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

By Burkhart Schraven,* Sheldon Ratnofsky,† Yvette Gaumont,‡ Heike Lindegger,* Henning Kirchgessner,* Eddy Bruyns,* Ulrich Moebius,* and Stefan C. Meuer*

From *the Department of Applied Immunology, Deutsches Krebsforschungszentrum, D-69120 Heidelberg, Germany; and the †Badische Anilin und Soda Fabrik (BASF) Bioresearch Corporation, Worcester, Massachusetts 01605

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 (SH2) of the protein tyrosine kinases p56lk and p59fn 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-α, -β, -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 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 (p56lk and p59fn) (9, 10) as well as the recently cloned src-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 p59fn (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 p56lk and p59fn 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-
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 x 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 2A2D2A2 (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-c chain 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 reactive with mAbs directed at the TCR, the CD3-ε molecule as well as CD4 and CD8, respectively.

mAbs, Antisera, and Glutathione S-transferase (GST) 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. The antisera used for reprecipitation experiments were: anti-p56 


t(kindly provided by Dr. A. Veillette, McGill Cancer Center, McGill University, Montreal, Canada [28, 34]), anti-p56 



t(donated by Dr. Doreen Cantrell, Imperial Cancer Research Fund, London, UK [28, 35]), and anti-pp52 (directed at the first 10 NH2-terminal amino acids of the human pp52 molecule [34]. GST fusion proteins of complete or, alternatively, isolated src homology type 2 (SH2) domains of p56 



SAP70 (NH2 + COOH-terminal), and p75 



SH2, src homology type 2.

Abbreviations used in this paper: GST, glutathione S-transferase; IEF, isoelectric focusing; LPC, l-α-lysophosphatidylcholine; Pl., 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 repurification 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-γ; (B) anti-CD3-ε; (C) anti-pp32; (D) anti-CD5; (E) anti-p56lck; and (F) anti-p59fom. 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\textsubscript{ck} and p59\textsubscript{frD} (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\textsubscript{frD} 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\textsubscript{frD} (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\textsubscript{ck} and, moreover, its isoelectric point (pI) almost exactly matched the pI of p56\textsubscript{ck} (5.3), we reasoned that pp29/30 comigrates with in vitro-labeled p56\textsubscript{ck} under nonreducing conditions. To prove this point, spots 1 and 2 shown in Fig. 2, A and B, were excised and subjected 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 post-lytical artifact (not shown).

Previous data had shown that the protein tyrosine kinases p56\textsubscript{ck} and p59\textsubscript{frD}, 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).
These data indicate that pp29/30 associates with 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...
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 ε 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 ε, 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 125I- 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 non-reducing/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 ε 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-32P]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-32P]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-32P]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 ε 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 p56lck 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-p56lck and anti-pp32 rabbit antisera (not shown). Collectively, the data shown in Fig. 5 strongly suggest that pp29/30 preferentially associates and copurifies 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 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 \( \zeta \) 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\( ^{ck} \) and p59\( ^{fyn} \). As demonstrated in Fig. 1, panel 2 boxes E and F, anti-p56\( ^{ck} \) 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\( ^{ck} \) 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\( ^{ck} \) 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\( ^{ck} \), p59\( ^{fyn} \), and ZAP70 coupled to agarose beads.

or, alternatively, GST fusion proteins of isolated SH2 domains of p56\( ^{ck} \), p59\( ^{fyn} \), ZAP70, and p72\( ^{yk} \).

Fig. 7 demonstrates that in vitro-labeled pp29/30 and \( \zeta \) chains were specifically reprecipitated by either intact GST-p56\( ^{ck} \) and GST-p59\( ^{fyn} \) fusion proteins or, alternatively, isolated GST-SH2 domains of the p56\( ^{ck} \) and p59\( ^{fyn} \) protein tyrosine kinases. In marked contrast, intact GST-ZAP70 and GST-SH2-ZAP70 fusion proteins selectively reprecipitated \( \zeta \) chains but not pp29/30. The same was true for the GST-SH2-p72\( ^{yk} \) 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\( ^{ck} \) and p59\( ^{fyn} \) but not of ZAP70 and p72\( ^{yk} \).

