MOLECULAR AND CELLULAR BIOLOGY, Dec. 1992, p. 5548–5554
0270-7306/92/125548-07$02.00/0

Vol. 12, No. 12

Association of Src-Like Protein Tyrosine Kinases with the CD2 Cell Surface Molecule in Rat T Lymphocytes and Natural Killer Cells

GREGORY M. BELL,[1] JOSEPH B. BOLEN,[2] AND JOHN B. IMBODEN[1]*

Department of Medicine, Veterans Affairs Medical Center, and The University of California, San Francisco, California 94121,[1] and Department of Molecular Biology, Bristol-Meyers Squibb Pharmaceutical Research Institute, Princeton, New Jersey 08543-4000[2]

Received 27 April 1992/Returned for modification 1 June 1992/Accepted 17 September 1992

The cell surface molecule CD2 has a signaling role in the activation of T lymphocytes and natural killer cells. Because perturbation of CD2 leads to the appearance of tyrosine-phosphorylated proteins, we investigated the possibility that CD2 associates with cytoplasmic protein tyrosine kinases. As determined by in vitro kinase assays and phosphoamino acid analysis, protein tyrosine kinase activity coprecipitated with CD2 from rat T lymphocytes, T lymphoblasts, thymocytes, interleukin-2-activated natural killer cells, and RNK-16 cells (a rat natural killer cell line). In each case, both $p56^{lck}$ and $p59^{fyn}$ were identified in the CD2 immunoprecipitate. In the thymus, the association between CD2 and these kinases occurred predominately in a small subset of thymocytes that had the cell surface phenotype of mature T cells, indicating that the association is a regulated event and occurs late in T-cell ontogeny. The finding that CD2 is associated with $p56^{lck}$ and $p59^{fyn}$ in detergent lysates suggests that interactions with these Src-like protein kinases play a critical role in CD2-mediated signal transduction.

CD2, a 55-kDa cell surface glycoprotein, has both adhesion and signaling functions in the activation of T lymphocytes and natural killer (NK) cells (18, 30, 31). Perturbation of CD2 by the combination of its natural ligand (LFA-3) and certain monoclonal antibodies (MAbs) provides a potent stimulus for T-cell activation, triggering proliferative responses comparable to those elicited by either mitogenic lectins or MAbs to the T-cell receptor (TCR)-CD3 complex (18, 31). CD2 also has the capacity to activate NK cells, as evidenced by the ability of CD2 MAbs to stimulate lysis of NK cell-resistant targets (30).

The mechanism by which CD2 couples to signal transduction pathways is uncertain. As with the TCR-CD3 complex, appropriate stimulation of CD2 induces tyrosine phosphorylations and stimulates phosphatidylinositol turnover (3, 17, 20, 23). These CD2-induced signaling responses depend on the cytoplasmic domain of CD2, which is relatively large (117 amino acids) and highly conserved among humans, rats, and mice (10, 16, 26–28, 38). CD2-mediated signaling also requires coexpression of molecules that are restricted in their tissue expression (2, 13, 16). CD2, for example, does not signal when it is expressed in fibroblasts by gene transfer (13, 16). One apparent requirement for CD2-mediated signaling is the coexpression of receptors that contain members of the CD3 ζ-chain family (19). Thus, CD2 cannot signal in T-cell mutants that lack TCR-CD3 but is functional in NK cells, which express ζ in association with CD16, and signals when transfected into mast cells, which express the ζ-related γ chain in association with the Fcε receptor (1, 4, 6, 19, 35). The molecular basis by which ζ-chain expression permits CD2 to signal remains uncertain.

Recent studies of lymphocyte activation have focused on the role played by the Src-like protein tyrosine kinases (PTKs) in coupling cell surface receptors to signaling pathways. $p56^{lck}$, for example, associates with the cytoplasmic domain of CD4 and CD8, and the cross-linking of MAbs to CD4 leads to an increase in the kinase activity of $p56^{lck}$ (22, 33, 34). On the other hand, $p59^{fyn}$ coprecipitates with TCR-CD3 when T cells are solubilized in digitonin (24). Studies of transgenic mice that overexpress either $p59^{fyn}$ or $p59^{fyn}$ mutants support the possibility that this PTK participates in TCR-CD3-mediated signaling (14).

As with CD4, CD8, and the known components of the TCR-CD3 complex, the cytoplasmic domain of CD2 does not have intrinsic PTK activity. The remarkable capacity of CD2 to activate T cells and NK cells, however, raises the possibility that, like TCR-CD3 and the CD4-CD8 coreceptors, CD2 interacts with cytoplasmic PTKs. Herein we demonstrate that CD2 is associated with $p56^{lck}$ and $p59^{fyn}$ in rat T lymphocytes and NK cells. In the thymus, the association of CD2 and PTKs occurs predominately in a small subset of thymocytes that express the cell surface phenotype of mature T cells, indicating that the association is a developmentally regulated event that occurs late in T-cell ontogeny.

MATERIALS AND METHODS

Cells and antibodies. RNK-16 cells that had been adapted for in vitro growth were passaged as described previously (5). Nylon wool nonadherent mononuclear cells (T cells) were isolated from the spleens of Fischer 344 rats as described previously (5). T lymphoblasts were generated by incubating splenic T cells with concanavalin A (Sigma Chemical Co., St. Louis, Mo.) at 1 μg/ml for 72 h. Splenic T cells and T lymphoblasts were approximately 90 and 99% pure, respectively, as assessed by flow cytometric analysis after staining with MAb R73. Interleukin-2 (IL-2)-activated NK cells were obtained from rat spleens by the method of Vujanovic et al. (37). A single-cell suspension of fresh thymocytes was obtained by harvesting thymuses from

* Corresponding author.

