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Association of the fyn protein-tyrosine kinase with the T-cell antigen receptor

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ABSTRACT **Activation of the T-cell antigen receptor (TCR) results in tyrosine phosphorylation of the TCR ζ chain and other intracellular substrates. Two other T-cell integral membrane proteins, CD4 and CD8, are associated with the protein-tyrosine kinase (PTK), lck. Despite evidence that activation of this enzyme results in TCR-ζ chain phosphorylation, it has not been shown that the TCR activates lck. We have sought evidence that the TCR is associated with a PTK. In this study we use digitonin to solubilize a murine T-cell hybridoma and demonstrate that antibodies binding extracellular but not intracellular domains of the TCR specifically coprecipitate only the fyn PTK and not lck or yes, two other kinases found in these cells. The association of the fyn PTK with the TCR might enable the T cell to independently regulate two PTKs through surface receptors.**

Over the past several years there has been increasing interest in the regulation of tyrosine phosphorylation in T lymphocytes. Our laboratory demonstrated that activation of T cells by way of the multisubunit T-cell antigen receptor (TCR) results in tyrosine phosphorylation on the TCR ζ chain (TCRζ) (1, 2). Subsequently, we have shown that additional cellular substrates are tyrosine phosphorylated within seconds of activation, more rapidly than phosphorylation of the ζ chain (3, 4). The protein-tyrosine kinase(s) [PTK(s)] responsible for these phosphorylations have not yet been identified. However, since the TCR subunits lack kinase consensus sequences, it is likely that this receptor is coupled to a nonreceptor PTK. Two other receptor structures on T cells, the CD4 and CD8 molecules, present together on immature T cells and present individually on nonoverlapping populations of mature T cells, are also coupled to tyrosine kinase activity. CD4 and CD8 molecules are noncovalently associated with lck, a src-like T-cell specific PTK (5, 6). Antibody-mediated cross-linking of CD4 activates lck and the TCR ζ chain is phosphorylated (7). Despite evidence that lck can phosphorylate TCRζ, it is not clear that this kinase is responsible for ζ chain tyrosine phosphorylation after TCR engagement. T cells lacking CD4 or CD8 molecules are capable of TCR-mediated tyrosine kinase activation (L.E.S., unpublished data), TCR crosslinking fails to activate lck (7), and the patterns of intracellular substrate phosphorylation observed with CD4 and TCR engagement are different, suggesting that different kinases are involved (4). We, therefore, sought evidence for an additional PTK activity associated with the TCR. In these studies solubilization of the murine T-cell hybridoma, 2B4 under nondissociating conditions followed by an immune-complex kinase assay revealed the association of the TCR with the fyn PTK (8, 9) and *in vitro* tyrosine phosphorylation of the TCR.

MATERIALS AND METHODS

Cell and Antibodies. The murine T-cell hybridoma, 2B4, proliferates continuously in culture and was grown as described (10). The following monoclonal antibodies (and their specificity) were used: 145-2C11 (hamster anti-CD3ε) (11), F23.1 (a mouse monoclonal anti-TCR antibody not binding the 2B4 TCR) (12), K204 (rat anti-H2) (13), FD 196.14 [rat anti-lymphocyte function-associated antigen 1 (LFA-1)] (14), M1/9 (rat anti-CD45) (15), and A2B4-2 (mouse anti-2B4 TCRα) (10). The following rabbit anti-peptide antisera (and specificity) were used: R9 (C-terminal sequence of CD3δ) (16), anti-fyn (residues 29–48 of the fyn protein) (17), anti-lck (residues 39–64 of the lck protein) (6), anti-src (C terminus of src shared between src and fyn) (6), and anti-yes (residues 5–50 of the yes protein). All anti-peptide antisera that recognize PTKs were kindly provided by J. B. Bolen (National Cancer Institute). The specificity of the anti-yes sera has been determined in his laboratory (J. B. Bolen, personal communication).