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,
plex with the CD2, CD4, CD5, and CD8 receptors that comodulates with the TCR/CD3 complex. Its presence in coprecipitation of components of the TCR/CD3 complex has previously been demonstrated by a number of different laboratories (24-29). Our recent analysis of a large panel of comodulates on a TCR/CD3-modulated human T cell line suggests that 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, pp29/30 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 4-1BB1 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.

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, pp29/30 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 4-1BB1 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 p56k and p59fn but not by SH2 domains of the src unrelated protein tyrosine kinases ZAP70 or, alternatively, p72sk (Fig. 7). Since pp29/30 is not found to be associated with either p56k or, alternatively, p59fn 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 p56k and/or p59fn 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<sup>lk</sup> 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 phospheptide 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<sup>lk</sup> and p59<sup>Ilk</sup> indicates that the protein sequence of p29/30 likely contains such motifs.

The biochemical properties of pp29/30 show striking similarities to the Src chains. First, both pp29/30 and the 29/30 represent nonglycosylated dimeric molecules that preferentially associate and comodulate with the TCR/CD3 complex. Second, expression of pp29/30 and the 29/30 are not restricted to TCR/CD3-positive lymphocytes since they are also expressed in NK cells. Third, in their tyrosine phosphorylated state pp29/30 and the 29/30 specifically interact with the SH2 domains of protein tyrosine kinases (p56<sup>lk</sup>, p59<sup>Ilk</sup>, 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. Brahim, and Ms. Engelhardt for excellent photographic assistance.

Address correspondence to Dr. Burkhard Schraven, DFKZ, FSP4, Angewandte Immunologie (0440), Im Neuenheimer Feld 280, D-69120 Heidelberg, Germany.

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

References

1. Meuer, S.C., O. Acuto, R.E. Hussey, J.C. Hodgson, K.A. Fitzgerald, S.F. Schlossman, and E.L. Reinherz. 1983. Evidence for the T3-associated heterodimer as the T-cell antigen receptor. Nature (Lond.). 303:808.
2. Meuer, S.C., D. Cooper, J.C. Hodgson, R.E. Hussey, K.A. Fitzgerald, S.F. Schlossman, and E.L. Reinherz. 1983. Identification of the receptor for antigen and major histocompatibility complex on human inducer T lymphocytes. Science (Wash. DC). 222:1239.
3. Meuer, S.C., K.A. Fitzgerald, R.E. Hussey, J.C. Hodgson, S.F. Schlossman, and E.L. Reinherz. 1983. Clonotypic structures involved in antigen-specific human T cell function. Relationship to the T3 molecular complex. J. Exp. Med. 157:705.
4. Clevers, H., B. Alarcon, T. Wilemann, and C. Terhorst. 1988. The T3 cell receptor/CD3 complex: a dynamic protein ensemble. Annu. Rev. Immunol. 6:629.
5. Samelson, L.E., J.B. Harford, and R.D. Klausner. 1985. Identification of the components of the murine T cell antigen receptor complex. Cell. 43:223.
6. Baniyash, M., P. Garcia-Morales, J.S. Bonifacino, L.E. Samelson, and R.D. Klausner. 1985. Dissociable linkage of the 29 and 30 chains of the T cell receptor. J. Biol. Chem. 263:9874.
7. Anderson, P., M. Caligiuri, J. Ritz, and S.F. Schlossman. 1989. CD3-negative natural killer cells express the 29 and 30 chains as part of a novel molecular complex. Nature (Lond.). 341:159.
8. June, C., M.C. Fletcher, J.A. Ledbetter, and L.E. Samelson. 1990. Increases in tyrosine phosphorylation are detectable before phospholipase C activation after T cell receptor stimulation. J. Immunol. 144:1591.
9. Strauss, B.D., and A. Weiss. 1992. Genetic evidence for the involvement of the lck tyrosine kinase in signal transduction through the T cell antigen receptor. Cell. 70:585.
10. Stein, P.L., H.-N. Lee, S. Rich, and P. Soriano. 1992. pp59<sup>Ilk</sup> mutant mice display differential signaling in thymocytes and peripheral T cells. Cell. 70:741.
11. Chan, A.C., B.A. Irving, J.D. Fraser, and A. Weiss. 1991. The 29 chain is associated with a tyrosine kinase and upon T-cell antigen receptor stimulation associates with ZAP-70, a 70-kDa tyrosine phosphoprotein. Proc. Natl. Acad. Sci. USA. 88:9166.
12. Chan, A.C., M. Iwashima, C.W. Turck, and A. Weiss. 1992. ZAP-70: a 70 kDa protein tyrosine kinase that associates with the 29 chain. Cell. 71:649.
13. Samelson, L.E., A.F. Phillips, E.T. Luong, and R.D. Klausner. 1990. Association of the fyn protein tyrosine kinase with the T cell antigen receptor. Proc. Natl. Acad. Sci. USA. 87:4358.
14. Iwashima, M., B.A. Irving, N.S.C. van Osers, A.C. Chan, and A. Weiss. 1994. Sequential interactions of the TCR with two distinct cytoplasmic tyrosine kinases. Science (Wash. DC). 263:1136.
15. Wegener, A.-M.K., F. Letourner, A. Hoeveier, T. Brocke, F. Luton, and B. Mallisen. 1992. The T cell receptor/CD3 complex is composed of at least two autonomous transduction modules. Cell. 68:83.
16. Irving, B., and A. Weiss. 1991. The cytoplasmic domain of the T cell receptor 29 chain is sufficient to couple to receptor-associated signal transduction pathways. Cell. 64:891.
17. Romeo, C., M. Amiot, and B. Seed. 1992. Sequence requirements for induction of cytolysis by the T cell antigen/Fc receptor 29 chain. Cell. 68:889.
clonal anergy. *Science (Wash. DC).* 248:1349.

