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Immunology

Rapid T-cell receptor-mediated tyrosine phosphorylation of p120, an Fyn/Lck Src homology 3 domain-binding protein

(T-cell activation/signal transduction/protein-tyrosine kinase/Src-family kinases)

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ABSTRACT **Tyrosine phosphorylation of cellular proteins is the earliest identifiable event following T-cell antigen receptor (TCR) stimulation and is essential for activating downstream signaling machinery. Two Src-family protein-tyrosine kinases, the TCR-associated p59^{fyn} (Fyn) and the CD4/8-associated p56^{lck} (Lck), have emerged as the likely mediators of early tyrosine phosphorylation in T cells. Here, we show direct binding of a 120-kDa TCR-induced phosphotyrosyl polypeptide, p120, to glutathione *S*-transferase fusion proteins of the Src homology 3 (SH3) domains of Fyn, Lck, and p60^{src} (Src) but not other proteins. While binding of p120 to Fyn SH2 domain was phosphotyrosine-dependent as expected, its binding to the SH3 domain was independent of tyrosine phosphorylation, as shown by lack of competition with a phosphotyrosyl competitor peptide. In contrast, an SH3-specific proline-rich peptide completely abolished p120 binding to SH3. p120 was tyrosine-phosphorylated within 10 sec following stimulation of Jurkat cells with anti-CD3 monoclonal antibody, with maximal phosphorylation at 30 sec. Importantly, p120 was found associated with Fyn and Lck proteins in unstimulated Jurkat cells and served as an *in vitro* substrate for these kinases. These results provide evidence for a role of the SH3 domains of Fyn and Lck in the recruitment of early tyrosine-phosphorylation substrates to the TCR-associated tyrosine kinases.**

Engagement of the T-cell antigen receptor (TCR) induces a rapid tyrosine phosphorylation of cellular proteins as an obligatory step in T-cell activation (1–3). While the TCR components lack intrinsic tyrosine kinase domains, TCR ζ/η and CD3 γ and ε chains physically complex with the unique N-terminal domain of the Src-family protein-tyrosine kinase p59^{fyn} (Fyn) (4, 5). A role for Fyn in TCR-induced tyrosine phosphorylation was suggested by enhanced antigen-induced or anti-TCR monoclonal antibody (mAb)-induced tyrosine phosphorylation in a T-cell hybridoma overexpressing an activated Fyn and by increased or decreased TCR signaling, respectively, in thymocytes of mice expressing activated or dominant-negative Fyn transgenes (6, 7). Defective responses of thymocytes in *fyn*$^{-/-}$ knockout mice further underscore the critical role of Fyn in TCR signaling (8, 9). p56^{lck} (Lck) interacts with the cytoplasmic tails of the CD4 and CD8 coreceptors and participates in CD4/8-dependent enhancement of antigen stimulation (10–12). In addition, Lck plays a critical role in early T-cell development (3).

The Src-family kinases contain the noncatalytic Src homology 3 and 2 (SH3 and SH2) domains that are required for negative regulation of kinase activity and are essential for mediating T-cell activation as shown with Lck transfectants (13, 14). The SH2 domains of signaling proteins bind to phosphotyrosyl (pY) peptide motifs and mediate assembly of signaling complexes (15, 16). The SH3 domains bind to small proline-rich peptide motifs to mediate protein–protein interactions (17, 18). A clear physiological role for SH3 domains of the Sem-5/Grb-2 protein in linking receptor tyrosine kinases to the Ras pathway has been demonstrated (19). Recently, the SH3 domains of Src-family kinases have been shown to bind to the p85 subunit of phosphatidylinositol (PI) 3-kinase (3-K) (20, 21), paxillin (a focal adhesion protein) (22), actin-associated protein p110 (23), and microtubule-associated GTPase dynamin (24). Thus, SH3 domains may couple tyrosine kinases to multiple signaling pathways. Here we provide evidence for a role of SH3 domains in recruiting a cellular substrate to TCR-associated tyrosine kinases.

