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The CD4 receptor is complexed in detergent lysates to a protein-tyrosine kinase (pp58) from human T lymphocytes

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ABSTRACT The CD4 (T4) antigen is a cell-surface glycoprotein that is expressed predominantly on the surface of helper T cells and has been implicated in the regulation of T-cell activation and in the associative recognition of class II antigens of the major histocompatibility complex. In addition, the CD4 antigen appears to serve as a receptor for the human immunodeficiency virus (HIV). An important question has been whether the CD4 receptor is linked to an intracellular mediator that could regulate the activation of the CD4+ subset. In this paper, we provide preliminary evidence that the CD4 receptor is complexed in detergent lysates to a protein-tyrosine kinase (PTK) of 55–60 kDa, which is expressed specifically in T cells. The PTK is the human analogue of the murine pp56Lck (pp56Lck) and has significant homology with c-src, c-yes, and other members of the src family. The identification of the PTK associated with CD4 receptor was made by use of an antisera to a synthetic peptide that was deduced from the DNA sequence of PTK. Two-dimensional nonequilibrium pH gradient gel electrophoresis/NaDodSO4/PAGE revealed the kinase to focus as a heterogeneous collection of spots in the pH range of 4.0–5.0. Furthermore, in vitro phosphorylation revealed the phosphorylation of two additional polypeptides at 40 and 80 kDa, in addition to the autophosphorylation of the PTK at 55–60 kDa. The potential importance of the association between the CD4 receptor and the PTK of T cells is discussed in relation to T-cell activation and HIV infectivity.

The CD4 (T4) antigen is a polypeptide of 55 kDa that is expressed predominantly on the surface of helper T cells and has been implicated in association with the T3 (CD3)-Ti complex in the recognition of class II antigens of the major histocompatibility complex (1, 2). The CD4 antigen also appears to serve as a receptor for the human immunodeficiency virus (HIV) [the acquired immunodeficiency syndrome (AIDS) virus] (3–5). Structurally, the CD4 antigen is a member of the immunoglobulin superfamily (6) and, as such, appears capable of regulating the proliferation of the CD4 subset of T cells (7–11). Monoclonal antibodies to the CD4 antigen were initially reported to inhibit specific T-cell functions by reducing the strength of interaction of the T cell with its target (1, 2, 7). However, it also appears that monoclonal antibodies to the CD4 receptor can inhibit or potentiate the activation of T lymphocytes in a manner independent of the recognition of HLA-D region antigens (8–11). The inhibitory effect of antibodies to CD4 appears to preferentially influence the T4+ 2H4+ subset within the CD4 population, a subset defined by the differential expression of the L-C/T200 (CD45) antigens (12, 13). These observations have implied that the CD4 receptor may regulate the activation of T cells, either singularly or in conjunction with the antigen receptor [T3 (CD3)-Ti] on the surface of T cells.

Protein-tyrosine kinases (PTKs) have been found to play crucial roles in the activation and transformation of mammalian cells. Oncogenic transformation by Rous sarcoma virus was first shown to be mediated by pp60csrc and its ability to phosphorylate was at a tyrosine residue (14, 15). The immune complexes containing pp60csrc have been found to possess phosphotransferase activity capable of transferring the γ-phosphate of ATP to an exogenous substrate (16). Tyrosine phosphorylation is thought to be a relatively rare modification to proteins; however, the past few years have seen a rapidly expanding number of retroviral oncoproteins and cellular receptors within the src family. These include the retroviral oncoproteins such as v-abl, v-erb, v-fes/lps, v-fgr, v-src, and v-yes, as well as the cellular receptors for epidermal growth factor, platelet-derived growth factor, and insulin (17, 18). Surprisingly, little is known regarding the role of PTKs in lymphoid cells. Murine T cells possess relatively high levels of PTK activity, and distinct patterns of tyrosine phosphorylation have been noted in T and B cells (19–25). In particular, a protein kinase termed pp56Lck has been identified in murine T cells (26, 27) and, recently, in human T lymphocytes (28–30). The rather dramatic decrease of pp56Lck within T cells after activation is suggestive of an important role of this enzyme in the activation process (31). One putative substrate of PTKs is the p21 subunit of the Ti-T3 complex (32, 33). However, the identity and role of PTKs in the activation of T cells and their relationship to receptors on the cell surface remain elusive.

In this paper, we provide preliminary evidence that the T4 (CD4) receptor is complexed in detergent lysates from human T lymphocytes to a T-cell-specific PTK of 55–60 kDa with homology to c-src and c-yes. The PTK was identified by use of an antisera against a synthetic peptide that was deduced from the DNA sequence of the kinase. A number of polypeptides of 40 and 80 kDa were detected in an in vitro kinase assay in addition to the autophosphorylated band of PTK at 55–60 kDa. The identity of this PTK and its association with the T4 (CD4) receptor may provide a crucial link in understanding the mechanism by which T cells are activated and can be infected with the AIDS virus (HIV).