19. June, C.H., J.A. Ledbetter, P.S. Linsley, and C.B. Thompson. 1990. Role of the CD28 receptor in T cell activation. *Immunol. Today.* 11:211.

20. Schwartz, R.H. 1992. Costimulation of T lymphocytes: the role of CD28, CTLA-4, and B7/BBI in interleukin-2 production and immunotherapy. *Cell.* 71:1065.

21. Selvaraj, P.M., L. Plunkett, M. Dustin, M.E. Sanders, S. Shaw, and T.A. Springer. 1987. The T lymphocyte glycoprotein CD2 (LFA-2/T11/E-rosette receptor) binds the cell surface ligand CFA-3. *Nature (Lond.)* 326:400.

22. Dustin, M.L., M.E. Sanders, S. Shaw, and T.A. Springer. 1987. Purified lymphocyte function–associated antigen 3 binds to CD2 and mediates T lymphocyte adhesion. *J. Exp. Med.* 165:677.

23. Van de Velde, H., N. von Hoegen, W. Lou, J.R. Parnes, and K. Thielemans. 1991. The B-cell surface protein CD72/Lyb-2 is the ligand for CD5. *Nature (Lond.)* 351:662.

24. Beyers, A.D., L.L. Spruyt, and A. Williams. 1992. Molecular associations between the T lymphocyte antigen receptor complex and the surface antigens CD2, CD4, or CD8 and CD5. *Proc. Natl. Acad. Sci. USA.* 89:2945.

25. Burgess, K.E., M. Yamamoto, K.V.S. Prasad, and C.E. Rudd. 1992. CD5 acts as a tyrosine kinase substrate within a receptor complex comprising T-cell receptor 6 chain/CD3 and protein tyrosine kinases p56lck and p59fyn. *Proc. Natl. Acad. Sci. USA.* 89:9311.

26. Osman, N., S.C. Ley, and M.J. Crumpton. 1992. Evidence for an association between the T cell receptor/CD3 antigen complex and the CD5 antigen in human T lymphocytes. *Eur. J. Immunol.* 22:2995.

27. Burgess, K.E., A.D. Odyssos, C. Zalvan, B.J. Druker, P. Anderson, S.F. Schlossman, and C.E. Rudd. 1991. Biochemical identification of a direct physical interaction between the CD4:p56lck and T(Leu)/CD3 complexes. *Eur. J. Immunol.* 21:1663.

28. Schraven, B., M. Wild, H. Kirchgessner, B. Siebert, R. Wallich, S. Henning, Y. Samstag, and S.C. Meuer. 1993. Alterations of CD2 association with T cell receptor signaling molecules in “CD2 unresponsive” human T lymphocytes. *Eur. J. Immunol.* 23:119.

