Identification of a Novel Dimeric Phosphoprotein (PP29/30) Associated with Signaling Receptors in Human T Lymphocytes and Natural Killer Cells

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Summary

Two-dimensional gel electrophoresis of in vitro phosphorylated proteins coprecipitated by CD2 monoclonal antibody (mAb) from Brij58 lysates of resting human T lymphocytes and natural killer (NK) cells resulted in the identification of a novel 29/30-kD disulfide-linked dimer (pp29/30). Comparative two-dimensional analysis of CD2, CD3, CD4, CD5, and CD8 immunoprecipitates revealed that pp29/30 associates with these signaling receptor complexes but not with CD18, CD27, and CD29 in human T lymphocytes. Analysis of CD2 immunoprecipitates prepared from T cell antigen receptor/CD3-modulated T lymphocytes indicated that pp29/30 preferentially associates and comodulates with the human T cell antigen receptor (TCR). Since tyrosine phosphorylated pp29/30 selectively interacts with the Src homology type 2 domains (SHZ) of the protein tyrosine kinases $p56^{lck}$ and $p59^{fyn}$ but not ZAP70 the present data suggest that pp29/30 represents a novel signaling receptor associated phosphoprotein likely involved in the activation of human T lymphocytes and NK cells.

The primary signal that initiates human T cell activation is delivered through the interaction of the TCR with its natural ligand, antigen/MHC. The TCR is composed of disulfide-linked polymorphic heterodimers (α/β, γ/δ) that noncovalently associate with the CD3 molecules (CD3-γ, -δ, -ϵ) as well as with ζ chains (1–5). Whereas the individual components of CD3 are only expressed as single chain polypeptides in T lymphocytes, ζ chains are expressed as disulfide-linked homodimers that associate with the TCR/CD3 complex in T lymphocytes and the CD16 molecule in NK cells (6, 7).

The earliest biochemical events after external binding of antigen/MHC to the to the TCR are activations of protein tyrosine kinases that induce tyrosine phosphorylation of a number of intracellular proteins (8). The major candidates for these protein tyrosine kinases are members of the src family ($p56^{lck}$ and $p59^{fyn}$) (9, 10) as well as the recently cloned syk-related kinase ZAP70 (11, 12). Since neither the TCR nor the CD3/ζ complex possess intrinsic tyrosine kinase activity, it was postulated that the TCR/CD3/ζ complex associates physically with protein tyrosine kinases. Such a view is supported by the recent observations that the CD3 molecules associate noncovalently with $p59^{fyn}$ (13) whereas phosphorylated ζ chains associate with ZAP70 (11, 12, 14).

With regard to the signaling function of the TCR/CD3/ζ complex, CD3-ϵ and ζ chains are of particular interest since both molecules alone are capable of transmitting external signals into the intracellular environment independent of expression of CD3-γ and CD3-δ (15, 16). This applies to activation of the tyrosine kinase pathway as well as to further downstream events, e.g., lymphokine secretion. A short peptide motif (Y-xx-L-x(6-8)-Y-xx-L) present in the cytoplasmic domains of both CD3-ϵ and -ζ has been defined which seems to be relevant for the triggering capacity of these molecules (17).

Besides the TCR/CD3/ζ-mediated primary signal, secondary stimuli are required for resting T cells to become fully activated (18). Such secondary signals result from interactions of accessory receptors (e.g., CD2, CD4, CD5, CD8, and CD28) with their respective ligands (e.g., CD58/CD2, MHC class II/CD4, CD72/CD5, MHC class I/CD8, and CD80/CD28) (19–23). How accessory receptors transduce their signals into the intracellular environment is as yet poorly understood. Since the intracytoplasmic domains of accessory molecules do not bear sequence similarities to typical signaling receptors it has been proposed that they, like the TCR, transmit signals via noncovalently associated molecules.

In this regard the recent demonstration that the CD2, CD4, CD5, and CD8 coreceptors associate with the protein tyrosine kinases $p56^{lck}$ and $p59^{fyn}$ as well as with CD3-ϵ and the CD3-associated portion of ζ (24–29) was of particular importance. Moreover, coexpression of CD2 and at least ζ ap-

 J. Exp. Med. © The Rockefeller University Press • 0022-1007/94/09/0897/10 $2.00
Volume 180 September 1994 897–906

pears to be necessary for the signaling function of CD2 (30). The latter observations for the first time provided a molecular basis for the known CD2 unresponsiveness of experimentally anergized T lymphocytes that lack expression of the TCR/CD3 molecular complex (31).

Employing two-dimensional analysis of in vitro–labeled immunoprecipitations, we here describe a novel dimeric phosphoprotein, pp29/30, that coprecipitates with the CD2, CD4, CD5, and CD8 accessory receptor complexes as well as with the TCR/CD3 complex under mild detergent conditions. Comparative analysis of immunoprecipitates obtained from untreated and TCR/CD3-modulated T lymphocytes revealed that pp29/30 preferentially associates and apparently comodulates with the CD3 molecular complex.

Materials and Methods

Cells. Resting human T lymphocytes were prepared from heparinized whole blood of healthy donors as described (32). To induce modulation of the TCR/CD3 complex, 2×10^6 freshly prepared T lymphocytes/ml were cultured for 18 h at 37°C and 100% humidity in a tissue culture supernatant of anti-CD3-ϵ mAb 2Ad2A2 (28) (IgM, kindly provided by Dr. E. Reinherz, Dana Farber Cancer Institute, Boston, MA). The HPB-ALL cell line was maintained in RPMI 1640 supplemented with 10% FCS, 2% Glutamine, and 1% Penicillin-Streptomycin (all from Gibco, Eggenstein, Germany). The human NK clone 7408 was propagated as described (33). It expresses the TCR-β gene in germline configuration (33) and mRNA for CD3-ϵ and CD3-δ but no mRNA for CD3-γ. It expresses the CD56 (NKH1) antigen and is unreactive with mAbs directed at the TCR, the CD3-ϵ molecule as well as CD4 and CD8, respectively.

mAbs, Antisera, and Glutathione S-transferase (GST)[1] Fusion Proteins. Protein A purified mAbs employed for primary CD2 and CD3 immunoprecipitates were CD2 mAb ICRFCD2.1.1A (IgG2a [32]) and CD3 mAb OKT3 (IgG2a; Ortho, Neckargemünd, Germany). Other mAbs used for immunoprecipitation experiments were obtained through the Fourth (Vienna, Austria, 1989) and Fifth (Boston, MA, 1993) International Conference On Human Leucocyte Differentiation Antigens and were as follows: CD5 (M-T505), CD18 (BA12), CD29 (K20), and CD27 (MT271). The latter mAbs were used at a 1:500 vol/vol dilution in immunoprecipitation experiments. Antisera used for reprecipitation experiments were: anti-$p56^{lck}$ (kindly provided by Dr. A. Veillette, McGill Cancer Center, McGill University, Montreal, Canada [28, 34]), anti-$p56^{fyn}$ (a gift from Dr. Sarah Courtneidge, European Molecular Biology Laboratory, Heidelberg, Germany [28]), anti-ζ (donated by Dr. Doreen Cantrell, Imperial Cancer Research Fund, London, UK [28, 35]), and anti-pp32 (directed at the first 10 NH_2-terminal amino acids of the human pp32 molecule [34]. GST fusion proteins of complete or, alternatively, isolated src homology type 2 (SH2) domains of $p56^{lck}$, $p59^{fyn}$, ZAP70 (NH_2 + COOH-terminal), and $p75^{syk}$ (COOH-terminal) were prepared and purified as described (36).