8-week-old Fisher 344 rats, passing the thymuses through no. 44 mesh, and washing the thymocytes in complete RPMI medium. CD53-positive thymocytes were isolated by magnetic bead immunoadsorption. First, thymocytes were labelled with anti-rat CD53 (MAb OX-44). The cells were washed with phosphate-buffered saline (PBS) containing 1% bovine serum albumin (BSA), incubated with rabbit anti-mouse immunoglobulin (Ig) antiserum, washed again, and incubated with magnetic beads coated with a goat anti-rabbit Ig antiserum (Dynabeads; Bioproducts for Science, Indianapolis, Ind.). The cells were placed in a magnetic field, thereby removing CD53-positive cells from the suspension. The remaining CD53-depleted cells were analyzed by fluorescence-activated cell sorting. The CD53-positive cells were washed once in PBS-BSA and then lysed. MAbs OX-18 (IgG1; anti-rat major histocompatibility complex class I antigen), OX-54 (IgG1; anti-rat CD2), OX-55 (IgG1; anti-rat CD2), OX-1 (IgG1; anti-rat CD45), OX-44 (IgG1; anti-rat CD53), and W3/25 (IgG1; anti-rat CD4) were obtained from Bioproducts for Science, Inc. MAbs OX-34 (IgG2a; anti-rat CD2), OX-8 (IgG1; anti-rat CD8 α chain), and R73 (IgG1; anti-rat TCR) were kindly provided by A. Williams and T. Hunig. Rabbit antiserum against mouse Ig was from Cappel Laboratories (Malvern, Pa.). Antibodies against *src* PTKs were obtained from rabbits immunized with synthetic peptides corresponding to amino acid sequences in the unique domain of the individual *src* family members as described previously (9).

Antiphosphotyrosine immunoblotting. Fresh T cells were suspended at 10^7 cells per ml in complete medium (RPMI, 10% fetal calf serum, L-glutamine [0.3 mg/ml], penicillin K [100 U/ml], streptomycin sulfate [0.1 mg/ml]) at 37°C, stimulated with MAb at 1 μg/10^6 cells for the indicated periods, immediately pelleted, and then resuspended for 30 min on ice in lysis buffer containing 1% Nonidet P-40 (NP-40), 150 mM NaCl, 50 mM HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid) (pH 7.4), 0.1 mM Na vanadate, 20 mM EDTA, and 1 mM phenylmethylsulfonyl fluoride. Postnuclear supernatant (100 μg) was mixed with 6× Laemmli reducing sample buffer, boiled for 2 min, resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), and transferred to Immobilon-P (Millipore Corp., Bedford, Mass.). The blot was blocked with 1% gelatin in Tris-buffered saline (TBS; 10 mM Tris [pH 8.0], 150 mM NaCl) and blotted for 2 h at 25°C at 1 μg/ml with antiphosphotyrosine MAb 4G10 (IgG2b; Upstate Biologicals, Inc., Lake Placid, N.Y.) in TBS with 0.05% Tween (TBST). The blot was washed three times in TBST and incubated for 1 h at room temperature with a 1:1,000 dilution of an alkaline phosphatase-conjugated rat anti-mouse MAb (PharMingen, San Diego, Calif.) that recognizes murine antibodies of the IgG2b subclass. The blot was washed three times in TBST and then developed with nitroblue tetrazolium and 5-bromo-4-chloro-3-indolylphosphate toluidinium (GIBCO-BRL, Frederick, Md.) according to the manufacturer's instructions.

Immunoprecipitation, immune complex kinase assays, and surface labelling. Cells were washed twice in PBS and solubilized in lysis buffer (1% NP-40, 150 mM NaCl, 50 mM HEPES [pH 7.4], 0.1 mM Na vanadate, 20 mM EDTA, 1 mM phenylmethylsulfonyl fluoride). The protein concentration of the postnuclear supernatants was adjusted to 1 mg/ml. The lysates were precleared for 45 min with preformed complexes of Pansorbin (Calbiochem, La Jolla, Calif.) and rabbit anti-mouse Ig antiserum. Immunoprecipitations from 500 to 1,000 μg of lysate with specific MAb adsorbed to the Pansorbin rabbit anti-mouse IgG complex were performed at 4°C for 1.5 h. Immune complexes were then washed three times in lysis buffer and resuspended in a kinase buffer (20 mM HEPES [pH 7.5], 100 mM NaCl, 5 mM $MnCl_2$, 5 mM $MgCl_2$) containing 20 μCi of [γ-^{32}P]ATP (>5,000 Ci/mmol; Amersham Corp., Arlington Heights, Ill.) at 4°C for 10 min. The immune complexes were washed once more with lysis buffer, eluted with 1× Laemmli sample buffer, and analyzed under reducing conditions by SDS-PAGE on 8 or 10% polyacrylamide gels. Gels were either transferred to Immobilon-P or fixed in 10% isopropanol–10% acetic acid for 1 h. To reduce nonspecific background serine-threonine phosphorylations, some gels were treated with 1 M KOH for 1 h at 55°C. This treatment was not required to visualize $p56^{lck}$ or $p59^{fyn}$. Standard molecular weight markers do not tolerate the KOH treatment; therefore, prestained molecular weight markers (Bio-Rad Laboratories, Richmond, Calif.) were used. Reprecipitation studies were performed in one of two ways. After the kinase reaction, immune complexes were resuspended in 1% deoxycholate–10 mM Tris (pH 8.0)–20 mM NaCl at 37°C for 30 min and pelleted, and reprecipitations were done on the supernatant. Alternatively, the immune complex was solubilized in 100 μl of 1% SDS–20 mM HEPES (pH 7.4)–150 mM NaCl–0.1 μM Na orthovanadate, boiled, and pelleted. The supernatant was diluted with lysis buffer to a final concentration of 0.1% SDS and 1.0% NP-40. Antiserum addition to the lysate for 45 min was followed by the addition of Pansorbin for an additional 45 min. The complexes were washed twice, eluted in 1× Laemmli sample buffer, and analyzed under reducing conditions by SDS-PAGE on 8% polyacrylamide gels. Surface labelling of both T cells and T lymphoblasts by the lactoperoxidase method was performed as described previously (5).

