferation was measured by [^{3}H[thymidine incorporation 72 h after stimulation. The ratio of [^{3}H]thymidine incorporation of stimulated clones to unstimulated controls was calculated and presented as stimulation index. Background incorporation into irradiated spleen cells was 300–1200 c.p.m.

[2]Production of IFN-γ 24 h after stimulation in CD3-antibody coated wells. Clones cultured in uncoated wells did not produce detectable IFN-γ. The range of IFN-γ production by T cell clones that express CD3-γ plus CD3-δ was 1000–15 000 IU/ml.

Mls^a: minor lymphocyte stimulating antigen. GLT: random polymer of Glu, Lys and Tyr. RGG: rabbit immunoglobulin G. ND: not determined.

Discussion

In this paper we have provided evidence that supports the notion that the TCR–CD3 complex can be expressed on the cell surface as two different receptors. One contains CD3-γ and lacks CD3-δ and the other receptor complex contains CD3-δ and lacks CD3-γ. There are three lines of evidence which support this proposed view: (i) several anti-CD3-γ and anti-CD3-δ antibodies immunoprecipitated TCR–CD3 complexes which lack CD3-δ and CD3-γ respectively. On the contrary, anti-CD3-ε antibodies, which have traditionally been used to study the CD3 complex, co-immunoprecipitated both CD3-γ and CD3-δ; (ii) CD3-γ and CD3-δ competed for the binding to CD3-ε in transfected COS cells, suggesting that CD3-ε forms alternate complexes with CD3-γ and CD3-δ and (iii) CD3-γ and CD3-δ were expressed at variable ratios on the surface of human and murine T cells, suggesting that CD3-γ and CD3-δ are expressed independently from each other. The finding of a human T cell line which expresses high levels of a CD3-δ containing complex ($\alpha\beta\delta\epsilon\zeta$) in the absence of any CD3-γ expression also strongly supports this view (Pérez-Aciego *et al.*, 1991).

It could be argued that the anti-CD3-γ antibody dissociated CD3-δ from the complex and, *vice versa*, the anti-CD3-δ antibodies dissociated CD3-γ. This possibility was ruled out by the immunodepletion experiments in which CD3-γ precleared supernatants were shown to contain CD3-δ in association with CD3-ε and TCR heterodimer rather than in an isolated form. The reverse experiment, using first an

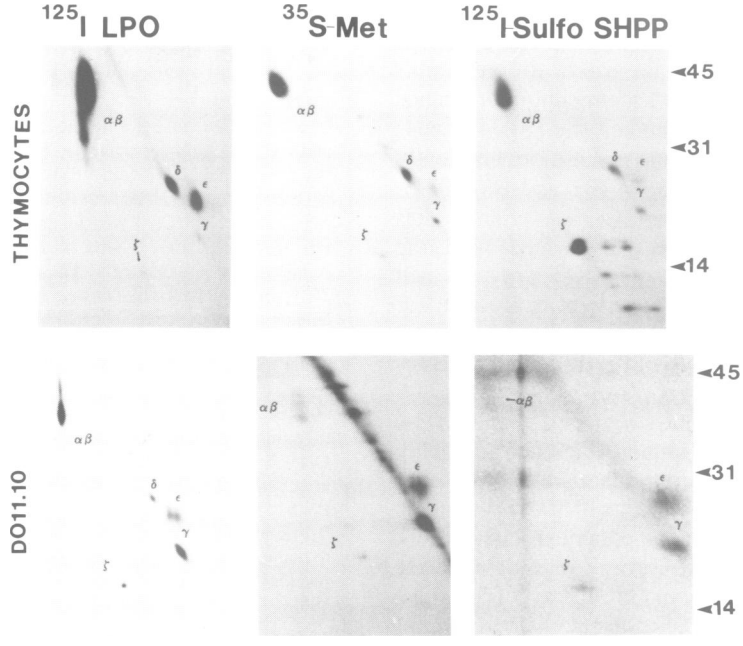

Fig. 7. Expression of the TCR–CD3 complex in murine thymus. 2 × 10^7 DO11.10 cells and 2 × 10^7 thymocytes from a 3 week old male CBA mouse were surface ^{125}I-labeled by the lactoperoxidase method (^{125}I-LPO) or by a modification of the Bolton–Hunter method (^{125}I-sulfo-SHPP) and immunoprecipitated with an anti-CD3-ε antibody (145-2C11). Alternatively, surface TCR–CD3 complexes were immunoprecipitated from metabolically labeled cells (^{35}S-Met) with antibody 145-2C11 as described in Materials and methods. Note that CD3-ζ is labeled very efficiently by [^{125}I]sulfo-SHPP and the association of CD3-ζ with other polypeptides of lower molecular weight.

anti-CD3-δ antibody to deplete the CD3-δ containing complexes, also demonstrated that CD3-γ was not dissociated, since CD3-γ was found in association with the TCR heterodimer and CD3-ε. The immunodepletion experiment and the competition experiment in COS cells suggested that the CD3-γ-ε pair interacts with the TCR heterodimer and CD3-ζ independently from the CD3-δ-ε pair. In other words they form complexes which are composed of αβγεζ and αβδεζ, respectively.