Immunoprecipitation, In Vitro Kinase Reaction, and Reprecipitation Experiments. 10^7 lymphocytes/sample were harvested, washed once in ice-cold TBS, and then lysed for 1 h in ice-cold lysis buffer (28) supplemented with 1% Brij58 detergent (Pierce, Oud Beijerland, The Netherlands). In general, antigens were immunoprecipitated from postnuclear cell lysates employing 25 μl of packed CNBr Sepharose beads coupled with protein A purified mAbs. The immunoprecipitates shown in Fig. 3 were prepared in 96-well microtiterplates. Briefly, plates were precoated over night at 4°C with 100 μl polyclonal goat anti–mouse antiserum (1:400 vol/vol in PBS; Dianova, Hamburg, Germany). Plates were washed three times with PBS/1% BSA (Sigma Chemical Co., St. Louis, MO) and blocked for 2 h with 200 μl of PBS/BSA. Subsequently 100 μl mAb diluted 1:500 vol/vol in PBS/BSA was added to each well and incubated for at least 1 h. Plates were washed again with PBS/BSA and 100 μl of cell lysate corresponding to 2×10^6 T cells was added to each well and incubated at 4°C for 1 h. Plates were washed four times and then subjected to in vitro kinase assay. In vitro kinase reaction of the washed immunoprecipitates, elution of coprecipitated proteins employing 1% Triton X-100 lysis buffer, and reprecipitation experiments were essentially performed as previously described (28, 34). Reprecipitation experiments employing GST fusion proteins were performed with 25 μl of packed glutathion-agarose coupled GST fusion proteins.

Biotinylation Experiments and Nonreducing/Reducing SDS-PAGE. Before biotinylation, 5×10^7 cells were washed twice in PBS and resuspended in 1 ml of ice cold PBS. 50 μl of a 1-mg/ml solution of L-α-lysophosphatidylcholine (LPC; Sigma Chemical Co.) in ice-cold ethanol was added to the cell suspension, mixed, and allowed to incubate for 5 min at 4°C. Subsequently cells were pelleted for 1 min at 2,500 rpm and washed twice in ice cold PBS. Permeabilized cells were resuspended in 250 μl of a biotinylation solution that consisted of 2.5 mg/ml sulfo-NHS-LC-biotin (Pierce) in PBS. LPC-treated cells were labeled with biotin for 10 min at room temperature. Subsequently 1 ml of ice-cold culture medium was added and incubated for further 2 min to quench unbound biotin. Biotinylated cells were washed twice in PBS, lysed in Brij58 containing lysis buffer, and immunoprecipitated as above with the exception that 50 μl of packed beads were employed for immunoprecipitation. After immunoprecipitation, beads were washed as above, and precipitated proteins released from the beads in 30 μl Triton X-100 lysis buffer supplemented with 8 M urea for 10 min at 37°C. Subsequently beads were spun down, the supernatant aspirated, and 6 μl of 5 × SDS-sample buffer was added. The solution was then subjected to a 6.3-cm-long tube gel (a 5.3-cm long 12% SDS-gel and a 1-cm-long stacking gel) casted in a 1.5-mm diameter glas tube. The tube gel was run with 80 V until the dye front had reached the bottom of the glas tube, equilibrated in 1 × reducing SDS-sample buffer for 45 min at room temperature and layered on top of a second 1.5-mm-thick 12% mini-SDS slab gel. The second dimension was also run at 80 V until the dye front had reached the bottom of the gel. Proteins were electrophoretically transferred to nitrocellulose sheets (Amersham, Braunschweig, Germany) using a semidry blotting apparatus (Pharmacia) and the blots were blocked over night in TBS supplemented with 5% nonfat dried milk. Blots were incubated with a 1:3,000 vol/vol dilution of biotin/streptavidin/peroxidase-complex (Amersham) in TBS/0.2% Tween 20/5% milk for 1 h at room temperature and then washed for four times with TBS/0.2% Tween 20. Biotinylated proteins were subsequently detected using the Amersham chemiluminescence (ECL) detection system.

Two-dimensional Gel Electrophoresis. In vitro phosphorylated proteins were eluted from primary or secondary immunoprecipitates for 10 min at 37°C in 50 μl of 1% Triton X-100 lysis buffer supplemented with 8 M urea (Gibco) ± 50 mM dithiothreitol (Sigma Chemical Co.). Subsequently beads were spun down and the supernatant loaded on isoelectric focusing (IEF) tube gels. When immunoprecipitates were prepared in 96-well microtiterplates, 25 μl

[1] *Abbreviations used in this paper:* GST, glutathione S-transferase; IEF, isoelectric focusing; LPC, L-α-lysophosphatidylcholine; PI., isoelectric point; SH2, src homology type 2.

of the above buffer was added to each well to release coprecipitated proteins. IEF and two-dimensional SDS-PAGE was performed as described (37) with the exception that the two-dimensional SDS-PAGE was prerun for 8 h in SDS-running buffer supplemented with thioglycolic acid (1:2,500 vol/vol dilution; Sigma Chemical Co.). The position of radiolabeled proteins was determined by autoradiography of the dried gels.

Results

Identification of Novel CD2-associated Phosphoproteins by Means of Two-dimensional Gel Electrophoresis. To identify proteins that copurify with the human CD2 molecule under mild detergent conditions, in vitro kinase assays of primary CD2 immunoprecipitates obtained from Brij58 lysates of resting human T lymphocytes were performed. The pattern of in vitro phosphorylated proteins was subsequently analyzed by means of two-dimensional gel electrophoresis with IEF in the first dimension followed by reducing 10% SDS-PAGE in the second dimension.

Fig. 1, *left* shows that at least nine distinct proteins coprecipitate with CD2 under these experimental conditions. These include a series of distinct spots migrating at 18–20 kD and 20–22 kD, respectively. Additional phosphoproteins migrate at, respectively, 29, 30, 32, 56, 59, 68, and 85 kD. Phosphoamino acid analysis of the different spots revealed that all proteins are exclusively phosphorylated on tyrosine residues (data not shown).