MATERIALS AND METHODS

Peptides. The pY peptide including Tyr^{324} of the hamster medium-sized tumor antigen (EPQ**pYEEI**PIYL, or pYEEI; bold type indicates the SH2-binding motif) (16) and its unphosphorylated analog (YEEI) were synthesized and HPLC purified (25). The p85 SH3-N proline-rich peptide (PI 3-kinase p85α aa 83–101, ISPPTPKPRPPRPLPVAPG) (26) was synthesized with an Applied Biosystems peptide synthesizer.

Generation of Glutathione *S*-Transferase (GST) Fusion Proteins. Various domains of human Lck (27) (from R. Perlmutter, University of Washington, Seattle) or Fyn (28) (American Type Culture Collection) were amplified by PCR and cloned as *Bam*HI–*Eco*RI fragments into pGEX2T.K vector (from Bill Kaelin and Erik Flemington, Dana–Farber Cancer Institute, Boston) in-frame with GST (29, 30) (Fig. 1*A*). DNA sequences of all constructs were confirmed. The non-SH3 C-terminal extension of Fyn SH3 fusion protein carried a proline instead of serine (aa 143). The primer/vector sequences added glycine and serine to the N terminus and EFIVTD to the C terminus of fusion proteins. Murine c-Src, neuronal Src [Src(+)], Abl type IV (aa 84–138) and v-Crk (aa 372–428) SH3 domains encoded in pGEX2T were provided by Bruce Mayer (Children's Hospital, Boston), and human Lck SH3SH2 in pGEX3b by J. Shin (Dana–Farber Cancer Institute).

Fusion proteins were affinity purified on glutathione-Sepharose beads (Pharmacia) from the Triton X-100-soluble fraction of isopropyl β-D-thiogalactopyranoside-induced *Escherichia coli* (strain DH5α or NB42) (29, 30), quantitated by Bradford assay (Bio-Rad; bovine γ-globulin standard),

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Abbreviations: TCR, T-cell antigen receptor; SH*n*, Src homology *n*; GST, glutathione *S*-transferase; mAb, monoclonal antibody; PI, phosphatidylinositol.

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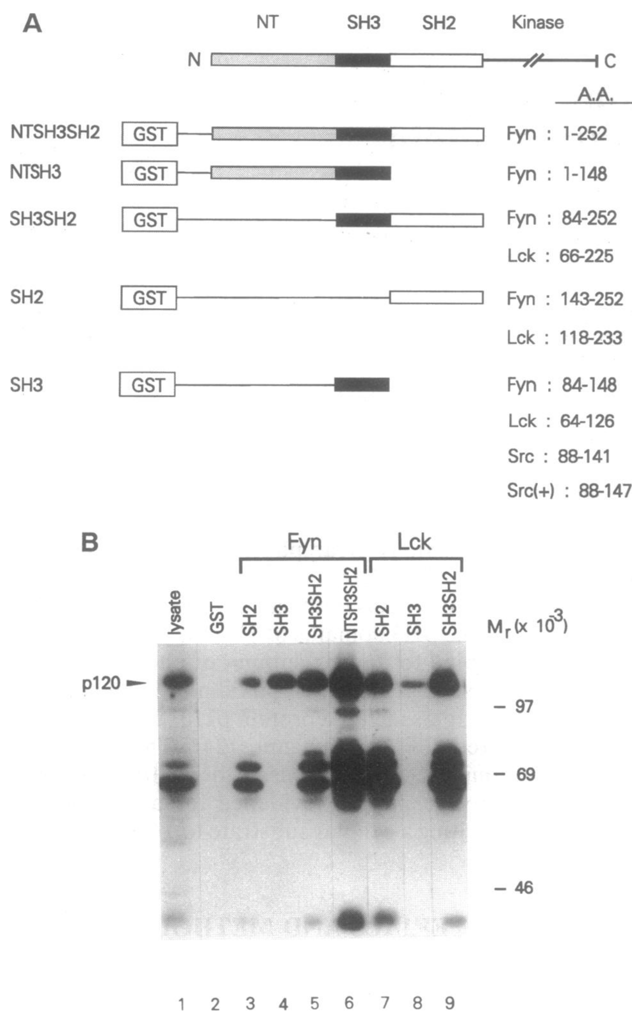