The immunodepletion experiments do not however exclude the possibility that the CD3-γ and CD3-δ specific antibodies could disrupt a putative interaction between the αβγεζ and αβδεζ complexes. The experiments shown in this paper do not support an interaction between the complexes containing αβγεζ and αβδεζ. However, recent experiments performed in our laboratory involving the transfection of the human CD3-ε chain in murine T cells imply the existence of more than one CD3-ε chain at least in part of the TCR−CD3 complexes (Blumberg *et al.*, 1990). This finding opens the possibility that CD3-ε or other subunit like CD3-ζ which is dimeric could bridge different αβγεζ and αβδεζ complexes.

Since this paper was submitted, Koning *et al.* (1990) have published some findings similar to ours although they reached different conclusions. They proposed a model where γε and δε dimers interact via CD3-ε and share a unique αβ heterodimer. Their model would be basically identical to the primary αβγδεζ model but incorporating an additional CD3-ε subunit and a restriction that CD3-γ and CD3-δ do not interact directly. Our immunodepletion experiments, however, suggest that γε and δε pairs are independently associated with different heterodimers. We have found a human PBL-derived T cell line which express high levels of a TCR−CD3 complex lacking CD3-γ on the cell surface as well as internally (Pérez-Aciego *et al.*, in press). The αβδεζ complex displayed by this cell line has been shown to promote proliferation upon stimulation with anti-CD3 antibodies but showed neither IL-2 secretion nor a raise in intracellular Ca^{2+}. Interestingly, the use of different anti-TCR−CD3 antibodies for indirect immunofluorescence analysis revealed that some antibodies (i.e. SP34, Leu4) stained the CD3-γ negative cell line much more efficiently than other antibodies (i.e. OKT3, WT31 and BMA031). These data show, perhaps, that this last class of antibodies recognize conformational epitopes associated with the expression of CD3-γ. In addition to this CD3-γ negative cell line, the finding of very different levels of expression for CD3-γ and CD3-δ in a panel of human and murine T cell lines, described in this paper, allows to generalize that CD3-γ and CD3-δ are independently expressed.

In addition to the results obtained with CD3-γ and CD3-δ specific antibodies which immunoprecipitated mutually exclusive complexes, we have found that a CD3-ε specific antibody, SP34, preferentially precipitated CD3-δ containing complexes versus CD3-δ containing complexes. This result suggests that CD3-γ and CD3-δ form different complexes with CD3-ε, otherwise SP34 should be able to coprecipitate CD3-γ and CD3-δ with identical efficiency. Interestingly, Marano *et al.* (1989) have found two different binding sites for monovalent fragments of OKT3 on Jurkat cells. These two binding sites were represented by an approximately equal number of molecules. Since OKT3 did not immunoprecipitate isolated CD3-ε chains (Figure 1) but immunoprecipitated dimers of CD3-ε with CD3-γ or CD3-δ (not shown), it is tempting to speculate whether the two binding sites for OKT3 are formed by the αβγεζ and αβδεζ complexes.

Given the homology between the CD3-γ and CD3-δ amino acid sequences (Krissansen *et al.*, 1986), it is not surprising that both chains could share similar mechanisms of assembly and function. The competition of CD3-γ with CD3-δ for the binding to CD3-ε (Figure 4) further supports this idea. It would also be possible that during ontogeny CD3-γ is expressed before CD3-δ or vice versa. In this regard, it would be interesting to explore the expression of CD3-γ and CD3-δ in fetal liver since CD3-ε can be detected very early in this organ (Campana *et al.*, 1989). It can also be hypothesized that looking backwards in the phylogenetic scale, before the CD3-γ/δ duplication took place (Krissansen *et al.*, 1986), an ancestral vertebrate had a single gene equivalent to CD3-γ and -δ whose protein product played the roles that CD3-γ and CD3-δ have in mice and humans.