To identify the proteins shown in Fig. 1, *left*, a primary CD2 immunoprecipitate was obtained from Brij 58 lysates of resting human T lymphocytes and subjected to in vitro kinase assay. After kinase reaction, phosphoproteins were eluted from the CD2 immunoprecipitate employing Triton X-100 lysis buffer. The released material was then incubated with individual polyclonal rabbit antipeptide sera or mouse mAb directed at known signaling molecules expressed in human

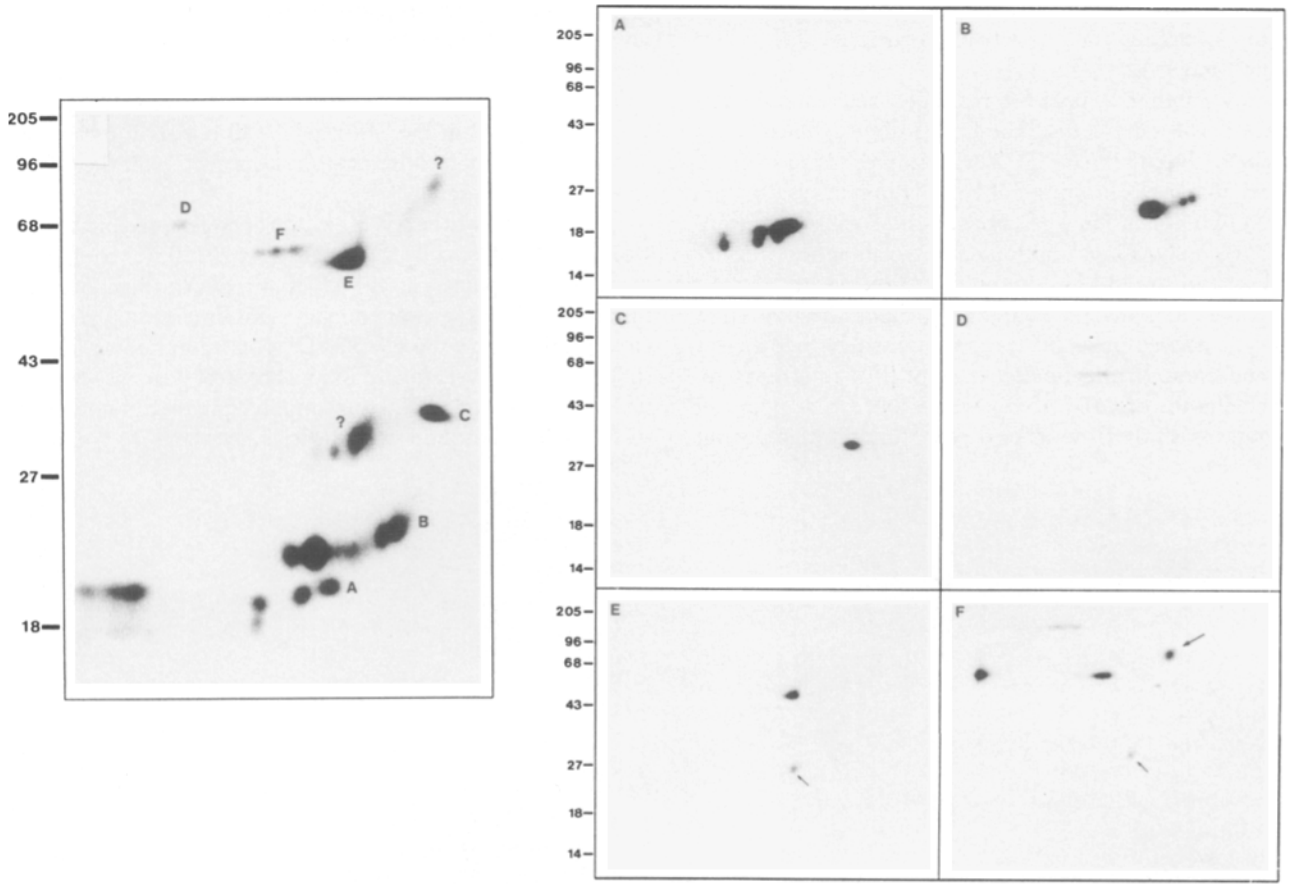

Figure 1. Analysis and identification of in vitro–labeled phosphoproteins that coprecipitate with CD2 mAb under mild detergent conditions. (*Left*) In vitro kinase assays were performed on CD2 immunoprecipitates obtained from Brij58 lysates of resting human T lymphocytes. After kinase reaction, phosphorylated proteins were eluted from the CD2 immunoprecipitates and subjected to two-dimensional gel electrophoresis with IEF in the first dimension followed by reducing 10% SDS-PAGE in the second dimension. (*Right*) Identification of the phosphoproteins shown *left* by means of reprecipitation experiments. In vitro–labeled phosphoproteins coprecipitated by CD2 mAb were released from the primary CD2 immunoprecipitate employing lysis buffer supplemented with 1% Triton X-100. Released proteins were then resubjected to secondary immunoprecipitations employing the following mouse mAb and polyclonal antipeptide sera: (*A*) anti-ζ; (*B*) anti-CD3-ε; (*C*) anti-pp32; (*D*) anti-CD5; (*E*) anti-p56^{lck}; and (*F*) anti-p59^{fyn}. The secondary immunoprecipitates were analyzed on two-dimensional gels as described for the *left* panel.

T lymphocytes. Secondary immunocomplexes were absorbed onto protein A–Sepharose beads, eluted with 8 M urea and subsequently analyzed by means of two-dimensional gel electrophoresis as above.

Fig. 1, *right*, panels *A* and *B* demonstrates that the 18–20-kD spots and the 20–22-kD spots represent in vitro–labeled ζ chains (Fig. 1, *right*, panel *A*) and CD3-ε (Fig. 1, *right*, panel *B*), respectively. The acidic 32-kD spot coprecipitated by CD2 mAb specifically reacts with an antipeptide serum directed at the CD45-associated phosphoprotein pp32 (Fig. 1, *right*, panel *C*) (34). The diffusely focused material at 68 kD was specifically reprecipitated by CD5 mAb (Fig. 1, *right*, panel *D*) while the 56- and 59-kD spots were identified as $p56^{lck}$ and $p59^{fyn}$ (Fig. 1, *right*, panels *E* and *F*). Two spots could not be identified, namely a 29/30-kD protein (pp29/30) as well as an acidic 85-kD phosphoprotein (see question marks in Fig. 1, *left*).

Fig. 1, *right*, panel *F* shows that the 85-kD protein (p85) was selectively reprecipitated by an antiserum directed at the $p59^{fyn}$ protein tyrosine kinase (upper arrow in Fig. 1, *right*, panel *F*). Preliminary experiments indicate that p85 represents a single chain polypeptide that is constitutively phosphorylated on tyrosine residues and selectively associates with $p59^{fyn}$ (data not shown).