FIG. 1. (*A*) Schematic representation and nomenclature of GST fusion proteins in relation to full-length Src kinases (top). NT, N-terminal unique domain. Amino acid residues (A.A.) are indicated. (*B*) Binding of Fyn and Lck GST fusion proteins to pY proteins in anti-CD3-stimulated Jurkat cell lysates. Triton X-100 lysates of 5 $\times 10^7$ Jurkat cells stimulated for 2 min with anti-CD3ε mAb SPV-T3b were incubated with 50 μg of indicated fusion proteins, and bound polypeptides were detected by anti-pY blot. Lane 1, whole cell lysate of 10^6 cells. p120 is indicated.

and assessed for purity (>95%) and lack of degradation by SDS/PAGE and staining with Coomassie blue R-250.

Activation of Jurkat Cells and Binding of Cellular Polypeptides to GST Fusion Proteins. Jurkat T leukemia cells (10^8 per ml) were incubated at 37°C for 2 min (or as specified) in RPMI 1640 medium with 20 mM Hepes, 0.2 mM Na_3VO_4, and either control mAb [OKT8 (anti-CD8) or W6/32 (anti-MHC class I), both IgG2a; American Type Culture Collection] or activating mAb [SPV-T3b (anti-CD3ε, IgG2a), 1:200 dilution of ascites]. Cells were lysed in cold lysis buffer [0.5% Triton X-100 (Pierce or Fluka)/50 mM Tris, pH 7.5/150 mM NaCl/1 mM phenylmethylsulfonyl fluoride/1 mM Na_3VO_4/10 mM NaF]. Lysate from 5 $\times 10^7$ cells was rocked for 1 hr at 4°C with 50 μg of fusion protein noncovalently coupled to glutathione-Sepharose beads. Beads were washed six times and bound proteins were resolved by SDS/PAGE and subjected to anti-pY blotting.

Anti-pY Immunoblotting. Polypeptides were transferred to poly(vinylidene difluoride) membranes (Immobilon-P; Millipore), which then were blocked with 2% gelatin (Bio-Rad) in Tris-buffered saline (TBS: 10 mM Tris, pH 8/150 mM NaCl/0.02% NaN_3) and serially incubated in TBS with 0.05% Tween 20 (Bio-Rad) containing anti-pY mAb 4G10 (31) at 1 μg/ml followed by TBS with ^{125}I-labeled protein A (Amersham) at 10 ng/ml (0.3 μCi/ml; 1 μCi = 37 kBq), with six washes in between. Autoradiograms were developed with Kodak XAR-5 film at −80°C.

***In Vitro* Kinase Reaction.** Unlabeled Jurkat cells were lysed in lysis buffer with 1% (vol/vol) Brij 96 (Fluka) instead of Triton X-100. Lysates from 5 $\times 10^7$ cells were immunoprecipitated with normal rabbit serum, anti-integrin β7-antiserum (aa 736–755, from Chris Parker, Harvard Medical School) anti-Lck (aa 22–51; Upstate Biotechnology, Lake Placid, NY), or anti-Fyn antiserum (aa 35–51, from Chris Rudd, Dana-Farber Cancer Institute) (32), and immunocomplexes were bound to protein A-Sepharose (Pharmacia). Immunocomplexes or products of fusion-protein binding reactions were washed once in kinase buffer without ATP and then incubated in 100 μl of kinase buffer (20 mM Hepes, pH 7.3/100 mM NaCl/5 mM $MgCl_2$/5 mM $MnCl_2$) in the absence or presence of 0.5 mM unlabeled ATP. After 20 min at 25°C, beads were washed three times with lysis buffer and anti-pY blotting was performed.