Solubilization and Kinase Reaction. Cells were harvested and solubilized at 10^7 cells per ml in either a digitonin buffer {digitonin [1%; prepared as described by Oettgen *et al.* (18)]/150 mM NaCl/50 mM Hepes, pH 7.5 with 1 mM sodium orthovanadate/1 mM EDTA/leupeptin (10 μg/ml)/aprotinin (10 μg/ml)/25 μM *p*-nitrophenyl *p*-guanidinobenzoate} or in a 0.5% Triton X-100 lysis buffer (0.5% Triton X-100/150 mM NaCl/50 mM Tris·HCl, pH 7.6 with the above inhibitors). Postnuclear supernatants from 5×10^6 cells were subjected to immunoprecipitations for 1 hr for antibodies preadsorbed to protein A-agarose or for rat monoclonal antibodies preadsorbed to protein G-agarose (Genex). Monoclonal antibodies were used as culture supernatants. After immunoprecipitation, the immune complexes were washed in lysis buffer with the appropriate detergent but without EDTA. Immune complex kinase assays were performed by incubation at 4°C to favor kinase autophosphorylation for 15 min in 50 μl of kinase buffer [100 mM NaCl/20 mM Hepes, pH 7.5/5 mM $MnCl_2$/5 mM $MgCl_2$/1 μM ATP/10–20 μCi of [γ-^{32}P]ATP (4500 Ci/mmol; 1 Ci = 37 GBq)]. The reaction was stopped with lysis buffer containing the appropriate detergent and 20 mM EDTA. Immune complexes were washed twice in lysis buffer and eluted in electrophoresis buffer.

Electrophoresis and Phosphopeptide Mapping. One-dimensional electrophoresis was performed on 7–12% polyacrylamide gradient gels. Molecular mass markers are as indicated. Two-dimensional diagonal gels were performed as described with 12% polyacrylamide in both dimensions (19). Two-dimensional nonequilibrium pH-gradient electrophoresis (NEPHGE)–SDS/PAGE was performed as described (19). The origin of the tube (H^+) is shown on the right of the autoradiograph. The second dimension was a 12% polyacrylamide gel. Gels were dried and subjected to Kodak XAR-2 film at −70°C. Partial peptide maps were performed by the method of Cleveland *et al.* (20).

RESULTS

TCR immune complexes isolated under detergent conditions (0.5% Triton X-100) that maintain the integrity of the seven-

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Abbreviations: TCR, T-cell antigen receptor; PTK, protein-tyrosine kinase; LFA-1, lymphocyte function-associated antigen 1.

chain TCR fail to demonstrate TCR-associated kinase activity (Fig. 1*A*). However, solubilization of the murine T-cell hybridoma 2B4 in digitonin followed by specific receptor immunopurification and *in vitro* kinase assay results in phosphorylation of a number of proteins. To confirm that precipitation of the TCR is required for this activity, a number of T-cell surface molecules were immunoprecipitated from digitonin lysates and subjected to kinase assays (Fig. 1*B*). There was no kinase activity associated with immunoprecipitated H-2 or CD45 molecules. Immunoprecipitations of the LFA-1 molecule and *in vitro* kinase assay resulted in phosphorylation of proteins of 180 and 100 kDa, the approximate molecular masses of the LFA-1 α and β chains, and an additional protein at 60 kDa, a pattern distinct from that observed with the TCR isolates. The specificity of immunoprecipitation was further tested using a panel of anti-TCR antibodies (Fig. 1*C*). A nearly identical pattern of phosphoproteins was observed after immunoprecipitation with antibodies binding external domains of the TCRα or CD3ε subunits. Shared phosphoproteins have molecular masses of 130, 120, 59, 56, and 21–28 kDa. The presence of an additional phosphoprotein of 64 kDa was detected with three anti-CDε reagents but not with the anti-TCRα antibody. However, use of antibodies binding the intracellular C terminus of the CD3 δ chain or the cytosolic domain of the TCR ζ chain (data not shown) failed to coprecipitate the same pattern of phosphoproteins as when the receptor complexes were isolated using antibodies directed against extracellular TCR epitopes. The presence of phosphoproteins in the anti-CD3δ immunoprecipitation might be nonspecific because the reagent is a rabbit polyclonal serum. Alternatively, these phosphoproteins represent phosphorylation from another TCR-associated kinase (see below). The failure of anti-CD3δ to precipitate the same pattern of phosphoproteins as anti-CD3ε or anti-TCRα suggests that the kinase activity associated with the TCR binds intracellular domains of the receptor and that this interaction is disrupted with the anti-CD3δ or anti-TCRζ antibodies.

