T Cell Receptor ζ/CD3-p59$^{fyn(T)}$–Associated p120/130 Binds to the SH2 Domain of p59$^{fyn(T)}$

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Summary

Intracellular signaling from the T cell receptor (TCR)ζ/CD3 complex is likely to be mediated by associated protein tyrosine kinases such as p59$^{fyn(T)}$, ZAP-70, and the CD4:p56^{lck} and CD8:p56^{lck} coreceptors. The nature of the signaling cascade initiated by these kinases, their specificities, and downstream targets remain to be elucidated. The TCR-ζ/CD3:p59$^{fyn(T)}$ complex has previously been noted to coprecipitate a 120/130-kD doublet (p120/130). This intracellular protein of unknown identity associates directly with p59$^{fyn(T)}$ within the receptor complex. In this study, we have shown that this interaction with p120/130 is specifically mediated by the SH2 domain (not the *fyn*-SH3 domain) of p59$^{fyn(T)}$. Further, based on the results of in vitro kinase assays, p120/130 appears to be preferentially associated with p59$^{fyn(T)}$ in T cells, and not with p56^{lck}. Antibody reprecipitation studies identified p120/130 as a previously described 130-kD substrate of pp60^{v-src} whose function and structure is unknown. TCR-ζ/CD3 induced activation of T cells augmented the tyrosine phosphorylation of p120/130 in vivo as detected by antibody and GST:*fyn*-SH2 fusion proteins. p120/130 represents the first identified p59$^{fyn(T)}$:SH2 binding substrate in T cells, and as such is likely to play a key role in the early events of T cell activation.

Engagement of the antigen–receptor complex (TCR-ζ/CD3) of T lymphocytes triggers a tyrosine phosphorylation cascade that is crucial to the activation process (1). Various candidate protein tyrosine kinases including three members of the src family of protein tyrosine kinases, p56^{lck}, p59$^{fyn(T)}$, and p60^{yes} (2), and related kinases such as ZAP-70 (3) and p72^{tsk} (4) are expressed in T cells. p56^{lck} can associate with the cytoplasmic tails of CD4 and CD8 (5, 6; for review see Rudd et al. [2]). In turn, CD4:p56^{lck} complexes can associate with the TCR-ζ/CD3 complex (7). p56^{lck} expression has been found crucial to TCR-ζ/CD3 mediated T cell activation (8, 9). By contrast, p59^{fyn} can be expressed by mutually exclusive splicing as two isoforms, a unique form (p59$^{fyn(T)}$) being expressed in T cells (10). p59$^{fyn(T)}$ is associated with the CD3 γ,δ,ε, chains and the TCR-ζ subunits of the TCR-ζ/CD3 complex (11), whereas ZAP-70 associates selectively with the ζ chain (12). p59$^{fyn(T)}$ associates constitutively with the TCR-ζ/CD3 complex (13), whereas ZAP-70 associates after ligation of the complex (12). Engagement of the TCR-ζ/CD3 complex with anti-CD3 Abs stimulates the activity of p59$^{fyn(T)}$ (14, 15). Overexpression of p59$^{fyn(T)}$ in transgenic mice acts to potentiate T cell proliferation, whereas the absence of the kinase causes partial defects in signaling (16–18).

A major challenge in understanding the mechanism by which these receptor-associated protein tyrosine kinases initiate T cell proliferation will be to define the downstream targets of an intracellular cascade. In this context, it is important to note that src-related kinases such as p59$^{fyn(T)}$ are comprised of src-homology 2 and 3 domains (SH2 and SH3) with the potential to bind to intracellular substrates (2). Intracellular proteins with enzymatic activities such as phospholipase Cγ (PLCγ),[1] the p85 subunit of PI-3 kinase and *ras*GTP'ase activating protein (*ras*GAP) carry related SH2 domains (19). SH2 domains can also be expressed within smaller noncatalytic adaptor proteins such as NCK and SHC (20, 21). Both catalytic and noncatalytic SH2-carrying proteins bind to tyrosine phosphorylated residues within the cytoplasmic regions of the receptor tyrosine kinases such as the platelet-derived growth factor (PDGF), epidermal growth factor (EGF), and CSF-1 receptors (22, 23). By comparison, the smaller SH3 domains bind proline-rich motifs first defined in the 3BP-1 protein (24, 25). Related motifs have been found in a variety of intracellular proteins such as formin and the m4 muscarinic acetylcholine receptor (25). Deletion of SH2 or SH3 domains results in defective T cell signals (26, 27). Thus, the SH2 and SH3 domains of p59$^{fyn(T)}$ are likely to be important mediators in the interaction of the TCR-ζ/CD3 complex with downstream messengers.

[1] *Abbreviations used in this paper:* GST, Glutathione *S*-Transferase; PDGF, platelet-derived growth factor; PLCγ, phospholipase Cγ.