As shown in Fig. 2, pp29/30 represents the reduced component of a 58–60-kD disulfide linked dimer. Thus, when an in vitro–labeled primary CD2 immunoprecipitate obtained from Brij58 lysates of resting human T lymphocytes was analyzed on two-dimensional gels under nonreducing conditions (Fig. 2 *B*), pp29/30 was no longer detectable (note the expected shift of the 18-kD ζ-monomer to the 36-kD dimeric form under nonreducing conditions). Since the molecular weight of reduced pp29/30 was approximately half that of $p56^{lck}$ and, moreover, its isoelectric point (PI.) almost exactly matched the PI. of $p56^{lck}$ (5.3), we reasoned that pp29/30 comigrates with in vitro–labeled $p56^{lck}$ under nonreducing conditions. To prove this point, spots 1 and 2 shown in Fig. 2, *A* and *B*, were excised and resubjected to one-dimensional reducing 10% SDS-PAGE. As shown in Fig. 2 *C*, pp29/30 was recovered from spot 2 (but not spot 1) when reanalyzed under reducing conditions. Identical results were obtained when the immunoprecipitates were prepared from cells lysed in presence of up to 20 mM iodoacetamide, which rules out the possibility that dimerization of pp29/30 is due to a postlytical artifact (not shown).

Previous data had shown that the protein tyrosine kinases $p56^{lck}$ and $p59^{fyn}$, CD3-ε, and ζ chains copurify with the accessory receptor molecules CD2, CD4, CD5, and CD8 as well as the TCR/CD3 complex in Brij lysates of rat and human T lymphocytes (24–29). It was therefore important to investigate whether pp29/30 copurifies also with these receptors. As shown in Fig. 3, *right*, in vitro–phosphorylated pp29/30 was detectable in CD3, CD4, CD5, and CD8 immunoprecipitates obtained from Brij58 lysates of HPB-ALL cells. (Note that similar results were obtained when the immunoprecipitates were prepared from resting human T lymphocytes or PHA-activated T cells, not shown). Although the relative intensities of the various phosphorylated protein spots differed among the respective immunoprecipitates (compare, for example, the relative intensities of the protein spots corresponding to CD3-ε and ζ chains in Fig. 3 *B*, to CD5 in Fig. 3 *D* and to pp32 in Fig. 3 *F*), pp29/30 is clearly present in all these immunoprecipitates with the exception of the CD45 immunoprecipitate.

The identity of the 29/30-kD proteins coprecipitated by CD3, CD4, CD5, and CD8 mAbs as pp29/30 was confirmed by peptide map analysis as well as analysis of the above immunoprecipitates by nonreducing two-dimensional gel electrophoresis where the 29/30-kD spots migrated at 58 kD (not shown). In vitro kinase assays of control goat anti–mouse, CD18, CD27, and CD29 immunoprecipitates did not result in detectable phosphoproteins (Fig. 3, *left*, lanes *1–3*, and *9*).

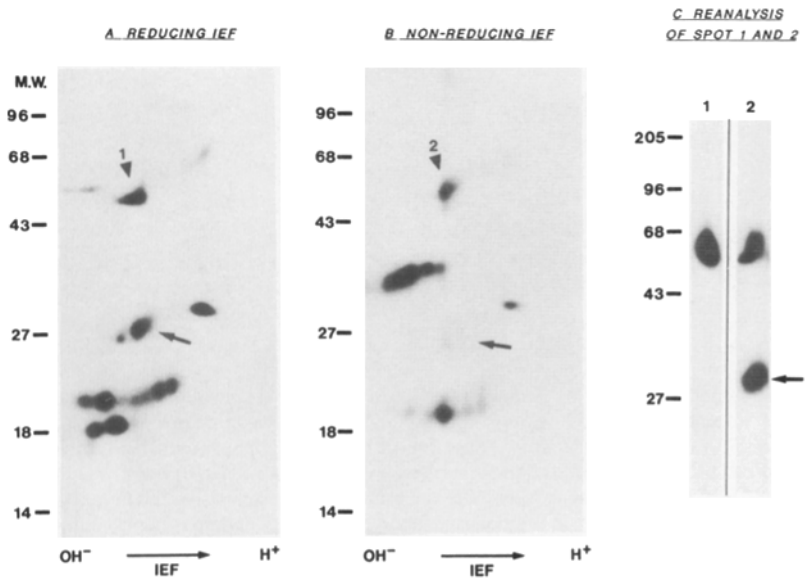

Figure 2. pp29/30 represents a disulfide linked dimer. Two-dimensional analysis of an in vitro–labeled CD2 immunoprecipitate under either (*A*) reducing or, (*B*) nonreducing conditions. (*C*) Reanalysis of spots 1 and 2 shown in *A* and *B* by means of reducing the one-dimensional SDS-PAGE. CD2 immunoprecipitates were obtained from resting human T lymphocytes lysed in 1% Brij58.

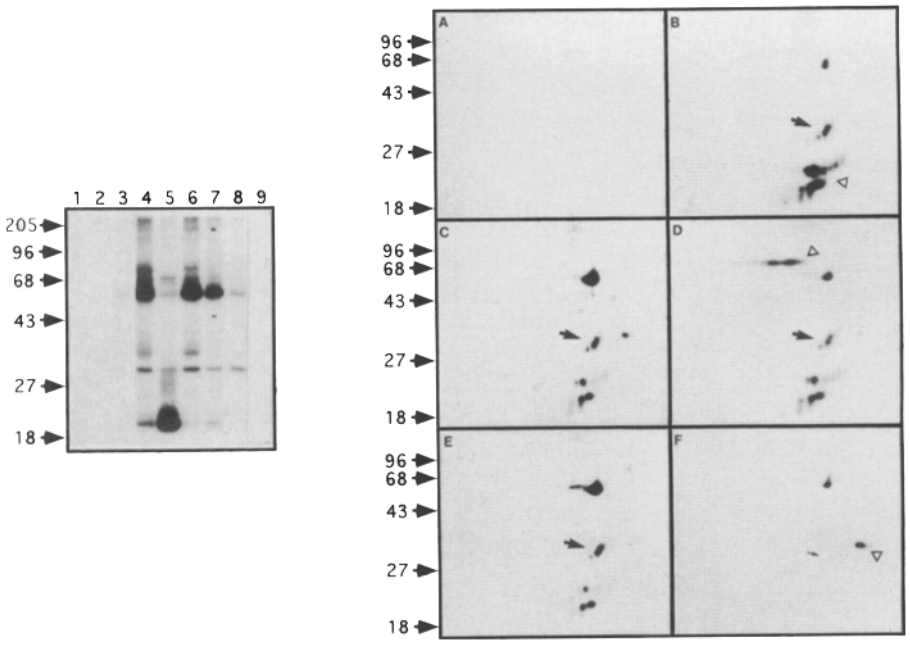

Figure 3. pp29/30 is a component of the CD3, CD4, CD5, and CD8 signaling receptor complexes. (*Left*) One-dimensional analysis of in vitro kinase assays performed on goat anti-mouse (lane *1*), CD29 (lane *2*), CD18 (lane *3*), CD2 (lane *4*), CD3 (lane *5*), CD4 (lane *6*), CD8 (lane 7), CD45 (lane *8*), and CD27 (lane *9*) immunoprecipitates prepared from Brij58 lysates of HPB-ALL cells. (*Right*) Two-dimensional analysis of in vitro–labeled goat anti-mouse (*A*), CD3 (*B*), CD4 (*C*), CD5 (*D*), CD8 (*E*), and CD45 (*F*) immunoprecipitates prepared from Brij58 lysates of HBP-ALL cells. The position of in vitro–labeled pp29/30 is indicated by an *arrow*. Positions corresponding to in vitro–labeled CD3-ϵ and ζ, CD5 and pp32 are indicated by *triangles* in *B*, *D*, and *F*, respectively.