The migration pattern of the lower molecular mass phosphoproteins appeared to be characteristic of phosphorylated TCR subunits. To confirm this identification two-dimensional electrophoresis was performed. Diagonal gels indicated that the prominent 21- to 23-kDa material migrated below the diagonal with the characteristics of phosphorylated TCR ζ chains (Fig. 2 *A* and *B*). These phosphoproteins are not isolated with the anti-CD3δ reagent (Fig. 1*C*). Additional phosphoproteins migrating with the properties of CD3γ, -δ, and -ε were observed (19). The 55- to 60-kDa coimmunoprecipitated phosphoproteins migrated on the diagonal and thus do not have the properties of the heterodimeric α and β chains of the TCR. Further analysis of the TCR phosphoproteins by nonequilibrium pH-gradient electrophoresis (NEPHGE)–SDS/PAGE demonstrated characteristic patterns of CD3 subunit migration and was remarkable for the complexity of the pattern of phosphorylation of the TCR ζ chain (Fig. 2*C*).

As a first step in characterizing the kinase activity in these experiments, phospho amino acid analysis was performed on the phosphoproteins. The TCR subunits immunoprecipitated with anti-TCRα or anti-CD3ε reagents were exclusively phosphorylated on tyrosine residues (data not shown). The complexity of phosphorylation of the ζ chain probably reflects the presence of six intracellular tyrosine residues on this chain, which can be variably and multiply phosphorylated *in vitro* (and to a lesser extent *in vivo*) (2, 21). The heavily phosphorylated band of 60 kDa coimmunoprecipitated with the anti-CD3δ antibody (Fig. 1*C*) was phosphorylated only on serine residues. This result further distinguishes the nature of the kinase activity coimmunoprecipitated with antibodies binding extra- and intracellular domains of the receptor. The additional phosphoproteins (130, 120, 59, and 56 kDa) were phosphorylated predominantly on tyrosine and, to a lesser extent, on serine. These phosphorylations suggested that both PTK and serine kinase activities were coprecipitating with the TCR. To identify the PTK, we asked whether any of the coimmunoprecipitated phosphoproteins represented a known nonreceptor PTK that was labeled as a result of autophosphorylation. T cells are known to express multiple nonreceptor tyrosine kinases (6). Antibodies specifically binding two of these kinases, lck and fyn, were compared