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Previously, we showed that the ligation of the TCR-ζ/CD3 complex induced the dramatic phosphorylation of a p120/130 polypeptide associated with $p59^{fyn(T)}$ and the TCR-ζ/CD3 complex (14). In this study, we demonstrate that p120/130 associates specifically with the *fyn*-SH2 domain (and not the *fyn*-SH3 domain) and corresponds to a previously identified p130 protein found associated with v-src in transfected chicken fibroblasts (28). However, in vitro kinase data suggested that in T cells, p120/130 is found preferentially associated with $p59^{fyn(T)}$, but not $p56^{lck}$. This introduces the possibility that p120/130 may serve as a marker for signals generated by the *fyn* kinase in response to antigen stimulation of the TCR-ζ/CD3 complex.

Materials and Methods

Cell Lines and Antibodies. The DC27.10 murine T cell hybridoma (29) was grown in RPMI supplemented with 5% FCS, 1% penicillin and Streptomycin, and 1% L-glutamine. Abs used include the hamster anti-murine CD3ε chain mAb 145-2C11 (gift of J. Bluestone, University of Chicago, Chicago, IL) (30), a rabbit anti-fyn antisera raised against a synthetic peptide containing residues 35-51 of the NH_2-terminal region of $p59^{fyn}$, the mouse anti-chicken p130 mAb 4F4 (IgM) (28), and the mouse anti-phosphotyrosine mAb 4G10 (gift of Drs. B. Druker and T. Roberts, Dana-Farber Cancer Institute) (31).

TCR-ζ/CD3 Stimulation, In Vitro Kinase Assays, and Immunoblotting. Cross-linking of the TCR-ζ/CD3 complex, in vitro kinase assays, and immunoblotting were carried out as previously described (14). For reprecipitation analysis, bands containing the specific phospholabeled proteins were excised from SDS-PAGE gels, the proteins were eluted for 8–12 h in 50 mM ammonium bicarbonate, 0.1% SDS, concentrated with Centricon filters (Amicon, Beverly, MA), and diluted to 1 ml of lysis buffer before immunoprecipitation with the Abs. The immunoprecipitates were then analyzed by SDS-PAGE and autoradiography.

GST-fyn Fusion Proteins. SH2 (residues 149-257), SH3 (82-148), and SH2/SH3 (82-257) encoding sequences from a cDNA clone for the mouse T cell–specific isoform of $p59^{fyn}$ (10) were subcloned into the pGEX-2T expression vector plasmid (Pharmacia, Uppsala, Sweden) and the resulting fusion proteins were purified and used in precipitations from cell lysates as previously described (32).

Results

In an attempt to identify a marker for signals specifically generated by TCR-ζ/CD3–associated $p59^{fyn(T)}$, we have attempted to identify TCR-ζ/CD3 and $p59^{fyn(T)}$ associated downstream substrates. As shown in Fig. 1, anti-CD3 precipitates derived from cell lysates from the mouse hybridoma DC27.10 and labeled in an in vitro kinase assay showed two heavily labeled sets of bands at 72 kD (p72) and 120/130 kD (p120/130) in addition to more faintly labeled bands corresponding to $p59^{fyn(T)}$ and the TCR-ζ/CD3 subunits (lane *2*). Anti-fyn serum precipitated a heavily labeled band at 59 kD corresponding to $p59^{fyn(T)}$, as well as the p120/130 doublet (lanes *3–5*). A faint amount of a 72-kD band was also visualized, but significantly less intensely than seen in the anti-CD3 precipitates. This anti-fyn and a portion of the CD3-associated p72 band corresponds to a serine/threonine phosphorylated