Phosphoamino acid analysis. ^{32}P-labeled proteins were eluted from Immobilon-P and then hydrolyzed in 6 N HCl for 1 h at 110°C as described previously (7). Phosphoamino acids were resolved on thin-layer cellulose plates by two-dimensional chromatography with a Hunter thin-layer electrophoresis system (HTLE-7000; CBS Scientific, Del Mar, Calif.).

Peptide mapping. Phosphoproteins were identified by autoradiography, carefully excised from the dried gel, rehydrated, and then treated with 1 μg of V8 protease (Calbiochem) per sample as described previously (12). Samples were analyzed on 15% polyacrylamide gels by SDS-PAGE and then autoradiographed.

RESULTS

The addition of agonist pairs of MAbs to human CD2 leads to the tyrosine phosphorylation of a variety of proteins in human T cells, and this response is comparable to the tyrosine phosphorylations that are elicited by stimulation of the TCR (20, 23). To confirm that a similar response is triggered in rat T cells, we stimulated nylon wool nonadherent rat spleen cells (>90% T cells by flow cytometric analysis) with the combination of OX-54 and OX-55. These MAbs recognize rat CD2 and together activate rat T cells and thymocytes to proliferate (11). As shown in Fig. 1, immunoblot analysis with an antiphosphotyrosine MAb detected a number of tyrosine-phosphorylated molecules that appeared as a result of stimulating cells with OX-54 plus OX-55. We have previously shown that these MAbs induce protein tyrosine phosphorylations in the rat NK cell line RNK-16 (5).

To address the possibility that CD2 associates with a PTK,

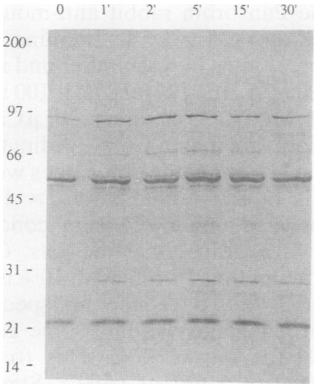

FIG. 1. Stimulation of rat splenic T lymphocytes by the combination of the CD2 MAbs OX-54 and OX-55 induces tyrosine phosphorylations. T lymphocytes were isolated from rat spleen as described in Materials and Methods and were stimulated with MAb OX-54 plus OX-55 at 1 μg/10^6 cells at 37°C for 1 to 30 min. At the end of the incubation period, the cells were immediately pelleted and lysed. Protein (100 μg) from each lysate was resolved by SDS-PAGE on a 5 to 15% gradient polyacrylamide gel, transferred to Immobilon-P, and analyzed by immunoblotting with the antiphosphotyrosine MAb, 4G10. Numbers on left show sizes in kilodaltons.

we performed in vitro kinase assays on CD2 immunoprecipitates from rat T cells, T lymphoblasts, thymocytes, IL-2-activated NK cells, and RNK-16 cells. In each case, protein kinase activity precipitated with CD2 (Fig. 2). The phosphoproteins shown in Fig. 2 were resistant to treatment with base, suggesting that they represented tyrosine phosphorylations. This conclusion was confirmed by phosphoamino acid analysis of bands excised from the CD2 kinase reaction (Fig. 3). The coprecipitation of PTK activity was selective for CD2. With the exception of CD4 and CD8, which are known to associate with $p56^{lck}$, we did not observe kinase activity in the immunoprecipitates of other cell surface molecules (Fig. 2 and data not shown). The absence of PTK activity in the TCR immunoprecipitates was probably a result of the relatively stringent detergent conditions (1% NP-40) used to solubilize cells in these experiments; coprecipitation of $p59^{fyn}$ with the TCR-CD3 complex requires the use of digitonin (24).

While a number of tyrosine-phosphorylated proteins were detected in the CD2 immune complex kinase reaction, the bands in the 56- to 60-kDa range were particularly prominent and were observed in each of the cell systems studied (Fig.