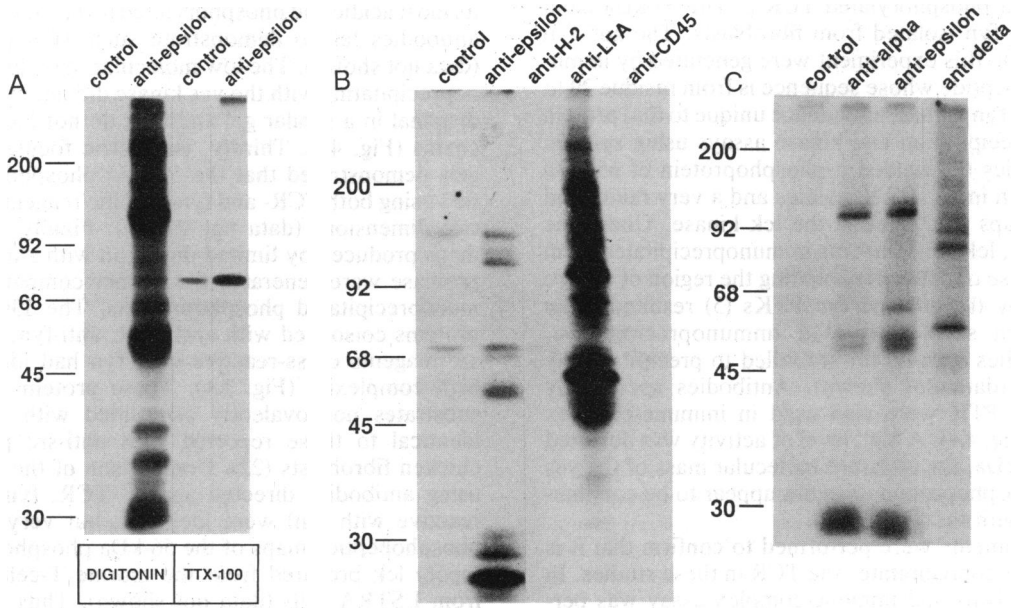

FIG. 1. *In vitro* immune complex protein kinase assays after immunoprecipitation from T-cell lysates. (*A*) Comparison of immune-complex assays after lysis in 1% digitonin or 0.5% Triton X-100 (TTX-100). The TCR was specifically immunoprecipitated using an anti-TCR/CD3-ε-specific monoclonal antibody. (*B*) Immune-complex assays after anti-TCR immunoprecipitation results in a unique pattern of phosphoproteins. The T cells were solubilized in digitonin and postnuclear lysates were subjected to immunopurification with antibodies binding to CD3ε, murine class I major histocompatibility complex molecules (H-2), the LFA-1 molecule (CD11a/CD18), or the leukocyte common antigen (CD45). (*C*) Comparison of immune-complex kinase assays using various anti-TCR antibodies, as indicated. Molecular masses in kDa are indicated.

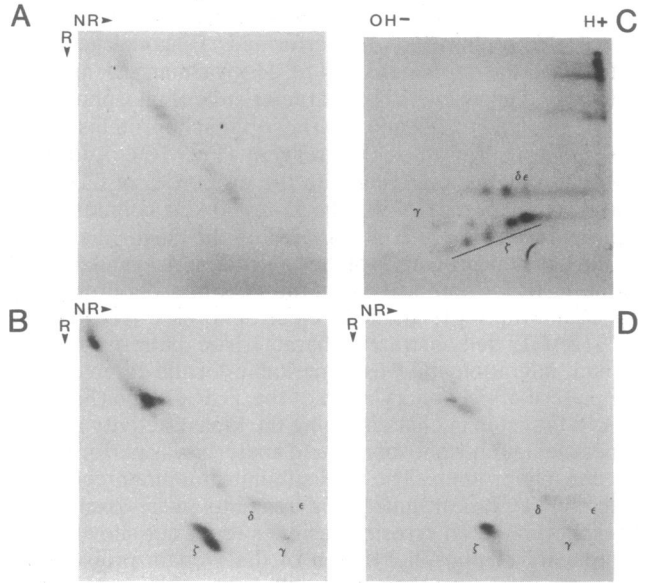

FIG. 2. Two-dimensional analysis of low molecular mass proteins generated on the TCR by *in vitro* kinase reactions. Two-dimensional diagonal gel after F23.1 (control) immunoprecipitation (*A*), 145-2C11 (CD3ε) immunoprecipitation (*B*), and anti-fyn immunoprecipitation using a rabbit serum directed against residues 29–48 of the fyn protein (*D*). (*C*) A two-dimensional nonequilibrium pH-gradient electrophoresis (NEPHGE)–SDS/PAGE analysis after 145-2C11 immunoprecipitation and the kinase reaction. Autoradiograph exposure was 2 days (*A* and *B*), 3 days (*C*), or 5 days (*D*). NR, nonreducing; R, reducing.