These data indicate that pp29/30, associates with the signaling receptors CD2, CD3, CD4, CD5, and CD8 in human T lymphocytes.

We have recently reported that CD3-ϵ and ζ chains are undetectable in CD2 immunoprecipitates obtained from TCR/CD3-modulated T lymphocytes because the proteins comodulate with the TCR/CD3 complex (28). We were next interested to investigate whether a similar behavior would be true for pp29/30. Thus, freshly prepared human T lymphocytes were incubated overnight in the presence of an anti-CD3-ϵ mAb of the IgM isotype known to induce modulation of the TCR/CD3 molecular complex (28, 31). Subsequently, two-dimensional gel electrophoresis was performed on in vitro–labeled CD2 immunoprecipitates obtained from Brij58 lysates of untreated vs. TCR/CD3-modulated cells.

Fig. 4 *A* shows the known IEF pattern of in vitro–labeled polypeptides coprecipitated by CD2 mAb from untreated human T lymphocytes lysed in Brij58. The positions and densitometric analyses of the pp29/30 and pp32 protein spots

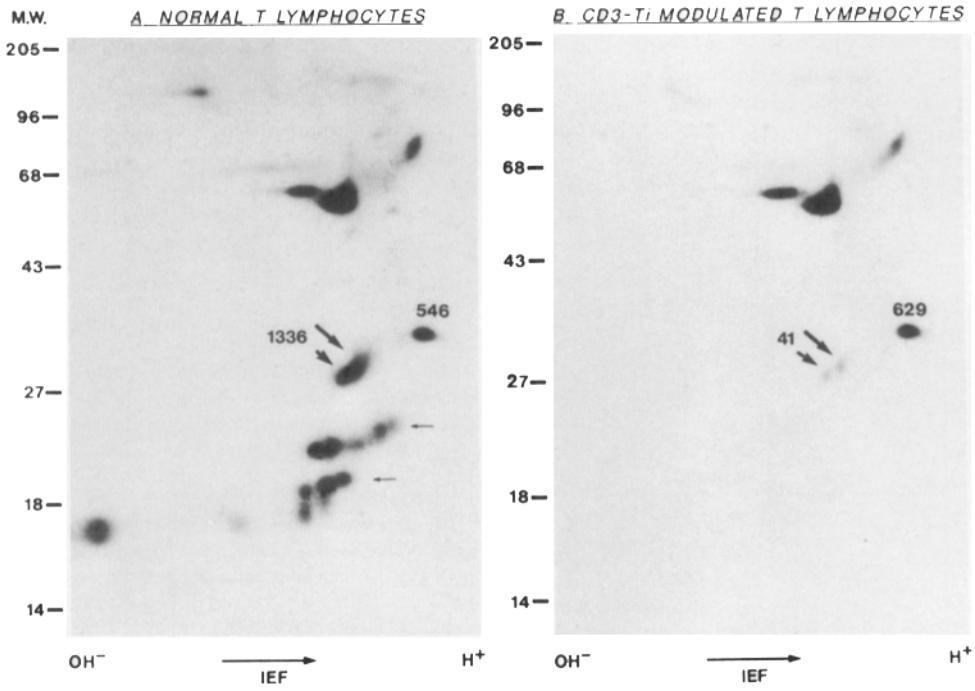

Figure 4. Impaired in vitro phosphorylation of pp29/30 in CD2 immunoprecipitates prepared from TCR/CD3-modulated T cells. Two-dimensional analysis of an in vitro–labeled CD2 immunoprecipitate obtained from resting (*A*) or TCR/CD3-modulated (*B*) human T lymphocytes. Cells were lysed in 1% Brij58. Numbers above the protein spots indicate the integrated densities of p29/30 and pp32 as judged from densitometric analysis. The decreased in vitro phosphorylation of the unknown 100-kD spot shown in *B* was not consistently observed and, therefore, was considered as nonspecific.

are indicated (1336 and 546 arbitrary units, respectively). The two-dimensional analysis of a CD2 immunoprecipitate obtained from TCR/CD3-modulated T lymphocytes shown in Fig. 4 *B* revealed the previously reported loss of ζ chains and CD3-ε in the CD2 immunoprecipitate (28). Perhaps more importantly in vitro-phosphorylation of pp29/30 was also strongly reduced (41 arbitrary units = 97% reduction compared to the nonmodulated cells) in CD2 immunoprecipitates obtained from CD3/TCR modulated cells. In contrast, the integrated density of pp32 rather increased (629 arbitrary units).

This finding suggested that pp29/30, like CD3-ε and -ζ, comodulates with the CD3/TCR complex. To exclude the possibility that reduced in vitro phosphorylation of pp29/30 in the CD2 immunoprecipitate obtained from TCR/CD3 modulated cells is due to comodulation/inhibition of a protein tyrosine kinase that is responsible for phosphorylation of pp20/30 rather than to comodulation of pp29/30 itself, we determined the amounts of pp29/30 protein coprecipitated by CD2 mAb from untreated vs. TCR/CD3 modulated T lymphocytes.

Since pp29/30 is not detectable in immunoprecipitates obtained from externally ^{125}I- or biotin-labeled T lymphocytes (not shown), we permeabilized T cells with LPC followed by biotinylation. CD2 and CD3 immunoprecipitations were subsequently obtained from untreated and TCR/CD3 modulated T lymphocytes and analyzed by two-dimensional nonreducing/reducing SDS-PAGE. The latter technique was chosen because analysis of the biotinylated immunoprecipitates by IEF/SDS page suffered from too high background.