MATERIALS AND METHODS

Monoclonal Antibodies and Antisera. The production and characterization of the monoclonal antibodies to the T4 (CD4) antigen, 19D, 1D11T4 (IgG1), and 1D11T4 (IgG1) have been described elsewhere (1). The antibodies W6/32 (a gift from W. F. Bodmer, Imperial Cancer Research Fund, London) against HLA-A, -B, -C antigens has also been described (34). The antibody 1F7 is

Abbreviations: PTK, protein-tyrosine kinase; HIV, human immunodeficiency virus; AIDS, acquired immunodeficiency syndrome; NEPHGE, nonequilibrium pH gradient gel electrophoresis.

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directed against a 120-kDa polypeptide on T cells (unpublished data). Lastly, the anti-PTK antiserum was generated against a synthetic peptide of the sequence Cys-Lys-Glu-Arg-Pro-Glu-Asp-Arg-Pro-Thr-Phe-Asp-Tyr-Leu-Arg-Ser-Val-Leu-Glu-Asp-Phe-Phe-Thr-Ala-Thr-Glu-Gly-Thr-Tyr-Gln-Pro-Gln-Pro, as described (29). The peptide was coupled to bovine serum albumin through the N-terminal cysteine at position 33 (Cys-Pro) by using 3-(2-pyridyl)dithiobiotin carboxyl N-hydroxysuccinimide ester as described (33).

Cells. Peripheral blood cells were isolated by Iso-Hypaque centrifugation and either used as resting cells or stimulated with the mitogen Con A (5 μg/ml; Sigma) for 24–36 hr. These cells, as well as the transformed T-cell lines HPB-MLT, REX, the B-lymphoblastoid cell line Raji, and the myeloid cell line U937, were cultured in RPMI 1640 medium with 10% (vol/vol) fetal calf serum and 1% (wt/vol) penicillin/streptomycin at 37°C in an atmosphere of 5% CO2/95% air.

Immunoprecipitation and Kinase Assay. Cells at 25 × 10⁶ per ml were solubilized in Nonidet NP-40 lysis buffer [1% (vol/vol) or 3% (vol/vol)] Nonidet P-40 in 20 mM Tris HCl buffer, pH 8.0/150 mM NaCl 1 mM phenylmethylsulfonyl fluoride] for 30 min at 4°C as described (36). The cells were initially lysed by 1% (vol/vol) Nonidet P-40; however, in later experiments, 3% (vol/vol) detergent was found to be a more efficient method of extraction of the kinase. The lysate was centrifuged at 12,000 × g and preclarified for 30 min with 50 μl of 10% (wt/vol) Staphylococcus Cowan strain 1. The lysate was then incubated for 1–2 hr at 4°C with either 5 μl of ascites fluid and 50 μl of 10% [wt/vol] protein A-Sepharose or 50 μl of 10% (wt/vol) protein A-Sepharose that had been crosslinked to antibody according to Schneider et al. (37). The immunoprecipitates were then washed three times with Nonidet P-40 lysis buffer prior to incubation with 30 μl of 25 mM Hepes containing 0.1% (vol/vol) Nonidet P-40, 10 μM ATP, and 1–10 μCi of [γ-³²P]ATP (1 Ci = 37 GBq; ICN Chemicals). After an incubation of 15–30 min at 25°C, the reaction mixture was subjected to NaDodSO₄/PAGE and autoradiography (38). For the analysis of reprecipitated antigen, the reaction mixture was supplemented to 1.0–2.0% (wt/vol) NaDodSO₄, boiled for 5 min, and then diluted to 0.1% NaDodSO₄ with a 1:10 or 1:20 dilution of lysis buffer. Two-dimensional nonequilibrium pH gradient gel electrophoresis (NEPHGE) NaDodSO₄/PAGE was conducted with an ampholine of a pH range between 2 and 11 as described (36, 39).

Analysis of Phosphorylated Amino Acids. Proteins were eluted from fixed polyacrylamide gels and precipitated with trichloroacetic acid as described (20, 40). The precipitate was washed in acetone and hydrolyzed in 6 M HCl at 100°C for 2 hr. The individual phosphorylated amino acids were separated by electrophoresis (2500 V, 30 min) at pH 3.5 in pyridine/acetic acid/water (1:1:10; vol/vol). Nonradioactive standards were detected with ninhydrin, while radiolabeled phosphorylated amino acids were observed by autoradiography.