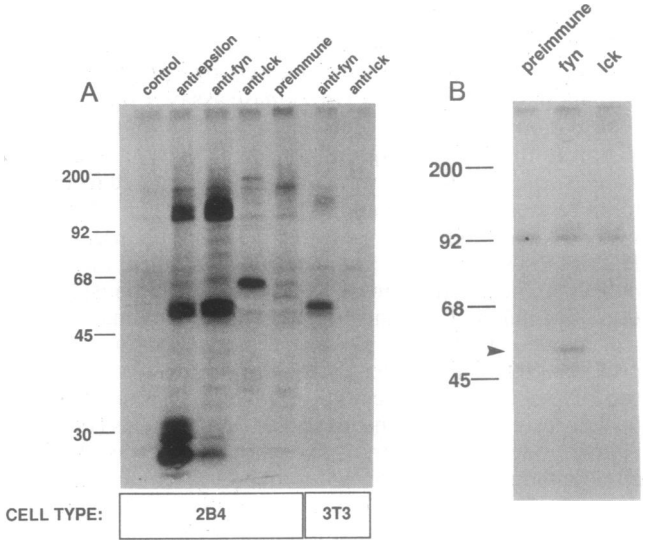

FIG. 3. Identification of the PTK associated with the TCR. (*A*) 2B4 cells were solubilized in digitonin and immunoprecipitated with F23.1 (control), 145-2C11 (anti-TCR CD3ε), rabbit antibodies specific for fyn (17) or lck (6) PTKs, or preimmune rabbit serum. 3T3 fibroblasts were solubilized in Triton X-100 and subjected to the indicated immunoprecipitations. (*B*) After a kinase reaction performed with immunopurified TCR, the immune complexes were incubated in Nonidet P-40. The eluted proteins were reimmunoprecipitated with the indicated sera. The position of the 59-kDa phosphorylated protein (pp59) fyn kinase is indicated with an arrowhead. The autoradiograph was exposed for 4 days. Molecular masses in kDa are indicated.

with anti-receptor antibodies in the immune-complex kinase assay (Fig. 3*A*). Again, the TCR–CD3ε-specific reagent resulted in phosphorylation of the 130/120-kDa doublet, the 59/56-kDa doublet, and the TCR. The kinase reaction with the fyn-specific immunoprecipitate produced a nearly identical pattern with exact comigration of the 130/120-kDa and 59/56-kDa doublets and a low level of phosphorylation comigrating with phosphorylated TCR ζ. The 59-kDa band comigrates with fyn isolated from fibroblasts. The anti-fyn antibodies used in this experiment were generated by immunization with a peptide whose sequence is from residue 29 to residue 48 of the fyn kinase, a sequence unique to that protein (17). Immunoprecipitation and kinase assays using specific anti-lck antibodies (6) yielded a phosphoprotein of 65 kDa that was not seen in anti-TCR isolates and a very faint band at 56 kDa perhaps representing the lck kinase. Under the same conditions, lck (56 kDa) was immunoprecipitated from LSTRA cells. Use of antibodies binding the region of the src kinase shared by the src and fyn PTKs (5) resulted in an identical pattern seen with TCR immunoprecipitation, whereas antibodies specific for src failed to precipitate any kinase activity (data not shown). Antibodies specifically binding the yes PTK were also used in immune-complex kinase assays (Fig. 4*A*). A high level of activity was detected migrating at 62 kDa, the apparent molecular mass of the yes kinase. This phosphoprotein does not appear to be coimmunoprecipitated with the TCR.