RESULTS

Fyn and Lck SH3 Fusion Proteins Bind to a TCR-Induced Tyrosine-Phosphorylated Protein of 120 kDa. Purified GST fusion proteins (Fig. 1*A*) noncovalently immobilized on glutathione-Sepharose beads were incubated with Triton X-100 lysates of Jurkat T cells stimulated with anti-CD3 mAb for 2 min, and the proteins adsorbed by the fusion proteins were analyzed by anti-pY immunoblotting. The Fyn SH2 fusion protein bound to essentially all induced pY proteins (Fig. 1*B*, compare lanes 1 and 3). Surprisingly, the binding of several phosphoproteins was increased when either the SH3 domain (lane 5) or the N-terminal and SH3 domains (lane 6) were included as part of the fusion protein. Increased phosphoprotein binding to SH3SH2 and NTSH3SH2 fusion proteins is partly due to enhanced affinity of the SH2 domain for pY peptide motifs (G.P., T.F., L. Stolz, G. Payne, K.A.R., S.E.S., Z. Songyang, L. Cantley, C. Walsh, and H.B., unpublished work). To test the additional possibility that the SH3 domain might directly bind to pY proteins, binding reactions were performed with GST–SH3 fusion protein. Indeed, a prominent 120-kDa pY polypeptide (p120) was observed to bind to Fyn SH3 (lane 4). p120 signals were consistently higher in SH3-binding reactions than in SH2-binding reactions (compare lanes 3 and 4; same amount of cell lysate). Lck SH3SH2 also showed higher p120 signals (lane 9) than the SH2 fusion protein (lane 7), and SH3 fusion protein itself showed binding to p120 (lane 8). Thus, p120 can directly bind to Fyn and Lck SH3 domains *in vitro*. Binding to p120 was not eliminated by boiling the cell lysates in 0.5% SDS to disrupt noncovalent protein–protein interactions, indicating that the interaction between p120 and the SH3 domain was direct rather than through an associated protein (data not shown).

p120 Is One of the Earliest Tyrosine-Phosphorylation Substrates After TCR/CD3 Triggering. The ability of p120 to bind to the SH3 domain suggested that it might associate with Fyn before activation and thus undergo tyrosine phosphorylation early after T-cell activation. To examine this possibility, we determined the time course of p120 tyrosine phosphorylation in whole cell lysates of anti-CD3-stimulated Jurkat cells. Control lysates (Fig. 2, lanes 1 and 2) showed a small basal phosphorylation of p120, p68 (which coimmunoprecipitates with TCR ζ and is likely to be ZAP-70, migrating just below the 69-kDa marker) (34), and additional minor proteins. Anti-CD3 stimulation induced the expected increase in phosphorylation of TCR ζ and ZAP-70 (maximal by 2 min) and other, unidentified cellular proteins (1, 2, 34). Phosphoryla-

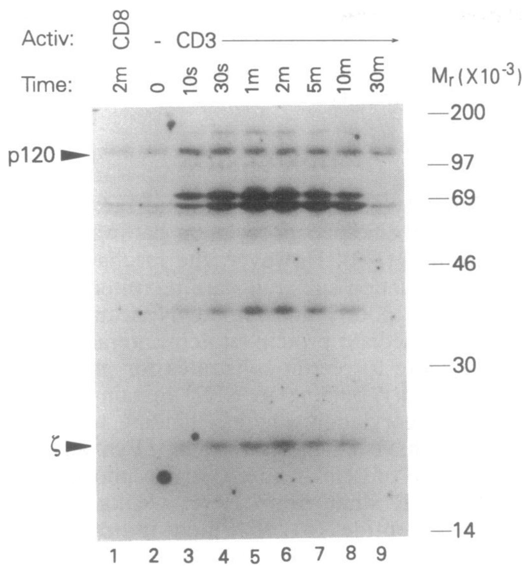

FIG. 2. p120 is tyrosine-phosphorylated very early after anti-CD3 stimulation of Jurkat T cells. Whole cell lysates of 10^6 Jurkat cells activated (Activ.) with anti-CD3ε mAb SPV-T3b (CD3) for the indicated times (m, minutes; s, seconds) were subjected to anti-pY blotting. Negative controls: lane 1, mAb OKT8 (CD8; Jurkat is CD8$^-$); lane 2, aliquot of cells removed before addition of anti-CD3. Note maximal phosphorylation of p120 by 30 sec, compared to about 2 min for TCR ζ.