The data shown in Fig. 5, *right*, panels *A* and *C* indicate that biotinylated off-diagonal 29/30 kD spots are visible in CD3 and to a fivefold lesser extent in CD2 immunoprecipitates obtained from untreated human T lymphocytes. In addition, a biotinylated 16-kD off-diagonal spot corresponding to ζ is coprecipitated by CD2 and CD3 mAbs, respectively. The 29/30 kD spot was proven to represent pp29/30 by (*a*) its comigration with a 29/30-kD off-diagonal spot released from an in vitro γ-[^{32}P]ATP–labeled CD2 immunoprecipitate (Fig. 5, *left*); (*b*) the observation that the in vitro–labeled off-diagonal 29/30 kD spot shown in Fig. 5, *left* produced an identical V8 protease peptide pattern as in vitro γ-[^{32}P]ATP–labeled pp29/30 separated on two-dimensional IEF/PAGE gels as above, and (*c*) the finding that the in vitro–labeled 29/30-kD spot shown in Fig. 5, *left* runs at exactly the same position as in vitro γ-[^{32}P]ATP–labeled pp29/30 when reanalyzed on two-dimensional IEF/PAGE (not shown).

Importantly, when the CD2 immunoprecipitate was prepared from TCR/CD3-modulated T lymphocytes and analyzed by Western blotting (Fig. 5, *right*, panel *D*), both ζ and pp29/30 were no longer observed. (Note that very low amounts of pp29/30 were detectable in the CD2 immunoprecipitate upon prolonged exposure of the shown gel.) Similar results were obtained when CD4 immunoprecipitates were prepared from untreated and TCR/CD3-modulated cells and analyzed on nonreducing/reducing SDS-PAGE. However, the amounts of biotinylated pp29/30 coprecipitated by CD4 mAb were approximately four times lower than in case of CD2. Note that the loss of pp29/30 in CD2 (and CD4) immunoprecipitates obtained from TCR/CD3 modulated cells was specifically due to TCR/CD3 modulation and not the result of altered reactivity of the mAbs with their respective antigens since the amounts of p56^{lck} and pp32 coprecipitated by CD2 and CD4 mAb did not differ in untreated and TCR/CD3-modulated cells as judged from reanalysis of the shown blots employing anti-p56^{lck} and anti-pp32 rabbit antisera (not shown). Collectively, the data shown in Fig. 5 strongly suggest that pp29/30 preferentially associates and comodulates with the TCR/CD3 complex.

Pp29/30 Is Expressed and Coprecipitates with CD2 in NK Cells. Given that pp29/30 comodulates with the TCR/CD3 complex, we were interested in proving whether expression of the molecule is restricted to TCR/CD3 expressing T lymphocytes. To this end we performed in vitro kinase assays of CD2 immunoprecipitates obtained from Brij58 lysates of the TCR/CD3-negative NK clone 7408 (33). The data shown in Fig. 6 *D* demonstrate that pp29/30 is expressed and copurifies with CD2 in the NK clone. Since CD3-ε mAb did not coprecipitate detectable material from clone 7408 (Fig. 6 *B*) one can exclude the possibility that coprecipitation of

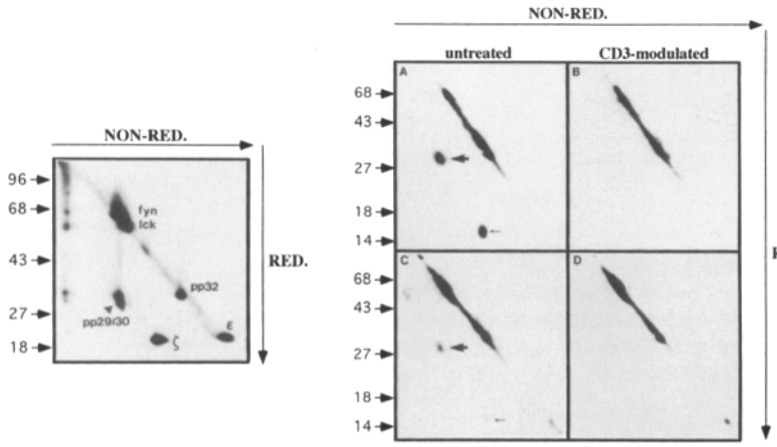

Figure 5. Evidence that pp29/30 comodulates with the TCR/CD3 molecular complex. (*Left*) Analysis of an in vitro–labeled CD2 immunoprecipitate on two-dimensional nonreducing/reducing gel electrophoresis. The identity of the individual in vitro phosphorylated spots is indicated. (*Right*) Loss of biotinylated pp29/30 in CD2 immunoprecipitates prepared from TCR/CD3 modulated T cells. CD3 (*A* and *B*) and CD2 (*C* and *D*) immunoprecipitates were obtained from untreated (*A* and *C*) or TCR/CD3-modulated (*B* and *D*) T lymphocytes that had been permeabilized with LPC before biotinylation and lysis in Brij58 detergent. Biotinylated molecules coprecipitated by CD2 and CD3 mAbs were blotted onto nitrocellulose and detected with the ECL chemiluminescence system.