In conclusion, we have shown that the TCR−CD3 complex in both human and murine T cells appears to be composed of mutually exclusive CD3-γ containing and CD3-δ containing complexes. We are now trying to determine the functional role of each type of complex. The data presented here together with the data of Blumberg *et al.* (1990) on the existence of two CD3-ε subunits in at least part of the TCR/CD3 complexes allow to propose models of stoichiometry in which two CD3-ε chains are associated to two CD3-γ chains or to two CD3-δ chains. The existence of mixed TCR−CD3 complexes containing one CD3-γ chain and one CD3-δ chain could also be possible (i.e. γεεγ, δεεδ and γεεδ). The association to each one of these tetramers of one TCR heterodimer or two (i.e. αβδεεδ, αβδεεδαβ etc) could also open new possibilities. We further propose that these complexes, αβγεζ and αβδεζ be named as the CD3-γ type and CD3-δ type TCR−CD3 complexes.

Materials and methods

Cells

The human leukemic T cell lines PEER, HPB-ALL, Jurkat, CEM T3.3, and HUT 78 and the murine T−T hybridomas 41A11. DO11.10 and BY were maintained at 37°C in RPMI 1640 medium (Whittaker, Walkersville, MD, USA) supplemented with 10% fetal bovine serum (Gibco, Grand Island, NY, USA). The COS-7 African green monkey cell line was obtained from Drs A.Aruffo and B.Seed and was maintained in DME medium supplemented with 5% FBS. The murine T cell clones E10, CDC25, and 2G5 were maintained by periodic stimulation with appropriate antigens and antigen-presenting cells, followed by the addition of 5 IU/ml of recombinant IL-2 (Amgen). CDC25 is a T cell line responsive to $F(ab')_2$ fragments from rabbit IgG in the presence of $I\text{-}A^k$ (Tony *et al.*, 1985). E10 is a T cell clone responsive to poly-(Glu,Lys,Tyr) in the context of $I\text{-}E^d$ (DeKruyff *et al.*, 1987; Ju *et al.*, 1988). 2G5 is a T cell clone from lpr/lpr mice responsive to minor lymphocyte stimulating antigen (Mls^a) (Strack *et al.*, 1989). DN.1.1 is a $CD3^+CD4^-CD8^-$ NK clone derived from lpr/lpr mice (Ju, unpublished). 41A11 is a T cell hybridoma derived from the fusion of BW5147 with lpr/lpr lymph node T cells (Ley *et al.*, 1989). BY is a T−T hybridoma resulting from fusing BW5147 with a T cell clone responsive to human JY cells (Sleckman *et al.*, 1987). DO11.10 is a T-cell hybridoma responsive to ovalbumin peptides in the context of $I\text{-}A^d$ (Haskins *et al.*, 1983).

Antibodies

Monoclonal antibodies of the SP series (SP8, SP19, SP34, SP64) were obtained by immunization of Balb/c mice with purified human CD3 proteins (Pessano *et al.*, 1985). HMT-3.1 and HMT-3.2 were obtained by immunization of Armenian hamsters with purified human proteins and were generously donated by Dr Ralph Kubo (National Jewish Center, Denver,

USA). Monoclonal antibodies APA 1/1 and APA 1/2 were obtained from Balb/c mice immunized with purified CD3 proteins isolated from human thymuses. J31 is an antipeptide serum obtained by immunization of New Zealand rabbits with a peptide corresponding to the last 13 amino acids (amino acids 138–150) of the human CD3-δ cDNA sequence (van den Elsen *et al.*, 1984). The anti-CD3 antibody OKT3 was a generous gift from Dr G.Goldstein (Ortho Pharmaceuticals, Raritan, NJ, USA). The anti-murine CD3-ε monoclonal antibody 145-2C11 was a generous gift of Dr Jeffrey Bluestone (University of Chicago, USA) (Leo *et al.*, 1987).

Radiolabeling

For metabolic labeling, 10^7 cells were collected, washed once with 10 ml of phosphate buffered saline, resuspended in 1 ml of DME medium without methionine or cysteine and incubated in a 10% CO_2 incubator for 1 h. After depletion of the intracellular pool of methionine and cysteine, 0.5 mCi of a [^{35}S]methionine and [^{35}S]cysteine mixture (1100 Ci/mmol; ICN Biomedicals, Irvine, CA, USA) were added and cells were incubated for the times indicated in the figures. After labeling, cells were collected by centrifugation at 1500 *g* for 5 min, and lysed in a buffered solution of either 1% Nonidet P40 (NP40, Sigma, St. Louis, MO, USA) or 1% digitonin (Aldrich, Milwaukee, WI) plus 0.12% Triton X-100 (Aldrich) (Alarcon *et al.*, 1988b). The buffer solution consisted in both cases of 150 mM NaCl, 10 mM triethanolamine pH 7.8, 10 mM iodoacetamide, plus 1 mM phenylmethylsulfonyl fluoride (PMSF) and 1 μg/ml each of leupeptin, antipain, pepstatin and chymostatin (all purchased from Sigma).