RESULTS AND DISCUSSION

In an attempt to ascertain whether the CD4 antigen was a monoclonal antibody against the CD4 antigen (1987h3D7) was used to precipitate the antigen from unlabeled peripheral blood lymphocytes and a number of transformed cell lines. The immunoprecipitates were then assessed for their ability to phosphorylate polypeptides during an incubation with [γ-³²P]ATP. As shown in Fig. 1A, the anti-CD4 antibody precipitated several polypeptides of 55–60 kDa from both resting (lane 2) and Con A-activated cells (lane 4), which were phosphorylated in the presence of [γ-³²P]ATP. The bands at 55/60 kDa were found to be more intense on a per cell basis from Con A-activated cells than from resting lymphocytes. The anti-CD4 antibody was also found to precipitate a similar pattern of bands within the 55- to 60-kDa range from the human T-lymphoblastoid cell lines REX (lane 6) and HPB-MLT (lane 8). However, in this case, the presence of the polypeptide at 40 kDa was somewhat variable. In addition, an extra band at ≈80 kDa was often observed. Neither the myeloid cell U937 (lane 10) nor the B-lymphoblastoid cell Raji (lane 12), which have been reported to express the CD4 antigen (41), were found to precipitate significant amounts of material labeled by [γ-³²P]ATP. A faint amount of material at 50 kDa was occasionally observed from Raji cells (lane 12); however, the position of this polypeptide was different from that observed from the T lymphocytes. As a negative control, the monoclonal antibody W6/32, which reacts with class I antigens of the major histocompatibility complex at 45 and 12 kDa, was unable to precipitate material capable of being labeled from the various cell lines (lanes 1, 3, 5, 7, and 9). Other monoclonal antibodies against antigens on T cells such as the T3 (CD3) complex, the T4 (CD4) antigen, HLA-D region antigens, and fibronectin receptor/vLA antigens (4B) also failed to precipitate material capable of being labeled under this regime (data not shown).

Analysis of phosphorylated amino acids was then carried out to investigate the nature of amino acid residues that were phosphorylated with [γ-³²P]ATP. Fig. 1B reveals that the bands at 55/60 kDa, which were precipitated by the anti-CD4 antibody, were heavily phosphorylated at tyrosine residues and to a much lesser extent at serine residue(s). The presence of a phosphoserine residue on the CD4 antigen has been detected by in vivo labeling techniques (42) and is consistent with the presence of a serine in the cytoplasmic tail of the antigen (6). However, in contrast, the cytoplasmic tail of CD4 lacks a site for tyrosine phosphorylation as well as a tyrosine kinase domain, as based on its DNA sequence. These data argued that the 55- to 60-kDa band was unlikely to correspond to the CD4 receptor, and, indeed, it was found that the 55- to 60-kDa band was a PTK and/or a substrate for the protein kinase that coprecipitated with the CD4 antigen.

Tyrosine kinases, such as the various receptors for growth factors on mammalian cells, have been found to possess the property of autophosphorylation (17, 18). Indeed, a family of putative kinases in B and T lymphocytes at ≈55 kDa have recently been described (19–25). In particular, a tyrosine kinase (PTK) of 58 kDa has been reported that appears to be expressed specifically in human T cells (28, 29). The CDNA for this T-cell kinase encodes a human analogue of pp55p60 src with extensive homology to the catalytic domain of pp60 src (28). To establish whether this PTK was associated with the

![Fig. 1. Phosphorylation by [γ-³²P]ATP of the CD4 antigen from peripheral blood lymphocytes and various cell lines. (A) Lanes: 1, 3, 5, 7, 9, and 11, anti-W6/32 antibody; 2, 4, 6, 8, 10, and 12, anti-CD4 antibody (1987h3D7). Resting cells (lanes 1 and 2); Con A-activated (112 hr) cells (lanes 3 and 4); REX (lanes 5 and 6); HPB-MLT (lanes 7 and 8); U937 (lanes 9 and 10); and Raji (lanes 11 and 12). (B) Phospho amino acid analysis of the individual 55/60-kDa bands precipitated by anti-CD4 antibody.](http://www.jimmunol.org/DownloadedFrom/...org/)
CD4 antigen, an antisemirum that had been raised against a synthetic peptide (Cys-Lys-Glu-Arg-Pro-Glu-Asp-Arg-Pro-Thr-Phe-Gly-Pro-Leu-Glu-Asp-Phe-Phe-Val-Ala-Thr-Glu-Gly-Gln-Tyr-Gln-Pro-Gln-Pro), corresponding to the C-terminus of the tyrosine kinase, was used in immunoprecipitation analysis and compared with precipitates formed by a monoclonal antibody against the CD4 antigen. Fig. 2A shows a composite of patterns of the polypeptides phosphorylated in immunoprecipitates of the anti-PTK antibody (lane 1) and in immunoprecipitates formed by two monoclonal antibodies to the CD4 antigen, termed 12T4D11 (lane 2) and 197hysD7 (lane 3). In each case, the exposure of immunoprecipitates produced a similar spectrum of phosphorylated bands with molecular sizes of about 40, 55, and 60 kDa. However, differences were noted in the relative intensities of the bands in the pattern. The anti-CD4 immunoprecipitates showed greater amounts of phosphorylation of the 55- and 60-kDa bands relative to the 40-kDa band, while the anti-PTK immunoprecipitates showed greater amounts of phosphorylation of the 40-kDa band than the 55- and 60-kDa bands. The anti-PTK antibody was unable to precipitate material from B cells (data not shown). As an internal control for tyrosine phosphorylation, each of the immunoprecipitates was found to label enolase, which was added as substrate during the labeling procedure (arrow). Thus, the similarity of patterns suggested that a common spectrum of polypeptides was associated with the anti-CD4 and the PTK immunoprecipitates.