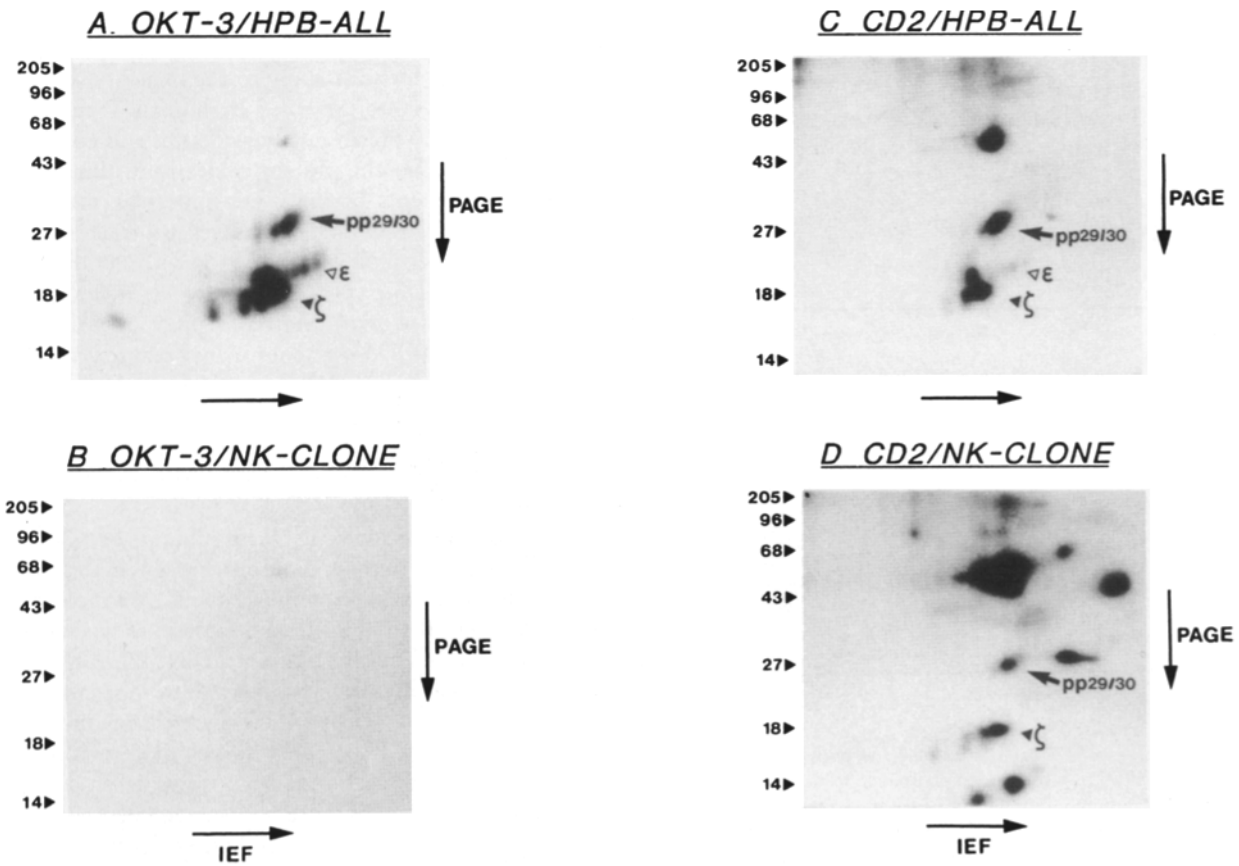

Figure 6. Expression and CD2 association of pp29/30 in Brij 58 lysates of NK cells. In vitro kinase assays of CD3 (*A* and *B*) or CD2 (*C* and *D*) immunoprecipitates were obtained from Brij 58 lysates of either HPB-ALL (*A* and *C*) or NK clone 7408 (*B* and *D*) and analyzed by means of two-dimensional gel electrophoresis. Note the coimmunoprecipitation and in vitro phosphorylation of ζ chains by CD2 mAb in the NK clone (*solid triangle*).

pp29/30 by CD2 mAb was due to contaminating CD3 positive feeder cells. The identity of the 29/30-kD protein coprecipitated by CD2 and CD3 mAb from HPB-ALL as well as the NK clone with pp29/30 was confirmed (*a*) by V8 protease peptide map analysis of the individual 29/30-kD spots; and (*b*) analysis of the individual CD2 and CD3 immunoprecipitates under nonreducing conditions where the protein migrated at 58–60 kD (not shown).

In Vitro–labeled pp29/30 Interacts with the SH2 Domains of p56^{lck} and p59^{fyn}. As demonstrated in Fig. 1, panel 2 boxes E and F, anti-p56^{lck} and p59^{fyn} antisera both coprecipitated low amounts of in vitro–labeled pp29/30 (arrow in Fig. 1, *right*, panel *E* and lower arrow in Fig. 1, *right*, and *F*). Since this was clearly not the case in primary p56^{lck} and p59^{fyn} immunoprecipitates obtained under identical experimental conditions (e.g., Triton X-100 lysis buffer, not shown), we reasoned that in its tyrosine phosphorylated state pp29/30 acquires the capacity to interact with p56^{lck} and p59^{fyn} most likely through binding to the SH2-domains of the kinases (36).

To investigate this possibility, in vitro–labeled phosphoproteins eluted from a primary CD2 immunoprecipitate obtained from Brij58 lysates of HPB-ALL were resubjected to immunoprecipitation employing GST fusion proteins of intact p56^{lck}, p59^{fyn}, and ZAP70 coupled to agarose beads or, alternatively, GST fusion proteins of isolated SH2 domains of p56^{lck}, p59^{fyn}, ZAP70, and p72^{syk}.

Fig. 7 demonstrates that in vitro–labeled pp29/30 and ζ chains were specifically reprecipitated by either intact GST-p56^{lck} and GST-p59^{fyn} fusion proteins or, alternatively, isolated GST-SH2 domains of the p56^{lck} and p59^{fyn} protein tyrosine kinases. In marked contrast, intact GST-ZAP70 and GST-SH2-ZAP70 fusion proteins selectively reprecipitated ζ chains but not pp29/30. The same was true for the GST-SH2-p72^{syk} fusion protein. The identity of the 29/30-kD spots shown in Fig. 7 as pp29/30 was proven by means of peptide map analysis and nonreducing IEF (not shown). Collectively, these data indicate that tyrosine phosphorylated pp29/30 interacts with the SH2 domains of p56^{lck} and p59^{fyn} but not of ZAP70 and p72^{syk}.

Discussion

Combining the sensitive technique of in vitro kinase assay with the resolution of two-dimensional gel electrophoresis we here identified an as yet unknown phosphoprotein, pp29/30, that coprecipitates with the signaling receptors CD2,

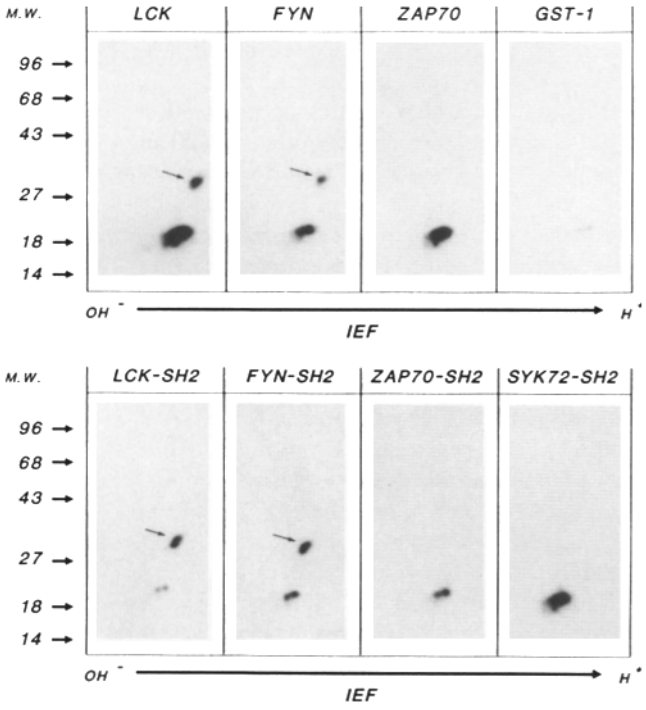

Figure 7. Interaction of in vitro-labeled pp29/30 with the SH2 domains of $p56^{lck}$ and $p59^{fyn}$. In vitro-labeled phosphoproteins were released from a primary CD2 immunoprecipitate obtained from Brij 58 lysates of HPB-ALL cells as described in Fig. 1. The released phosphoproteins were resubjected to secondary immunoprecipitation employing the agarose bead-coupled GST fusion proteins indicated on top of the individual panels. Secondary immunoprecipitates were then analyzed by means of two-dimensional gel electrophoresis. The position of pp29/30 is indicated by an *arrow*.