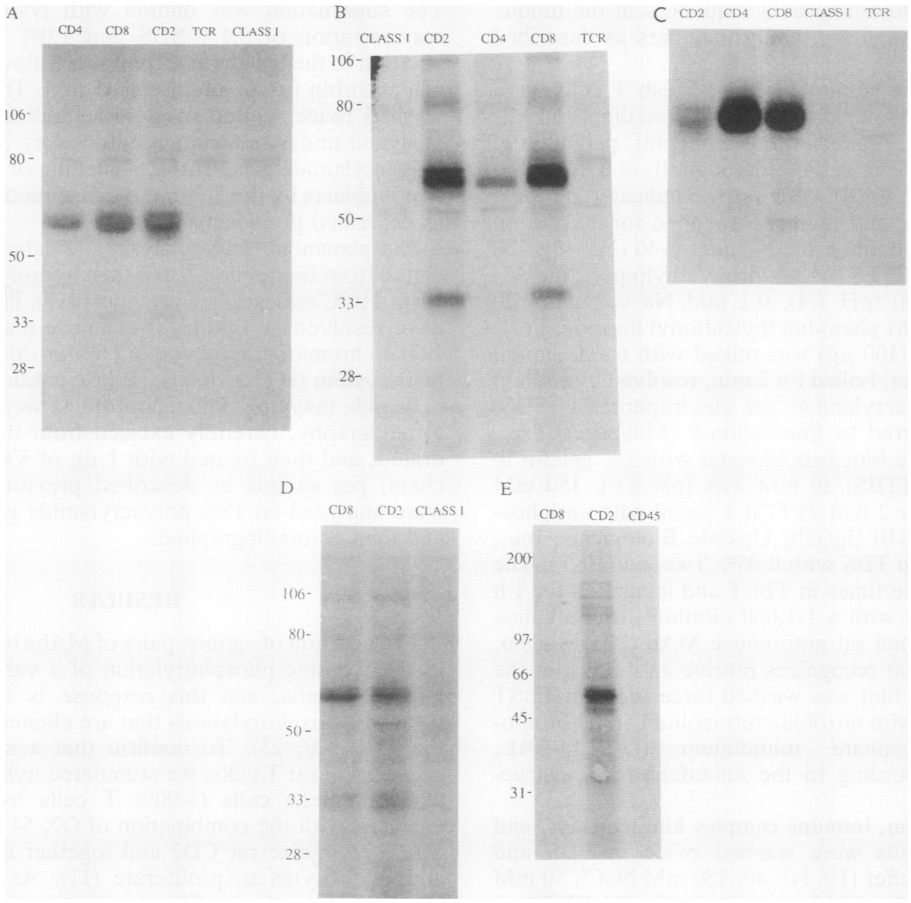

FIG. 2. In vitro protein kinase assays with immunoprecipitates from rat T cells (A), T lymphoblasts (B), thymocytes (C), IL-2-activated NK cells (D), and RNK-16 cells (E). Cells were lysed in a buffer containing 1% NP-40, and immunoprecipitations were performed with MAb OX-34 (anti-CD2) or MAbs recognizing the indicated cell surface molecules. The immune complexes were washed and incubated in a kinase reaction buffer containing 20 μCi of [γ-^{32}P]ATP. Phosphoproteins were separated by SDS-PAGE on either an 8% (A to D) or 10% (E) polyacrylamide gel, fixed (A to D) or transferred to Immobilon-P (E), treated with 1 M KOH, and evaluated by autoradiography. The low level of CD8 expression on RNK-16 may explain the absence of PTK activity in the CD8 immunoprecipitates from this cell line. Numbers on left show sizes in kilodaltons.

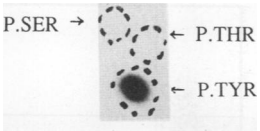

FIG. 3. Phosphoamino acid analysis of phosphoproteins generated by in vitro kinase assays with CD2 immune complexes. The 56- to 60-kDa ^{32}P-labelled bands from the experiment shown in Fig. 2E were excised, eluted, and subjected to acid hydrolysis as described previously (7). The phosphoamino acids were resolved by two-dimensional thin-layer electrophoresis and revealed by autoradiography. The positions of unlabelled standards are indicated. P.SER, phosphoserine; P.THR, phosphothreonine; P.TYR, phosphotyrosine.

2). The lower band comigrated with the p56^{lck} that coprecipitated with either CD4 or CD8. An additional band lay above p56^{lck} and had an approximate M_r of 59,000. To determine whether these bands were PTKs of the Src family, we performed kinase reactions with CD2 immune complexes and then dissociated the complexes with either 1% deoxycholate or 1% SDS and diluted them in NP-40. We subjected the resolubilized phosphoproteins to immunoprecipitation using antisera directed against Src-like PTKs. In each case, antisera to p56^{lck} and p59^{fyn} (but not to other PTKs) immunoprecipitated proteins of the appropriate M_r from the CD2 immune complex (Fig. 4).

Additional evidence for the presence of p56^{lck} and p59^{fyn} in the CD2 immunoprecipitates came from an analysis of the peptide maps of the 56- to 60-kDa phosphoproteins generated by the in vitro kinase reaction, using CD2 immunoprecipitates from RNK-16 cells. The map of the 59-kDa phosphoprotein from the CD2 kinase reaction was identical to that of directly immunoprecipitated p59^{fyn} (Fig. 5). Similarly, the peptide map of the 56-kDa CD2-associated band was identical to that of directly immunoprecipitated p56^{lck}

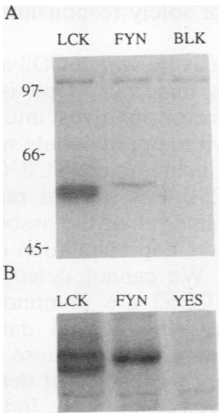

FIG. 4. Identification of p56^{lck} and p59^{fyn} in CD2 immune complexes. In vitro kinase assays with CD2 immune complexes from RNK-16 cells (A) or T cells (B) were performed as described in Materials and Methods, eluted in buffers containing either 1% deoxycholate (A) or 1% SDS followed by dilution in a buffer with NP-40 (B), and then subjected to immunoprecipitation with antiserum directed against p56^{lck}, p59^{fyn}, p56^{blk} (expressed by RNK-16), or p62^{yes} (expressed by T cells). In both cell systems, p56^{lck} and p59^{fyn} were specifically immunoprecipitated from the resolubilized CD2 immune complexes; identical results were obtained with thymocytes, T lymphoblasts, and IL-2-activated NK cells (data not shown). Numbers on left show sizes in kilodaltons.

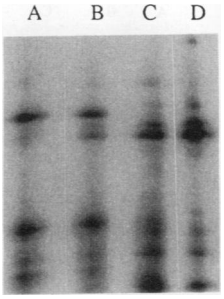

FIG. 5. Peptide maps comparing autophosphorylated p56^{lck} and p59^{fyn} with phosphoproteins from in vitro kinase assays with CD2 immune complexes. In vitro kinase assays were performed with CD2, p56^{lck}, and p59^{fyn} immunoprecipitated from RNK-16 cells. The reaction products were resolved by SDS-PAGE and identified by autoradiography. The bands corresponding to the 56-kDa (A) and 59-kDa (C) phosphoproteins from the CD2 reaction, to autophosphorylated p56^{lck} (B), and to autophosphorylated p59^{fyn} (D) were carefully excised from the gel, rehydrated, and then digested with 1 μg of V8 protease. The peptides were resolved by electrophoresis in 15% polyacrylamide gels and revealed by autoradiography. The peptide map of the 56-kDa band in the CD2 immunoprecipitate (A) matches that of p56^{lck} (B), and the peptide map of the 59-kDa band (C) matches that of p59^{fyn} (D). These results were confirmed by using CD2 immunoprecipitations from T cells, T lymphoblasts, and IL-2-activated NK cells (data not shown).