Several experiments were performed to confirm that it is the fyn PTK that coprecipitates the TCR in these studies. In one, a digitonin lysis and immune-complex assay was performed on TCR-associated proteins as above (Fig. 3*B*). The immune complex was then incubated in buffer containing Nonidet P-40, a detergent known to disrupt TCR subunit interactions. The eluted proteins were then subjected to reimmunoprecipitation. Specific anti-fyn but not anti-lck antibodies reprecipitated the 59-kDa phosphoprotein. Secondly, two-dimensional diagonal gels were performed after anti-fyn immunoprecipitates were subjected to kinase assays. This gel system demonstrates that the low molecular mass phosphoproteins coprecipitating with fyn are TCRζ and CD3 chains giving a pattern identical to that seen with the reciprocal anti-TCR precipitation (compare Fig. 2 *B* and *D*). In this experiment it appears that phosphorylated TCRζ precipitating with fyn is primarily migrating at 23 kDa, presumably in its most acidic and phosphorylated form. Assays with anti-lck antibodies fail to demonstrate such TCR phosphorylation (data not shown). The low molecular mass proteins coimmunoprecipitating with the yes kinase did not migrate below the diagonal in a similar gel and thus do not include the TCR ζ chains (Fig. 4*B*). Thirdly, isoelectric focusing–SDS/PAGE gels demonstrated that the 59-kDa phosphoproteins identified using both TCR- and fyn-specific reagents comigrated in two dimensions (data not shown). Finally phosphopeptide maps produced by limited digestion with staphylococcal V8 protease were generated for further comparison of the immunoprecipitated phosphoproteins. The 130- and 120-kDa proteins coisolated with anti-TCR, anti-fyn, and those anti-src reagents cross-reactive with fyn had identical maps of high complexity (Fig. 5*A*). These proteins may represent substrates noncovalently associated with fyn, similar or identical to those reported with anti-src precipitation of chicken fibroblasts (22). Comparison of the 56-kDa protein using antibodies directed against TCR, fyn or src (cross-reactive with fyn) were identical, but very different from phosphopeptide maps of the 56-kDa phosphorylated protein (pp56) lck prepared from the murine T-cell hybridoma or from LSTRA cells (data not shown). Thus both TCR- and fyn-specific antibodies coprecipitate identical substrates. Finally, comparison was made between maps of fyn isolated from fibroblasts and murine T cells with fyn-specific reagents and with the TCR-associated kinase (Fig. 5*B*). Fibroblast and T-cell fyn shared several bands, and several additional bands were seen in the T-cell fyn. It has been reported that fyn isolated from fibroblasts and T cells differ in primary se-