For the iodination of membrane proteins on the cell surface, 2×10^7 cells were washed with PBS, centrifuged and resuspended in 150 μl of PBS. 2 mCi of carrier free Na^{125}I (New England Nuclear, Boston, MA, USA) and 30 μl of a 140 IU/ml solution of lactoperoxidase (Sigma) were added (Oettgen *et al.*, 1986). Finally, 10 μl aliquots of a 0.06% H_2O_2 solution were added 4 times at 5 min intervals. The reaction was stopped by diluting the mixture in 10 ml of 20 mM potassium iodide in PBS containing a saturating concentration of tyrosine.

Radiolabeling of cell surface proteins by a modified Bolton–Hunter reagent

The method used has been described by Thompson *et al.* (1987). Briefly, 2 μl (400 ng) of a 0.2 mg/ml solution of sulfosuccinimidyl (hydroxyphenyl) propionate (sulfo-SHPP; Pierce, Rockford, IL, USA) were placed in a 1.5 ml microcentrifuge tube and the following reagents were added in succession and quickly: (i) 40 μl (2 mCi) of carrier free Na^{125}I (New England Nuclear); (ii) 20 μl of a 5 mg/ml solution of chloramine T (Sigma) in 0.5 M sodium phosphate pH 7.5; (iii) 200 μl of a 1 mg/ml solution of hydroxyphenylacetic acid (Merck, Darmstadt, FRG); (iv) 20 μl of a 12 mg/ml solution of sodium metabisulfite in 0.05 M sodium phosphate pH 7.5. All solutions were freshly prepared. The reaction mixture was immediately added to 200 μl suspension of 2×10^7 prewashed DO11.10 or murine thymocytes in PBS and incubated on ice for 30 min. The reaction was stopped by addition of 10 ml of a 1 mg/ml solution of L-lysine (Sigma) in PBS.

Immunoprecipitation and electrophoresis

Labeled cells were lysed by incubation with the appropriate immunoprecipitation buffer for 15 min on ice. From this point, all the immunoprecipitation steps were carried out at 0–4°C. The lysates were centrifuged at 12 000 *g* for 15 min in an Eppendorf centrifuge. After this, the supernatants were cleared by an additional centrifugation at 100 000 *g* for 30 min in a Beckman airfuge. The supernatant from the last centrifugation was precleared three times by incubation for 1 h each with protein A–Sepharose beads (Pharmacia, Piscataway, NJ, USA), previously coated with non-immune murine serum. The supernatant from the last preclearing was incubated for 4 h with 2.5 μl of protein A–Sepharose beads previously coated with the specific monoclonal or polyclonal antibody. After immunoprecipitation, the beads were washed 5 times with 1 ml of the corresponding immunoprecipitation buffer and boiled for 5 min in 30 μl of Laemmli sample buffer without reducing agents (Laemmli, 1970). Electrophoresis was performed on 12.5% polyacrylamide gels in the presence of sodium dodecyl sulfate according to Laemmli. For two dimensional electrophoresis, samples were run under non-reducing conditions on 12.5% polyacrylamide gels made in 200 μl capillary tubes (Fisher, Boston, MA, USA). After electrophoresis, gels were extruded from the tubes, soaked in Laemmli buffer with 5% 2-mercaptoethanol for 15 min and laid on a 12.5% polyacrylamide slab gel. When the second electrophoresis was completed, gels were dried and exposed to Kodak XAR-5 X-ray film. Gels with ^{35}S-labeled protein samples were fixed in a 7.5% acetic acid, 20% ethanol solution and soaked for 1 h in 1 M sodium salycylate (Sigma) before drying.