(Fig. 5). Immunoblot analysis of the CD2 immunoprecipitates with an antiserum to p56^{lck} confirmed this result and indicated that approximately 2% of cellular p56^{lck} coprecipitated with CD2 from RNK-16 (data not shown).

The well-characterized associations between p56^{lck} and either CD4 or CD8 require free sulfhydryl groups of the Cys-X-Cys motif found in these molecules and can be disrupted by treating lysates with iodoacetamide (Fig. 6) (29, 32). The Cys-X-Cys motif is not present in CD2. Treatment of lysates with iodoacetamide did not prevent the coprecipitation of p56^{lck} with CD2, indicating that the association between CD2 and p56^{lck} is independent of free sulfhydryl groups (Fig. 6). This result also argues strongly against the possibility that the p56^{lck} in the CD2 immune complexes reflects coprecipitation of either CD4 or CD8. Indeed, when we performed immunoprecipitations of ^{125}I-surface-labelled T cells and T lymphoblasts under the conditions used for the kinase reaction, the CD2 MAbs immunoprecipitated only a diffuse band with an apparent M_r of 50,000 to 60,000, which, after digestion with endo-β-*N*-acetylglucosaminidase F

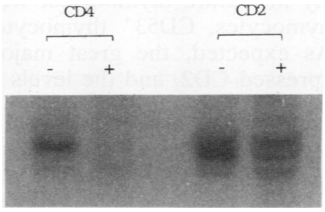

FIG. 6. Treatment with iodoacetamide does not prevent the coprecipitation of PTKs with CD2. CD2 and CD4 were independently immunoprecipitated from fresh T-cell lysates in the presence (+) or absence (−) of 20 mM iodoacetamide. In vitro kinase assays were performed, and the products were analyzed as described in the legend to Fig. 2. The presence of iodoacetamide did not affect the coprecipitation of p56^{lck} with CD2 but, as previously reported (29, 32), led to the dissociation of p56^{lck} from CD4.

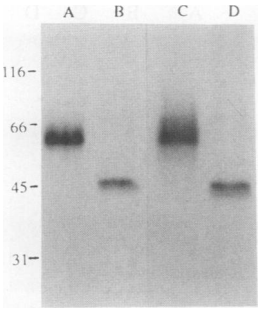

FIG. 7. Immunoprecipitation of CD2 from ^{125}I-surface-labelled T cells and T lymphoblasts. Fresh T cells (A and B) and T lymphoblasts (C and D) were surface labelled with ^{125}I and solubilized in lysis buffer containing 1% NP-40 as described in Materials and Methods. Immunoprecipitates with MAb OX-34 were resolved by SDS-PAGE on 10% gels and analyzed by autoradiography. Lanes B and D were treated with endo F before SDS-PAGE. Numbers on left show sizes in kilodaltons.

(endo F), migrated as a single band of 42 kDa (the M_r of deglycosylated CD2) (Fig. 7). Thus, the only detectable radiolabelled cell-surface molecule in the CD2 immunoprecipitates was CD2.

The level of kinase activity that coprecipitated with CD2 from thymocytes appears to be substantially less than that which coprecipitated with CD2 from mature T cells (Fig. 2). This finding suggests that the association between CD2 and these PTKs might be restricted to a subset of thymocytes and, in particular, might occur late in thymocyte ontogeny. During thymocyte maturation, cells progress from a stage in which they do not express the TCR, through a phase of intermediate TCR expression, and then to a stage of high-level TCR expression (TCR^{high}) (36). The mature TCR^{high} thymocytes can be further subdivided by the cell-surface molecule CD53. CD53, which is expressed by all mature T cells, is found on 5 to 10% of rat thymocytes, primarily those that express high levels of the TCR (Fig. 8) (5, 21). Patterson et al. (21) established that removal of the CD53$^+$ subset greatly reduces the proliferative response of rat thymocytes to alloantigen and mitogenic lectins, suggesting that CD53 identifies most functionally mature rat thymocytes.

CD2 and p56^{lck} are expressed relatively early in T-cell ontogeny and are coexpressed by the great majority of thymocytes. The level of p59^{fyn}, on the other hand, increases 10-fold as T cells progress into the TCR^{high} stage. To determine whether the association between CD2 and PTKs occurs primarily in mature thymocytes, we compared unfractionated thymocytes, CD53$^+$ thymocytes, and CD53$^-$ thymocytes. As expected, the great majority of CD53$^-$ thymocytes expressed CD2, and the levels of p56^{lck} in the CD53$^+$ and CD53$^-$ thymocytes were comparable (Fig. 8B and C, and 9A). When we compared kinase reactions generated from CD2 immunoprecipitates, however, there were dramatic differences between the thymocyte populations. The CD53$^+$ thymocytes had substantially more PTK activity associated with CD2 than did either the CD53$^-$ cells or the unfractionated thymocytes (Fig. 9B). Comparison of the unfractionated thymocytes and the CD53$^-$ cells, moreover, demonstrates that depletion of CD53$^+$ thymocytes substantially reduced CD2-associated PTK activity (Fig. 9B), suggesting that the CD53$^+$ subset accounts for most of the CD2-associated PTK activity in the thymus.