CD3, CD4, CD5, and CD8 under mild detergent conditions in human T lymphocytes (Figs. 1 and 3).

Analysis of in vitro-labeled pp29/30 under nonreducing vs. reducing conditions (Fig. 2) revealed that the apparent molecular mass of the nonreduced molecule is ~58–60 kD. This indicates that pp29/30 represents the reduced form of a 58–60-kD disulfide-linked dimer. Our observation that pp29/30 is not detectable after external labeling of T lymphocytes suggests that the protein is mainly localized intracellularly.

Importantly, in vitro phosphorylation of pp29/30 was strongly impaired in immunoprecipitates obtained from TCR/CD3-modulated T lymphocytes (Fig. 4). Together with the finding that the amounts of biotinylated pp29/30 protein coprecipitated by CD2 (and CD4) mAb were also strongly reduced in immunoprecipitates prepared from TCR/CD3-modulated cells (Fig. 5), these data suggest that pp29/30 represents a novel protein that preferentially associates and comodulates with the TCR/CD3 complex. Its presence in CD2, CD4, CD5, and CD8 immunoprecipitates likely results from coprecipitation of components of the TCR/CD3 complex with the CD2, CD4, CD5, and CD8 receptors that has previously been demonstrated by a number of different laboratories (24–29). Our recent analysis of a large panel of mAbs directed at human lymphocyte surface molecules demonstrated that this is a rather special feature of the above receptor complexes (29) and does not apply to the majority of additional receptor molecules expressed by human T cells.

Although pp29/30 preferentially associates and comodulates with TCR/CD3, the protein is clearly distinct from the known components of the CD3 molecular complex, CD3-γ, CD3-δ, CD3-ϵ, and CD3-ω as well as ζ chains. Thus, pp29/30 is not reprecipitated by mAbs or antisera directed at either CD3-γ, CD3-ϵ, or ζ chains (Fig. 1, *right*, panels *A* and *B* and data not shown). Moreover, pp29/30 is distinct from the CD3-γ and CD3-δ glycoproteins because it neither possesses *N*- nor *O*-linked carbohydrates (not shown). Finally, the identification of pp29/30 as a dimer (Fig. 2) rules out the possibility that pp29/30 is identical with the recently described 29-kD CD3-ω chain which represents a single chain polypeptide (38).

A number of dimeric molecules with subunits of ~30 kD have been described in human, mouse, or rat T lymphocytes. These include the CD8 glycoproteins, the CD69 molecule, the 4-1BB antigen, the NKR-P1 glycoprotein as well as the most recently described murine p32 protein (39–42). Although not shown here p29/30 is different from these molecules for the following reasons: (*a*) pp29/30 is expressed in CD8 depleted resting human T lymphocytes and, moreover, is not reprecipitated by CD8 mAb in CD8-positive cells; (*b*) several individual CD69 mAbs failed to reprecipitate pp29/30. Moreover, PMA-stimulation of resting human T lymphocytes, which induces modulation of the TCR/CD3 molecular complex, leads to comodulation and concomitant downregulation of pp29/30, whereas CD69 expression is upregulated (39). (*c*) The same applies for the recently described 4-BB1 antigen, which also represents a PMA-inducible mouse T cell antigen (40).

We cannot yet formally exclude the possibility that pp29/30 is homologous to the NKR-P1 or the murine p32 glycoprotein (41, 42). However, these possibilities seem unlikely since NKR-P1 is almost exclusively expressed in rat and mouse NK cells (42) and expression of p32 seems to be limited to murine pre-T lymphocytes (41). In contrast, pp29/30 is strongly expressed in mature human T lymphocytes. Finally, both NKR-P1 and p32 represent *N*-glycosylated proteins (41, 42), whereas pp29/30 represents a nonglycosylated polypeptide.

After in vitro tyrosine phosphorylation, pp29/30 is specifically reprecipitated by the SH2 domains of the src family members $p56^{lck}$ and $p59^{fyn}$ but not by SH2 domains of the src unrelated protein tyrosine kinases ZAP70 or, alternatively, $p72^{syk}$ (Fig. 7). Since pp29/30 is not found to be associated with either $p56^{lck}$ or, alternatively, $p59^{fyn}$ in primary immunoprecipitates obtained from nonstimulated T lymphocytes, this indicates that pp29/30 is most likely not constitutively phosphorylated on tyrosine residues in resting T lymphocytes in vivo and, therefore, does not bind to the SH2 domains of the kinases.

Although we have so far been unable to show in vivo tyrosine phosphorylation and interaction of pp29/30 with the SH2 domains of $p56^{lck}$ and/or $p59^{fyn}$ after CD2- or, alter-

natively, CD3-mediated T cell activation, preliminary data obtained in our laboratory indicate that tyrosine-phosphorylated pp29/30 coimmunoprecipitates with the SH2 domains of $p56^{lck}$ in pervanadate-treated T lymphocytes (43). Inasmuch as pervanadate is believed to mimic proximal T cell activation events (e.g., tyrosine kinase activation and tyrosine phosphatase inhibition, [43]) this indicates that under particular conditions pp29/30 undergoes tyrosine phosphorylation in vivo.

The phosphopeptide sequences of signaling proteins that preferentially bind to the SH2 domains of the src protein tyrosine kinases have recently been elucidated (36). Phosphopeptide *YEEI (*Y indicates a phosphorylated tyrosine residue) represents the motif that shows highest affinity for SH2 domains. Lower affinities were found for substitutions at *Y + 1 = D, T, Q, *Y + 2 = N, Y, D, Q, and *Y + 3 = M, L, V. The reprecipitation of pp29/30 with the SH2 domains of $p56^{lck}$ and $p59^{fyn}$ indicates that the protein sequence of p29/30 likely contains such motifs.

The biochemical properties of pp29/30 show striking similarities to ζ chains. First, both pp29/30 and ζ represent nonglycosylated dimeric molecules that preferentially associate and comodulate with the TCR/CD3 complex. Second, expression of pp29/30 and ζ is not restricted to TCR/CD3-positive lymphocytes since they are also expressed in NK cells. Third, in their tyrosine phosphorylated state pp29/30 and ζ specifically interact with the SH2 domains of protein tyrosine kinases ($p56^{lck}$, $p59^{fyn}$, ZAP70) known to play a key role in human T cell activation (9, 10). Based on these findings it is tempting to speculate that pp29/30 represents a novel receptor–associated protein that serves a signaling function in human T lymphocytes and NK cells.

We would like to thank Drs. A. Veillette, D. Cantrell, S. Courtneidge, and E. Reinherz for kindly providing antibodies. We gratefully thank Dr. R. Wallich for critically reading the manuscript. We also thank Ms. Jung, Ms. Brahimi, and Ms. Engelhardt for excellent photographic assistance.

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Received for publication 21 December 1993 and in revised form 11 April 1994.

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