tion of p120 increased within 10 sec (lane 3), was maximal at 30 sec (lane 4), and decreased after 10 min. Enhanced p120 phosphorylation was still noticeable at 30 min, by which time phosphorylation of other polypeptides had returned to near basal levels. Thus, p120 is one of the earliest tyrosine-phosphorylation substrates after anti-CD3 activation of Jurkat T cells.

p120 Binding to Fyn SH2 and SH3 Domains Is Blocked by Domain-Specific Competitor Peptides. To address the mechanism of p120 binding to Fyn SH2 and SH3 fusion proteins, we assessed binding in the presence of competitor peptides. As expected (33), 100 μM phosphopeptide (pYEEI) prevented the binding of p120 and other pY proteins to the SH2 fusion protein (Fig. 3*A*, compare lanes 3 and 5), whereas the unphosphorylated analog (YEEI, lane 4) had no effect. In contrast, proline-rich p85 SH3-N peptide inhibited p120 binding to SH3 fusion protein in a concentration-dependent manner (lanes 10–13). Effective inhibition was observed even at 20 μM (lane 12) and was complete at 100 μM concentration (lane 11). The selectivity of peptide inhibition was demonstrated by unaltered p120 binding when SH2 fusion protein was preincubated with 500 μM p85 SH3-N (lane 6) or when SH3 fusion protein was preincubated with 100 μM pYEEI (lane 9) or YEEI (lane 8). Thus, p120 interacts with the SH2 domain via pY binding, whereas it interacts with the SH3 domain via proline–peptide interactions.

Concurrent p120 Binding to Fyn SH3 and SH2 Domains. Since p120 showed independent binding to SH3 and SH2 fusion proteins (Fig. 3*A*), we examined whether it bound to both the SH3 and SH2 domains expressed in a single fusion protein (Fyn NTSH3SH2). Expected inhibition of p120 binding to Fyn SH2 (Fig. 3*B*, lane 4) or Fyn SH3 (lane 8) fusion proteins was observed with 100 μM pYEEI or p85 SH3-N peptide, respectively. In contrast, p120 binding to Fyn NTSH3SH2 showed only a small diminution at 100 or 500 μM concentrations of either pYEEI (lanes 10 and 13) or p85 SH3-N (lanes 11 and 14). Notably, pYEEI substantially reduced the binding of other (non-p120) pY proteins to NTSH3SH2 at 100 μM and nearly completely inhibited their binding at the 500 μM concentration (lane 13). The two peptides together mark-