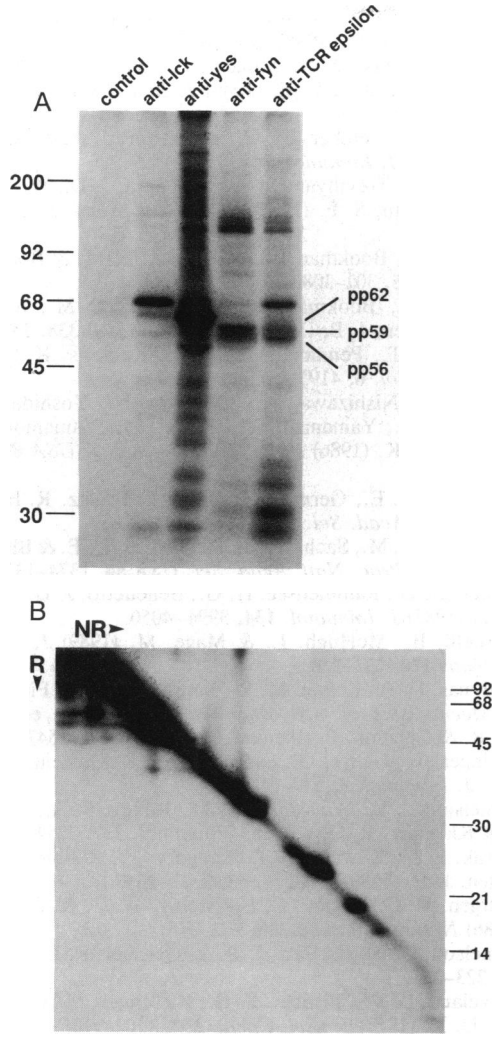

FIG. 4. Identification of T-cell tyrosine kinases and comparison with the TCR-associated kinase. (*A*) 2B4 cells were solubilized in digitonin and immunoprecipitated with F23.1 (control) or rabbit antibodies specific for lck, yes, or fyn, or 145-2C11 anti-CD3ε. Immune-complex kinase assays were performed as above. The positions of phosphorylated proteins pp62 yes, pp59 fyn, and pp56 lck are as indicated. Molecular masses in kDa are also indicated. (*B*) Two-dimensional diagonal gel after immune-complex kinase assay using anti-yes serum. NR, nonreducing; R, reducing.

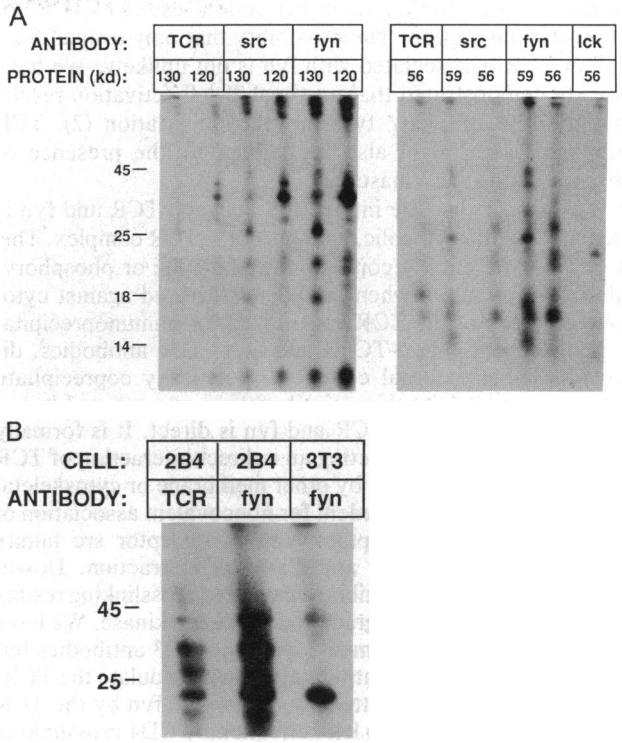

FIG. 5. Phosphopeptide maps of *in vitro* phosphorylated substrates and kinases. (*A*) Kinase reactions were performed using an anti-CD3ε monoclonal antibody, a rabbit anti-peptide serum specific for the C-terminal sequence of the src kinase shared by fyn (6), residues 29–48 of fyn, or residues 39–64 of lck. After the *in vitro* kinase reaction and electrophoresis, phosphoproteins of 130, 120, 59, and 56 kDa were isolated and subjected to partial digestion with staphylococcal V8 protease and electrophoresed again. (*B*) V8 phosphopeptide mapping of pp59 isolated from 2B4 T cells or 3T3 fibroblasts with anti-CD3 or anti-fyn and phosphorylated *in vitro*. Exposure was for 2 days (*A*) and 4 days (*B*). Molecular masses in kDa are indicated.

quence due to the use of an alternate exon within their kinase domains (23). This difference is one possible explanation for the distinct phosphopeptide patterns of fyn from the two sources. The 59-kDa phosphoprotein isolated with the TCR demonstrated a similar pattern of peptides as seen after immunoprecipitation with anti-fyn antibodies. Preliminary high-resolution tryptic phosphopeptide mapping indicates that both free and TCR-associated fyn share a tyrosine-phosphorylated peptide (the autophosphorylation site). Additional serine- and tyrosine-containing phosphopeptides are detected only in the TCR-associated fyn (data not shown).