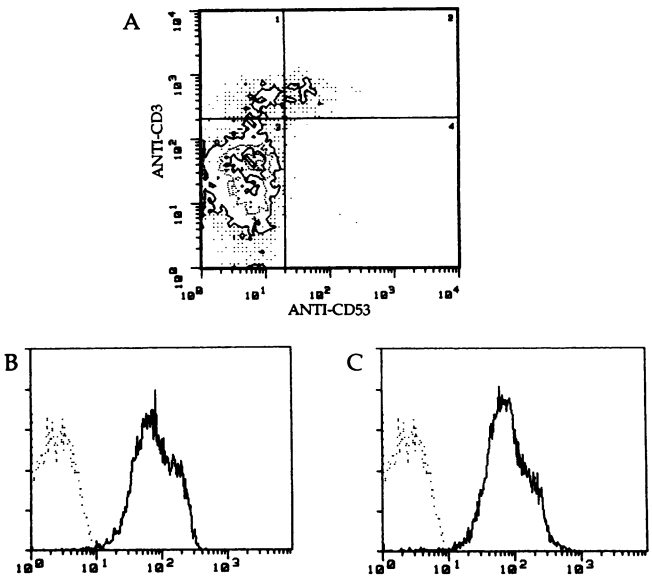

FIG. 8. Cell surface phenotype of rat thymocytes. After being stained with MAb to CD53 and to the CD3 component of the TCR, rat thymocytes were subjected to two-dimensional flow cytometric analysis (A). CD53 is expressed predominately by a subset of TCR^{high} cells. To study the expression of CD2, unfractionated rat thymocytes (B) or thymocytes depleted of CD53$^+$ cells by magnetic bead immunoadsorption (C) were stained with either the CD2 MAb OX-34 (solid line) or a nonbinding isotype-matched control MAb (dotted line).

DISCUSSION

In this report, we demonstrate that PTK activity coprecipitates with CD2 from rat T cells, T lymphoblasts, thymocytes, IL-2-activated NK cells, and RNK-16 cells. In each cell system studied, we identified both p56^{lck} and p59^{fyn} in the CD2 immunoprecipitates, indicating that these Src-like kinases are partly or solely responsible for the CD2-associated PTK activity.

The molecular basis by which CD2 associates with p56^{lck} and p59^{fyn} remains uncertain. One issue is whether this association is direct or involves intermediary molecules. CD2 has been shown to coprecipitate with several other cell surface molecules, including the TCR-CD3 complex, CD45, and CD53 (5, 8, 25). For several reasons, however, we believe that it is unlikely that the association between CD2 and the PTKs reflects coprecipitation of these or other cell surface molecules. We cannot detect PTK activity when TCR, CD45, and CD53 are immunoprecipitated directly from NP-40 lysates (Fig. 2 and data not shown). The associations between CD2 and these other structures are greatly influenced by the choice of detergent and generally are not preserved by NP-40 (5, 8). Indeed, CD2 is the only cell surface molecule that we detect in the CD2 immunoprecipitations. Thus, if another cell surface molecule is responsible for the coprecipitation of PTKs with CD2, that molecule must either label poorly or be present in very low amounts. CD4 and CD8 are potential sources of p56^{lck}, but the resistance of the CD2-p56^{lck} association to iodoacetamide argues strongly against the possibility that the presence of p56^{lck} in our CD2 immunoprecipitates is due to coprecipitation of either CD4 or CD8.

In thymocytes, the association between CD2 and PTKs occurs predominately in a small subset of thymocytes that

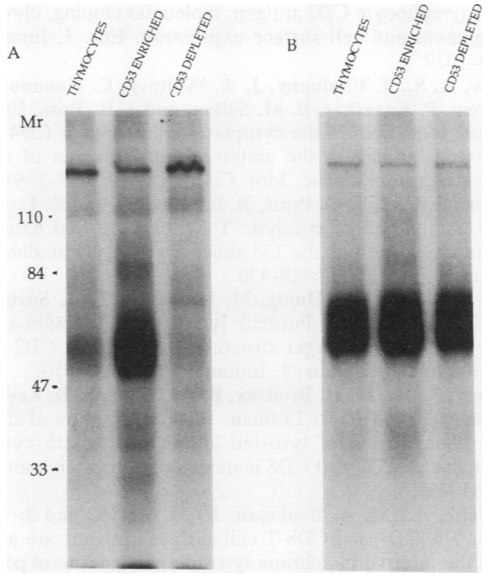

FIG. 9. CD2-associated kinase activity is enriched in the CD53$^+$ subset of thymocytes. NP-40 lysates were prepared from unfractionated rat thymocytes, from CD53$^+$ thymocytes, and from CD53$^-$ thymocytes. In vitro kinase reactions were performed with immunoprecipitates of either CD2 from 500 μg of lysate protein (A) or p56^{lck} from 200 μg of lysate protein (B).

have the cell surface phenotype of mature T cells. p59^{fyn} is expressed only at low levels in immature thymocytes, and its expression increases 10-fold as thymocytes mature into the TCR^{high} stage (14). Thus, the developmentally regulated expression of p59^{fyn} provides a likely explanation for the finding that CD2 associates with p59^{fyn} primarily in mature thymocytes. In contrast, CD2 and p56^{lck} are coexpressed in the majority of thymocytes. The failure of these two molecules to interact in immature thymocytes, therefore, implies that the CD2-p56^{lck} interaction is tightly regulated during T-cell ontogeny. It is not clear whether this involves negative regulation in immature cells or positive regulation in mature cells.