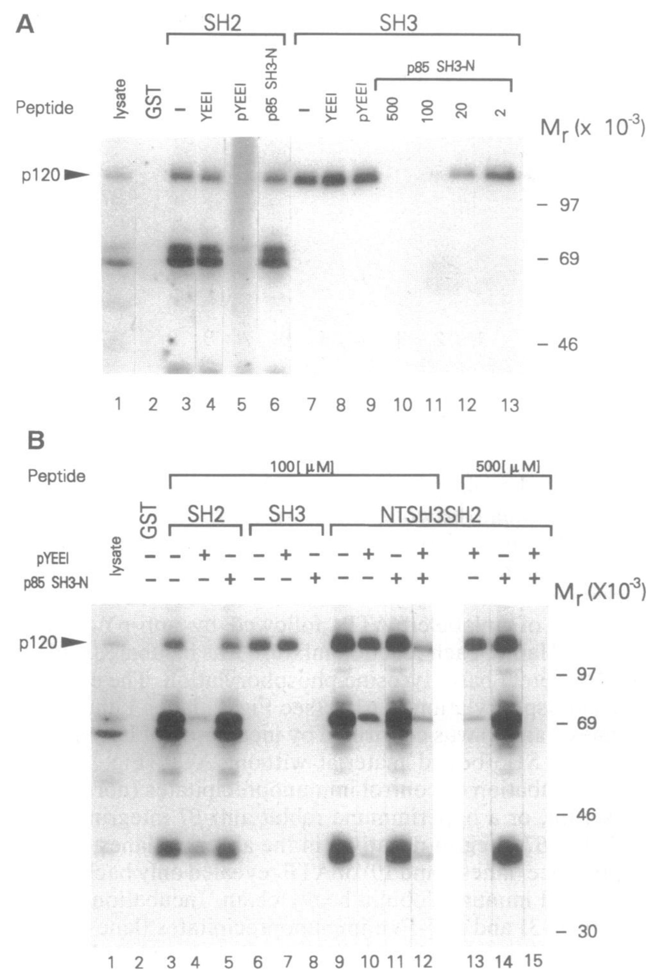

FIG. 3. Specific peptide competition of p120 binding to Fyn SH2 and SH3 domains. Assay conditions were similar to those for Fig. 1. Fusion proteins were preincubated with peptides for 30 min. (*A*) Binding of p120 to Fyn SH2 is inhibited by pY peptide whereas binding to Fyn SH3 is blocked by proline-rich peptide. Peptide concentrations (μM) are indicated. −, No peptide; pYEEI, pY-containing peptide; YEEI, unphosphorylated analog of pYEEI; p85 SH3-N, proline-rich peptide. (*B*) Both phosphopeptide and proline-rich peptide are required for effective competition of p120 binding to Fyn NTSH3SH2 fusion protein. Peptide concentrations are shown at the top; − and + indicate absence or presence of the respective peptides in the binding reaction mixture.

edly inhibited the p120 binding at 100 μM (lane 12) and completely abolished it at 500 μM (lane 15). These results are consistent with simultaneous binding of p120 to both the SH3 and the SH2 domain.

p120 Binds to a Subset of SH3 Domains. Higher p120 signals in Fyn compared with Lck SH3 binding reactions suggested a selectivity in binding (Fig. 1*B*, lanes 4 and 8; Fig. 4, lanes 4 and 5). The p120 binding to c-Src SH3 was comparable to the p120 binding of Fyn SH3 (lane 6), whereas neuronal Src SH3 [Src(+), lane 9], which has a 6-aa insertion compared with c-Src (35), showed no p120 binding. The SH3 fusion proteins of murine c-Abl (lane 7) and Crk (lane 8) and human α-spectrin (not shown) showed no detectable binding to p120, although Abl, spectrin, and Src(+) SH3 fusion proteins were able to bind to a number of cellular polypeptides in lysates of ^{35}S-labeled Jurkat cells (data not shown). Thus, p120 appears to preferentially bind to SH3 domains of Src-family proteins, although binding to other Src-family members remains to be examined.

Association of p120 with Fyn and Lck in Jurkat T Cells Prior to Activation. The pY-independent binding to SH3 domain

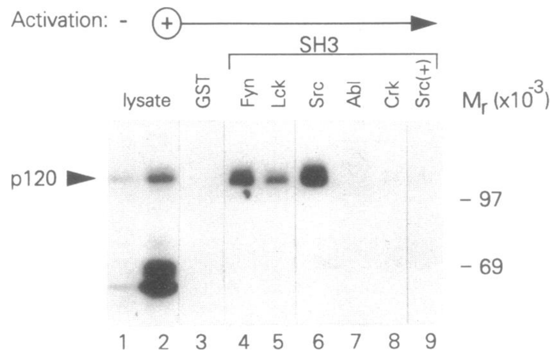

FIG. 4. Selectivity of p120 binding to various SH3 fusion proteins. Binding conditions were same as in Fig. 1. −, Anti-CD8 mAb; +, anti-CD3ε mAb.