29. Schraven, B., H. Kirchgessner, K. Rensch, and S.C. Meuer. 1994. Analysis of the signaling panel of monoclonal antibodies for coprecipitation of tyrosine kinase activity. In *Leucocyte Typing V. White Cell Differentiation Antigens.* Stuart Schlossman, editor. Oxford University Press, Oxford. In press.

30. Howard, F.D., P. Molineux, U. Moebius, D.J. McConkey, B. Yandava, T.E. Gennert, and E.L. Reinherz. 1992. The CD3® cytoplasmic domain mediates CD2-induced T cell activation. *J. Exp. Med.* 176:139.

31. Meuer, S.C., R.E. Hussey, M. Fabbi, D. Fox, O. Acuto, K.A. Fitzgerald, J.C. Hodgdon, J.P. Protentis, S.F. Schlossman, and E.L. Reinherz. 1984. An alternative pathway of T-cell activation: a functional role for the 50 kd TH1 sheep erythrocyte receptor protein. *Cell.* 36:897.

32. Schraven, B., Y. Samstag, P. Altevogt, and S.C. Meuer. 1990. Association of CD2 and CD45 on human T lymphocytes. *Nature (Lond.)* 344:71.

33. Moebius, U., M. Manns, G. Hess, G. Kober, K.-H. Meyer zum Büschenfelde, and S.C. Meuer. 1990. T cell receptor gene rearrangements of T lymphocytes infiltrating the liver in chronic active hepatitis B and primary biliary chirrosis. *Eur. J. Immunol.* 20:889.

34. Schraven, B., H. Kirchgessner, B. Gaber, Y. Samstag, and S. Meuer. 1991. A functional complex is formed in human T lymphocytes between the protein tyrosine phosphatase CD45, the protein tyrosine kinase p56lck and pp32, a possible common sulfate. *Eur. J. Immunol.* 21:2469.

35. Monostori, É., D. Desai, M.H. Brown, D.A. Cantrell, and M.J. Crumpton. 1990. Activation of human T lymphocytes via the CD2 antigen results in tyrosine phosphorylation of T cell antigen receptor 6-chains. *J. Immunol.* 144:1010.

36. Songyang, Z., S. Shoelson, M. Chaudhuri, G. Gish, T. Pawson, W.G. Haser, F. King, T. Roberts, S. Ratnofsky, R.J. Lechleider, et al. 1993. SH2 domains recognize specific phosphopeptide sequences. *Cell.* 72:767.

37. Samstag, Y., A. Bader, and S.C. Meuer. 1991. A serine phosphatase is involved in CD2-mediated activation of human T lymphocytes and natural killer cells. *J. Immunol.* 1991. 147:788.

38. Neisig, A., A. Vangsted, J. Zeuthen, and C. Geisler. 1993. Assembly of the T cell antigen receptor. Participation of the CD3ζ chain. *J. Immunol.* 151:870.

39. Fiebig, H., I. Behn, U. Hommel, H. Kupper, and W. Eichler. 1989. Characterisation of a new activation-dependent antigen (CD69) recognized by the monoclonal antibody A12 (BL48/p26) and equivalent workshop antibodies (A10, A40, A90, A91). In *Leucocyte typing IV. W. Knapp,* editor. Oxford University Press, Oxford. 439 pp.

40. Pollok, K.E., Y.-J. Kim, Z. Zhou, J. Hurtado, K.K. Kim, R.T. Pickard, and B.S. Kwon. 1993. Inducible T cell antigen 4-IBB. *J. Immunol.* 150:771.

41. Groettrup, M., K. Ungewiss, O. Azogul, R. Palacios, M.J. Owen, A.C. Hayday, and H. von Boehmer. 1993. A novel disulfide linked heterodimer on pre-T cells consists of the T cell receptor 6 chain and a 33 kd glycoprotein. *Cell.* 75:283.

42. Giorda, R., W.A. Rudert, C. Varassori, W.H. Chambers, J.C. Hiserot, and M. Trucco. 1990. NKR-P1, a signal transduction molecule on natural killer cells. *Science (Wash. DC).* 249:1298.

43. Secrist, J.P., L.A. Burns, L. Karnitz, G.A. Koretzky, and R.T. Abraham. 1993. Stimulatory effects of the protein tyrosine phosphatase inhibitor pervanadate, on T cell activation events. *J. Biol. Chem.* 268:5886.

906  A Novel Signaling Receptor-associated Phosphoprotein