Because p56^{lck} and p59^{fyn} appear to play critical roles in the activation of T lymphocytes, in vivo associations between CD2 and these PTKs are likely to be important for CD2-mediated signaling. Our results demonstrate that CD2 is associated with p56^{lck} and p59^{fyn} in detergent lysates of T cells and NK cells. The observation that this association is a regulated event that occurs late in T-cell development suggests that it is likely to be of importance for the function of mature T cells. The ability of CD2 MAbs to stimulate tyrosine phosphorylations, moreover, indicates that CD2 likely couples to PTKs in vivo (20, 23). Danielian et al. (15) recently demonstrated that stimulation of human T cells by agonist MAbs to CD2 leads to an increase in the kinase activity of p56^{lck}. Thus, as with CD4, perturbation of CD2 activates p56^{lck}. Our results suggest that a physical association between CD2 and p56^{lck} is responsible for this capacity of CD2 to regulate the enzymatic activity of p56^{lck} and raise the possibility that CD2 also regulates p59^{fyn}. CD2, therefore, may represent a mechanism for coupling cell surface events to the activation of Src-like PTKs.

ACKNOWLEDGMENTS

We thank W. Seaman for critical review of the manuscript, Gail Dethloff for technical assistance, and A. Williams and T. Hunig for the kind gifts of MAbs.

This work was supported by Public Health Service grants CA52669 and K11-AI00999 from the National Institutes of Health and by the Department of Veterans Affairs.

REFERENCES

1. **Alcover, A., C. Alberini, O. Acuto, L. K. Clayton, C. Transey, G. C. Spagnoli, P. Moingeon, P. Lopez, and E. L. Reinherz.** 1988. Interdependence of CD3-Ti and CD2 activation pathways in human T lymphocytes. EMBO J. **7**:1973–1977.
2. **Alcover, A., H. C. Chang, P. H. Sayre, R. E. Hussey, and E. L. Reinherz.** 1988. The T11 (CD2) cDNA encodes a transmembrane protein which expresses $T11_1$, $T11_2$ and $T11_3$ epitopes but which does not independently mediate calcium influx: analysis by gene transfer in a baculovirus system. Eur. J. Immunol. **18**:363–367.
3. **Alcover, A., M. J. Weiss, J. F. Daley, and E. L. Reinherz.** 1986. The T11 glycoprotein is functionally linked to a calcium channel in precursor and mature T-lineage cells. Proc. Natl. Acad. Sci. USA **83**:2614–2618.
4. **Arulanandam, A. R. N., S. Koyasu, and E. L. Reinherz.** 1991. T cell receptor-independent CD2 signal transduction in FcR$^+$ cells. J. Exp. Med. **173**:859–868.
5. **Bell, G. M., W. E. Seaman, E. C. Niemi, and J. B. Imboden.** 1992. The OX-44 molecule couples to signaling pathways and is associated with CD2 on rat T lymphoctes and a natural killer cell line. J. Exp. Med. **175**:527–536.
6. **Bockenstedt, L., M. A. Goldsmith, M. Dustin, D. Olive, T. A. Springer, and A. Weiss.** 1988. The CD2 ligand LFA-3 activates T cells but depends on the expression and function of the antigen receptor. J. Immunol. **141**:1904–1911.
7. **Boyle, W. J., P. van der Geer, and T. Hunter.** 1991. Phosphopeptide mapping and phosphoamino acid analysis by two dimensional separation on thin-layer cellulose plate. Methods Enzymol. **201**:110–120.
8. **Brown, M. H., D. D. Cantrell, G. Brattsand, M. J. Crumpton, and M. Gullberg.** 1989. The CD2 antigen associates with the T-cell antigen receptor CD3 antigen complex on the surface of human T lymphocytes. Nature (London) **339**:551–553.
9. **Burkhardt, A. L., M. Brunswick, J. B. Bolen, and J. J. Mond.** 1991. Anti-immunoglobulin stimulation of B lymphocytes activates src-related protein-tyrosine kinases. Proc. Natl. Acad. Sci. USA **88**:7410–7414.
10. **Chang, H., P. Moingeon, P. Lopez, H. Krasnow, C. Stebbins, and E. L. Reinherz.** 1989. Dissection of the human CD2 intracellular domain. J. Exp. Med. **169**:2073–2083.
11. **Clark, S. J., D. A. Law, D. J. Paterson, M. Puklavec, and A. F. Williams.** 1988. Activation of rat T lymphocytes by anti-CD2 monoclonal antibodies. J. Exp. Med. **167**:1861–1872.
12. **Cleveland, D. W.** 1983. Peptide mapping in one dimension by limited proteolysis of sodium dodecyl sulfate-solubilized proteins. Methods Enzymol. **96**:222–229.
13. **Clipstone, N. A., and M. J. Crumpton.** 1988. Stable expression of the cDNA encoding the human T lymphocyte-specific CD2 antigen in murine L cells. Eur. J. Immunol. **18**:1541–1545.
14. **Cooke, M. P., K. M. Abraham, K. A. Forbush, and R. M. Perlmutter.** 1991. Regulation of T cell receptor signaling by a *src* family protein-tyrosine kinase (p59^{fyn}). Cell **65**:281–292.
15. **Danielian, S., R. Fagard, A. Alcover, O. Acuto, and S. Fischer.** 1991. The tyrosine kinase activity of p56^{lck} is increased in human T cells activated via CD2. Eur. J. Immunol. **21**:1967–1970.
16. **He, Q., A. D. Beyers, A. N. Barclay, and A. F. Williams.** 1988. A role in transmembrane signaling for the cytoplasmic domain of the CD2 T lymphocyte surface antigen. Cell **54**:979–984.
17. **June, C. A., J. A. Ledbetter, P. S. Rabinovitch, P. J. Martin, P. G. Beatty, and J. A. Hansen.** 1986. Distinct patterns of transmembrane calcium flux and intracellular calcium mobilization after differentiation antigen cluster 2 (E rosette receptor) or

3 (T3) stimulation of human lymphocytes. J. Clin. Invest. **77**:1224–1232.