and early phosphorylation upon TCR stimulation suggested that p120 might be complexed with Fyn and Lck prior to T-cell activation. To detect such complexes, anti-Fyn and anti-Lck immunoprecipitates from lysates of unstimulated Jurkat cells were subjected to *in vitro* kinase reactions in the presence of unlabeled ATP, followed by anti-pY blotting (Fig. 5). Half of each reaction mixture was incubated without ATP to detect basal tyrosine phosphorylation. The expected basal phosphorylation of p120 (see Fig. 2, lanes 1 and 2; Fig. 4, lanes 1 and 2) was confirmed by incubation of Fyn SH2- or Fyn/Lck SH3-bound material without ATP (Fig. 5, lanes 5–7). Incubation of control immunoprecipitates (normal rabbit serum, or a hyperimmune rabbit anti-β7 integrin serum; Jurkat is β7 integrin-negative) in the absence (lanes 1 and 2) or presence (lanes 9 and 10) of ATP revealed only background bands and immunoglobulin heavy chain. Incubation of anti-Lck (lane 3) and anti-Fyn immunoprecipitates (lane 4) without ATP showed lack of basal tyrosine phosphorylation of Lck/Fyn or p120. In contrast, anti-Lck (lane 11) and anti-Fyn (lane 12) immunoprecipitates incubated with ATP showed readily detectable p120 that migrated identically with the SH2- and SH3-bound p120 (lanes 13–15). In addition, prominent tyrosine-phosphorylated Lck and Fyn proteins were detected (lanes 11 and 12). Thus, unphosphorylated p120 is associated with both Fyn and Lck and serves as an *in vitro* substrate for these kinases.

Interestingly, *in vitro* incubation of Fyn SH3-bound material with ATP resulted in an increase in p120 tyrosine phosphorylation and the appearance of an unidentified 65-kDa pY protein (compare lanes 6 and 14). These results indicate that one or more Fyn/Lck SH3-binding polypeptides possess tyrosine kinase activity.

DISCUSSION

The Src-family tyrosine kinases Fyn and Lck appear to play key roles in tyrosine-phosphorylation pathways initiated by T-cell activation (1–3). However, the mechanisms whereby early phosphorylation substrates are recruited to these tyrosine kinases are poorly understood. Noncatalytic SH2 and SH3 domains mediate protein–protein interactions (15) and hence may serve to recruit substrates for phosphorylation. Since high-affinity binding to SH2 domain requires prior tyrosine phosphorylation whereas binding to SH3 domain does not, we have examined whether Fyn and Lck SH3 domains bind to proteins whose tyrosine phosphorylation is induced by TCR stimulation. Here, we have identified a 120-kDa polypeptide (p120) which interacts in a pY-independent manner with the SH3 domains of Fyn and Lck. The specificity of SH3–p120 binding was demonstrated by its abrogation with a proline-rich competitor peptide at concentrations similar to those required to block binding to other SH3-specific proteins (33). Furthermore, p120 binding showed a selectivity toward the SH3 domains of Src-family kinases (Src, Fyn, and Lck).

Consistent with a role for Fyn/Lck SH3 domains in recruiting p120 as a substrate, this polypeptide was among the earliest tyrosine-phosphorylation substrates upon anti-CD3ε activation of Jurkat cells, and its maximal phosphorylation (30 sec) preceded that of TCR ζ and other polypeptides. Importantly, p120 was associated with Fyn and Lck in Jurkat cells even when it was not phosphorylated and served as an *in vitro* substrate for these tyrosine kinases. Although Jurkat is a transformed T-cell line, the Fyn/Lck-associated p120 showed little basal tyrosine phosphorylation, consistent with the presence of Fyn/Lck–p120 complexes before activation. In addition, we have observed increased phosphorylation of p120 in normal human T-cell lines (data not shown). Experiments with naive T cells will be required to establish this hypothesis definitively.