A direct demonstration of the association was shown by denaturing the phosphorylated immunoprecipitates in the presence of NaDodSO4 and then again using anti-CD4 antibody (Fig. 2B) shows a typical pattern in the immunoprecipitates formed by an anti-CD4 antibody subjected to in vitro phosphorylation with [32P]ATP (lane 1). The anti-CD4 immunoprecipitates were then denatured in either 1% (wt/vol) or 2% (wt/vol) NaDodSO4 and reprecipitated with the anti-PTK antibody (lanes 3 and 4, respectively). Importantly, the anti-PTK antibody recognized the labeled 55- to 60-kDa band from the anti-CD4 immunoprecipitate. Conversely, a cocktail of anti-CD4 antibodies (12T4D11, 18T3A9, 197hysD7) was found to reprecipitate a very faint band from the phosphorylated precipitates formed by the anti-PTK antisemur (lane 6). In neither case was the control antibody 1F7 found to reprecipitate antigen (lanes 2 and 5). The specificity of the recognition by the antibody of the polypeptides was shown by the ability of the synthetic peptide to block the precipitation by anti-PTK antisemur, but not by the anti-CD4 antibody (data not shown).

Subsequent phospho amino acid analysis of the reprecipitated PTK antigen by the anti-PTK antisemur detected radiolabel at a tyrosine residue (Fig. 2C). These data demonstrated that the 55- to 60-kDa bands corresponded primarily to the autophosphorylation of the T-cell-specific PTK, which coprecipitated with the CD4 receptor.

Lastly, two-dimensional NEPHGE/NaDodSO4/PAGE was conducted to confirm the identity in structure of the PTK associated with the CD4 antigen with that recognized directly by the anti-PTK antisemirum in cells (Fig. 3). The polypeptides reprecipitated by the anti-PTK antisemur from a denatured anti-CD4 immunoprecipitate focused as two separate series of spots of slightly different molecular sizes and isoelectric positions over a pH range of 4.0–5.0 (Lower). This pattern was similar to that observed when the anti-PTK antisemur was used to reprecipitate PTK from denatured anti-PTK immunoprecipitates (Upper). The only detectable difference in the two patterns was that the pattern derived from the anti-CD4 precipitate appeared to extend over a slightly smaller pH range than that recognized by the anti-PTK antisemur. These data confirm that the PTK associated with the T4 (CD4) receptor is a member of the series of spots recognized by the anti-PTK antisemur.

An association of the T-cell-specific PTK with the CD4 receptor is potentially of major importance to understanding

![Fig. 2. NaDodSO4/PAGE analysis of the association between the CD4 and PTK antigens.](image1)

![Fig. 3. Two-dimensional NEPHGE/NaDodSO4/PAGE of reprecipitated PTK polypeptides. The anti-PTK antisemur and the anti-CD4 antibody were used to precipitate antigen from Con A-stimulated peripheral blood lymphocytes. Immunoprecipitates were then subjected to labeling with [32P]ATP, as described in Fig. 1. The labeled polypeptides were eluted from protein A-Sepharose beads by boiling in the presence of 1% (wt/vol) NaDodSO4 for 5 min and diluting 1:10 in Nonidet P-40 lysis buffer. The anti-PTK antisemur was then used to precipitate antigen from these preparations followed by three washes in lysis buffer and acid concentration.](image2)
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the mechanism of T-cell activation. Tyrosine phosphorylation has been shown to play a key role in the activation of cells by surface receptors (epidermal growth factor, insulin, and platelet-derived growth factor receptors) and in the transformation of cells by various retroviral oncogenes (v-abl, v-erb-B, v-ras, v-yes, etc.) (17, 18). In fact, the amino acid sequence of the T-cell oncogene src kinase identified in this study shares some 70–80% similarity with the oncogenes c-src and v-yes in the region of the catalytic domain at the C terminus (29, 30). The overall sequence is homologous to the human YT16 gene (30) and appears to be the human analogue of the mouse pp53 