18. **Meuer, S. C., R. E. Hussey, M. Fabbi, M. Fox, O. Acuto, K. A. Fitzgerald, J. C. Hodgon, J. P. Protentis, S. F. Schlossman, and E. L. Reinherz.** 1984. An alternative pathway of T-cell activation: a functional role of the 50 kd sheep erythrocyte receptor protein. Cell **36**:897–906.
19. **Moingeon, P., J. L. Lucich, D. J. McConkey, F. Letourneur, B. Malissen, J. Kochan, H.-S. Chang, H.-R. Rodewald, and E. L. Reinherz.** 1992. CD3ζ dependence of the CD2 pathway of activation in T lymphocytes and natural killer cells. Proc. Natl. Acad. Sci. USA **89**:1492–1496.
20. **Monostori, E., D. Desai, M. H. Brown, D. A. Cantrell, and M. J. Crumpton.** 1989. Activation of human T lymphocytes via the CD2 antigen results in tyrosine phosphorylation of T cell antigen receptor ζ-chains. J. Immunol. **144**:1010–1014.
21. **Patterson, D. J., J. R. Green, W. A. Jeffries, M. Puklavec, and A. F. Williams.** 1987. The MRC OX-44 antigen marks a functionally relevant subset among rat thymocytes. J. Exp. Med. **165**:1–13.
22. **Rudd, C. E., J. M. Trevilyan, J. D. Dasgupta, L. L. Wong, and S. F. Schlossman.** 1988. The CD4 receptor is complexed in detergent lysates to a protein-tyrosine kinase (pp58) from human T lymphocytes. Proc. Natl. Acad. Sci. USA **85**:5190–5194.
23. **Samelson, L. E., M. C. Fletcher, J. A. Ledbetter, and C. A. June.** 1990. Activation of tyrosine phosphorylation in human T cells via the CD2 pathway. J. Immunol. **145**:2448–2454.
24. **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**:4358–4362.
25. **Schraven, B., Y. Samtag, P. Altevogt, and S. C. Meuer.** 1990. Association of CD2 and CD45 on human T lymphocytes. Nature (London) **345**:71–74.
26. **Seed, B., and A. Aruffo.** 1987. Molecular cloning of the CD2 antigen, the T-cell erythrocyte receptor, by a rapid immunoselection procedure. Proc. Natl. Acad. Sci. USA **84**:3365–3369.
27. **Sewell, W. A., M. H. Brown, J. Dunne, M. J. Owen, and M. J. Crumpton.** 1986. Molecular cloning of the T-lymphocyte glycoprotein surface CD2 (T11) antigen. Proc. Natl. Acad. Sci. USA **83**:8718–8722.
28. **Sewell, W. A., M. H. Brown, M. J. Owen, P. J. Fink, C. A. Kozak, and M. J. Crumpton.** 1987. The murine homologue of the T lymphocyte CD2 antigen: molecular cloning, chromosome assignment and cell surface expression. Eur. J. Immunol. **17**: 1015–1020.
29. **Shaw, A. S., J. Chalupny, J. A. Whitney, C. Hammond, K. E. Amrein, P. Kavathas, B. M. Sefton, and J. K. Rose.** 1990. Short related sequences in the cytoplasmic domains of CD4 and CD8 mediated binding to the amino-terminal domain of the $p56^{lck}$ tyrosine protein kinase. Mol. Cell. Biol. **10**:1853–1860.
30. **Siliciano, R. F., J. C. Pratt, R. E. Schmidt, and E. L. Reinherz.** 1985. Activation of cytolytic T lymphocyte and natural killer cell function through the T11 sheep erythrocyte binding protein. Nature (London) **317**:428–430.
31. **Tiefenthaler, G., T. Hunig, M. L. Dustin, T. A. Springer, and S. C. Meuer.** 1987. Purified lymphocyte function-associated antigen-3 and T11 target structure are active in CD2-mediated T-cell stimulation. Eur. J. Immunol. **17**:1847–1850.
32. **Turner, J. M., M. H. Brodsky, B. A. Irving, S. D. Levin, R. M. Perlmutter, and D. R. Littman.** 1990. Interaction of the unique N-terminal region of tyrosine kinase $p56^{lck}$ with cytoplasmic deomains of CD4 and CD8 is mediated by cysteine motifs. Cell **60**:755–765.
33. **Veillette, A., M. A. Bookman, E. M. Horak, and J. B. Bolen.** 1988. The CD4 and CD8 T cell surface antigens are associated with the internal membrane tyrosine-protein kinase $p56^{lck}$. Cell **55**:301–308.
34. **Veillette, A., M. A. Bookman, E. M. Horak, L. E. Samelson, and J. B. Bolen.** 1989. Signal transduction through the CD4 receptor involves the activation of the internal membrane tyrosine-protein kinase $p56^{lck}$. Nature (London) **338**:257–259.
35. **Vivier, E., P. M. Morin, C. O'Brien, S. F. Schlossman, and P. Anderson.** 1991. CD2 is functionally linked to the ζ-natural killer receptor complex. Eur. J. Immunol. **21**:1077–1080.
36. **von Boehmer, H.** 1988. The developmental biology of T lymphocytes. Annu. Rev. Immunol. **6**:309–325.
37. **Vujanovic, N. L., R. B. Herberman, A. A. Maghazachi, and J. C. Hiserodt.** 1988. Lymphokine-activated killer cells in rats. III. A simple method for the purification of large granular lymphocytes and their rapid conversion into lymphokine-activated killer cells. J. Exp. Med. **167**:15–29.
38. **Williams, A. F., A. N. Barclay, S. J. Clark, D. J. Paterson, and A. C. Willis.** 1987. Similarities in sequence and cellular expression between rat CD2 and CD4 antigen. J. Exp. Med. **165**:368–380.