DISCUSSION

Multiple src-like kinases have been demonstrated in hematopoetic cells. In the murine T-cell hybridoma used in these studies, we have been able to detect yes, fyn, and lck activity with anti-peptide antibodies specific for these kinases. Under mild lysis conditions, we observe that of these three PTKs, fyn is most efficiently coimmunoprecipitated with the TCR of the 2B4 cell. The sensitive immune complex assays that result, most likely, in the autophosphorylation of PTK, allowed us to detect the associated kinase. This activity is probably responsible for the *in vitro* phosphorylation of the TCR ζ chain, a normal substrate for PTK, other TCR subunits that are nonphysiologic PTK substrates, and the additional cellular substrates. The fact that both anti-TCR and anti-fyn antibodies immunoprecipitate this same set of *in vitro* phosphorylated substrates is strong evidence of this particular receptor–kinase association. It seems that fyn is directly immunoprecipitated as part of a complex of proteins that can be PTK substrates. It is interesting that the anti-TCR antibody coprecipitates the same complex of proteins along with fyn. Kinase activity also appears to be associated with the TCR on other T-cell tumors and clones, but the identity of the TCR-associated phosphoproteins observed in these experiments has not yet been studied.

We have not been able to determine what fraction of fyn protein in the cell is TCR-associated. This is because the current reagents available are of insufficient titer and affinity to immunoblot or immunodeplete fyn protein. We have, however, been able to determine what fraction of TCR is associated with the fyn kinase. T cells were metabolically labeled for 4 hr, extracted in the digitonin-based lysis buffer, and subjected to immunoprecipitation with anti-TCR, anti-fyn, or as a control anti-CD45 antibodies. A small fraction of TCRζ (0.1%) was coprecipitated with the anti-fyn antibodies whereas no ζ chain was detected on diagonal gels using preimmune or anti-CD45 reagents. This fraction of TCRζ associated with fyn represents a lower limit as we cannot be

certain whether the digitonin extraction results in TCRζ or TCRζ–fyn disruption. The possibility that only a small fraction of TCR is associated with fyn is not unlikely. We have already demonstrated that maximal T-cell activation results in only 10% of TCRζ tyrosine phosphorylation (2). TCR subpopulations might also be defined by the presence or absence of the fyn kinase.

We suspect that the interaction between TCR and fyn is mediated by the cytosolic domains of the TCR complex. This is because we cannot coprecipitate the PTK or phosphorylated TCR subunits when antibodies directed against cytosolic epitopes of the TCR are utilized for immunoprecipitation. In contrast, anti-TCRα and anti-CD3ε antibodies, directed against external epitopes, specifically coprecipitate the PTK activity. At this point however, we cannot be sure that the association of TCR and fyn is direct. It is formally possible that we are detecting an indirect interaction of TCR with the kinase mediated by other membrane or cytoskeletal structures. There is precedent for noncovalent association of integral membrane receptors with nonreceptor src family kinases in the CD4–lck and CD8–lck interaction. Down-modulation of CD4 by antibody-mediated crosslinking results in internalization and degradation of the lck kinase. We have attempted similar experiments with anti-CD3 antibodies but have been unable to quantitatively down-modulate the TCR. Moreover, preliminary attempts to activate fyn by the TCR in a manner analogous to lck activation by CD4 crosslinking have not been possible. However, insights from the study of the CD4/8–lck interaction may not perfectly apply to understanding other surface receptors or PTKs. The current results indicate that at least in these murine T cells, the antigen receptor and fyn are associated and can be coimmunoprecipitated. This observation serves as a major clue to our understanding TCR-mediated tyrosine phosphorylation. The nature of the TCR–fyn interaction, the regulation of fyn kinase activity, and the relationship between multiple receptors and tyrosine kinases in the T cell can now be approached.

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