Immunoprecipitation of cell surface proteins from metabolically labeled cells

2×10^7 DO11.10 cells or 2×10^7 thymocytes from a 3 week-old male CBA mouse were labeled by an overnight incubation with 0.5 mCi [^{35}S]methionine plus [^{35}S]cysteine mixture (ICN) in 2 ml of DME medium without unlabeled methionine or cysteine. Cells were collected, washed twice with 10 ml of ice-cold PBS containing 0.02% sodium azide and incubated with 10 μg of purified 145-2C11 antibody in 1 mg/ml of PBS-azide for 4 h at 4°C with gentle shaking. After this incubation, cells were collected by centrifugation, washed 4 times with 1 mg/ml of PBS-azide and were lysed by incubation with 1 ml of digitonin lysis buffer for 15 min on ice. The cell lysate was centrifuged at 12 000 *g* in an airfuge. The supernatant was incubated 4 h with 5 μl of packed protein A–Sepharose beads. After this, the beads were washed 7 times with 1 ml of digitonin lysis buffer and suspended in 30 μl of non-reducing Laemmli sample buffer.

Deglycosylation procedure

After immunoprecipitation, samples were dried by addition of 1 ml acetone and incubated for 1 h at −70°C. Samples were centrifuged at 12 000 *g* for 5 min at 4°C, the supernatant removed and the pellet was left to air dry. The dried immunoprecipitates were boiled for 3 min in 14 μl of a 0.5% SDS, 0.8% 2-mercaptoethanol solution. After this, the samples were allowed to cool and mixed with 28 μl of 0.25 M phosphate buffer (pH 8.6) containing 10 mM phenanthroline (Sigma). Half of each sample was incubated overnight at 30°C in the presence of 0.25 IU of *N*-glycosidase F (Genzyme, Boston, MA, USA) and the other half was left untreated.

Plasmids

The cDNA expression vectors MNC8 and pSRα have been described previously (Aruffo and Seed, 1987; Seed, 1987; Takebe *et al.*, 1988). MNC8-δ was obtained by introducing a *Kpn*I–*Xba*I fragment (nucleotides 5–630) of the human CD3-δ cDNA (van den Elsen *et al.*, 1984) into the polylinker of M13mp19 and recloning into MNC8 as a *Sac*I–*Pst*I fragment. pSRα-γ was constructed by inserting a partially digested *Xho*I–*Bam*HI fragment from pSVL-γh (Berkhout *et al.*, 1988; Krissansen *et al.*, 1986) in the *Xho*I–*Bam*HI sites of pSRα. To construct pSRα-ε, the *Xho*I fragment of pCDε h was inserted into the unique *Xho*I site of pSRα. pSRα-δ was constructed by removing the cDNA insert from pCD-δ with *Xho*I and ligating into the *Xho*I site of pSRα.

COS cells transfections

COS cell transfections were performed using the DEAE–Dextran method (Aruffo and Seed, 1987). Briefly, subconfluent cell monolayers in 100 mm Falcon Petri dishes (Becton Dickinson, Lincoln Park, NJ, USA) seeded the day before, were washed with 10 ml of PBS and covered with 2 ml of transfection medium: DME with 10% Nu serum (Collaborative Research, Lexington, MA, USA) and 100 μM chloroquine diphosphate (Sigma). Usually 2 μg of each plasmid DNA were added per plate. Afterwards, 0.5 ml of 2 mg/ml DEAE–Dextran (Pharmacia) in transfection medium were added dropwise and the plates were incubated for 4 h at 37°C. After this incubation, the medium was removed and the cells incubated with 2 ml of 10% DMSO (Fisher) in PBS at room temperature for 2 min. After washing the cells with PBS, 10 ml of DMEM containing 5% FBS were added and the cells incubated at 37°C for 48–72 h.

For radioiodination, the transfected cells were rinsed twice with 10 ml of PBS and incubated for 10 min at 37°C in 10 ml of 1 mM EDTA in PBS. After this, cells were removed by pipetting up and down, centrifuged at 1500 *g* for 5 min and radioiodinated as indicated above.

For metabolic labeling of transfected COS cells the monolayers were washed twice with 10 ml of PBS and incubated for 1 h at 37°C with 2 ml of DME without methionine or cysteine prior to addition of 0.5 mCi of [^{35}S]methionine and [^{35}S]cysteine mixture for 1 h.

Proliferation assays

Clones (3×10^4/well) were stimulated with antigens (40 μg/ml) and irradiated spleen cells of the appropriate strain. 1 μCi of [^{3}H]thymidine (ICN) was added at 64 h after stimulation and the incorporation of [^{3}H]thymidine was determined 8 h. Background incorporation of [^{3}H]thymidine was determined for unstimulated clones cultured in the absence of antigen.

IFN-γ production

Clones (10^6/well) were stimulated in wells (24 well, Costar, Cambridge, MA, USA) coated with anti-CD3 antibody 145-2C11 and supernatants were collected 24 h later. An assay for IFN-γ was carried out as previously described (Ju *et al.*, 1988).

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