In addition to anti-CD3ε mAb, p120 phosphorylation was also induced by anti-TCR β-chain variable region (V_β) stimulation of Jurkat cells and anti-CD8 activation of Jurkat cells transfected with a CD8/TCR ζ cytoplasmic domain chimera

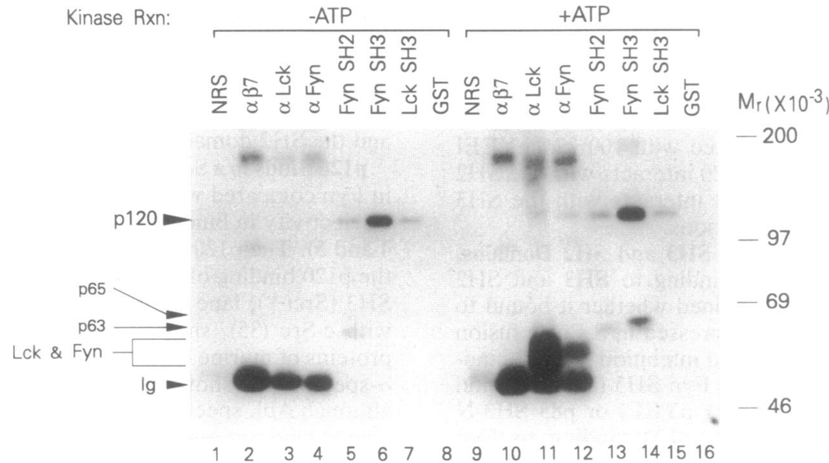

FIG. 5. Association of p120 with Fyn and Lck proteins in unstimulated Jurkat T cells. Immunoprecipitates or products of fusion-protein binding reactions (from lysates of 5 × 10^7 cells) were incubated in kinase buffer without (−ATP) or with (+ATP) unlabeled ATP and pY proteins were selectively visualized by anti-pY blot. Normal rabbit serum (NRS) and hyperimmune anti-β7 integrin serum are negative controls. Note p120 in anti-Lck (lane 11) and Fyn (lane 12) immunoprecipitates incubated with ATP; this polypeptide comigrates with SH2/SH3-binding p120 (lanes 5–7 and 13–15). Specific pY proteins and immunoglobulin (Ig) are indicated at left. α, Anti-.

(data not shown). Thus, p120 is a substrate for tyrosine phosphorylation triggered by at least two TCR signaling modules (ζ and ε). Although not assessed yet, p120 may also serve as a phosphorylation substrate upon triggering of other Lck/Fyn-associated membrane receptors on T cells (1–3).

While we suggest that the SH3 domain would recruit p120 as a substrate, once phosphorylated it may also bind to adjacent SH2 domains to establish a more stable two-site contact. The requirement for a combination of SH2- and SH3-specific blocking peptides to inhibit p120 binding to Fyn NTSH3SH2 fusion protein (Fig. 3*B*) is consistent with this proposal. Whether binding to Fyn/Lck SH2 domains alters p120 function, analogous to activation of the PI 3-kinase by phosphotyrosyl peptide binding to its SH2 domain (36), is an intriguing possibility that will require identification of p120.

The identity of p120 is unknown. p120 was unreactive with mAb against the 120-kDa Ras-associated GTPase-activating protein, which is tyrosine-phosphorylated in certain cells (15) (data not shown). The p120 appears to be distinct from the recently described Fyn SH2-binding p130, as the latter did not bind to the SH3 domain (32). Similarly, the binding pattern of p120 is distinct from that reported for the 110-kDa Src substrate (p110) which binds to Src SH3 when unphosphorylated but not when tyrosine-phosphorylated (23). Availability of reagents against the human homolog of p110 should allow its direct comparison with p120. The smaller size (100 kDa) and lack of proline-rich motifs in VCP (valosin-containing protein), which is tyrosine-phosphorylated in murine T cells, suggest that p120 is distinct from this protein (33). Fyn SH3 binding reaction mixtures contained a tyrosine kinase activity that led to increased tyrosine phosphorylation of p120 and appearance of another pY protein, p65 (Fig. 5). Since tyrosine kinases generally autophosphorylate (see Lck and Fyn in Fig. 5), it is possible that p120 or p65 itself may be a tyrosine kinase.

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