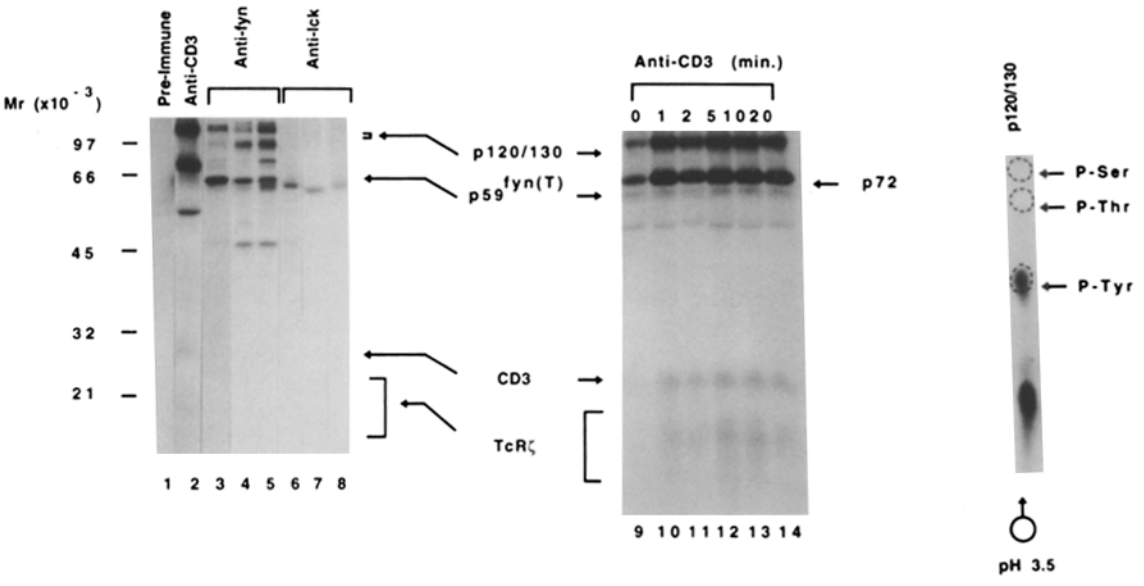

Figure 1. Engagement of TCR-ζ/CD3 complex stimulates detection of $p59^{fyn(T)}$ associated p120/130. Lysates from DC27.10 cells were prepared using 1% Brij 96 lysis buffer, immunoprecipitated with the indicated Abs and the immune complexes subjected to a kinase assay. (Lane *1*) Rabbit preimmune serum; (lanes *2* and *9–14*) anti-CD3; (lane *3*) anti-fyn rabbit antisera; (lane *4*) anti-fyn mouse monoclonal 33B1; (lane *5*) CST-1; (lanes *6–8*) three independently generated anti-lck rabbit antisera raised against the unique NH_2-terminal domain. (Lanes *9–14*) Intact cells were incubated at 37°C with anti-CD3 Ab to cross-link the TCR-ζ/CD3 complex for the indicated times (minutes) before lysis. Time 0 actually corresponds to an incubation with the rabbit anti-hamster Ab alone for 10 min at 37°C. The labeled samples were then separated by 10% SDS-PAGE, treated with 1 M KOH for 1 h at 56°C to preferentially remove serine/threonine labeling, and exposed to autoradiography. The right panel is a phosphoamino acid analysis of the CD3 associated p120/130, purified from a phospholabeled anti-CD3 precipitate and excised from a gel not treated with KOH.

isoform of the fyn kinase (33). A portion is also likely to correspond to ZAP-70 (3). Significantly, two other anti-fyn Abs, 33B1 (lane *4*), a fyn-specific mouse mAb against the *fyn*-SH3 domain (Janssen, O., and C. E. Rudd, unpublished results) and CST-1 (lane *5*), a pan-reactive rabbit antisera raised against a COOH-terminus of pp60$^{c\text{-}src}$ and which cross-reacts with p60^{yes} and p59^{fyn} (34), also precipitated a similar pattern of phosphoproteins. In contrast, repeated experiments using a panel of anti-lck antisera (lanes *6–8*) failed to precipitate detectable amounts of p120/130, indicating that p120/130 may preferentially associate with p59$^{fyn(T)}$ (lanes *3–5*). Ab-mediated cross-linking of the TCR-ζ/CD3 complex followed by an in vitro kinase assay of the anti-CD3 complexes purified from cell lysates showed the rapid phosphorylation of p120/130 (lanes *9–14*). More faintly labeled p59$^{fyn(T)}$, CD3, and TCR ζ chains were also visualized. Similar results were obtained with the human Jurkat T cell line and purified T cells ([14] and data not shown). Phosphopeptide map analysis of the p120 and p130 bands revealed that they were the same molecule (data not shown). Phosphoamino acid analysis revealed that p120/130 was phosphorylated in tyrosine residues (Fig. 1, *right*).

We were next interested in determining the region within p59$^{fyn(T)}$ to which p120/130 bound. Given the high degree of tyrosine phosphorylation of p120/130 and that the SH2 domains bind to various phosphotyrosine residues (35), the p59$^{fyn(T)}$-SH2 domain was of particular interest. Glutathione *S*-Transferase (GST) fusion proteins were constructed with *fyn*-SH2, *fyn*-SH3, or *fyn*-SH2/SH3 domains and were then used in precipitation experiments. p120/130 derived from in vitro–labeled anti-fyn precipitates were efficiently and equally recognized by the *fyn*-SH2 and the *fyn*-SH2/SH3 fusion proteins (Fig. 2 *A*, lanes *3* and *5*, respectively), but not by the *fyn*-SH3 domain (lane *4*) or GST alone (lane *2*). Identical results were obtained using p120/130 from anti-fyn precipitates from human T cells (data not shown). Anti-CD3–associated p120/130 was also recognized by the *fyn*-SH2 fusion protein (Fig. 2 *A*, lane *8*).

To further establish the interaction of the SH2 domain of p59$^{fyn(T)}$ with p120/130 as purified from freshly isolated lysates (without in vitro labeling), we carried out antiphosphotyrosine immunoblotting of *fyn*-SH2, *fyn*-SH3, and *fyn*-SH2/SH3 precipitates from total cell lysates (Fig. 2 *B*, lanes *2–5*). The *fyn*-SH2 domain precipitated a series of at least 10 polypeptides labeled on tyrosine residues (lane *3*). By contrast, the *fyn*-SH3 domain precipitated a distinct set of some four polypeptides (lane *4*), whereas the combination of *fyn*-SH2/SH3 proteins precipitated both sets of proteins (lane *5*). The p120/130 doublet was a prominent protein precipitated by the *fyn*-SH2 domain (lanes *3* and *5*).

In v-src transfected chicken embryo fibroblasts, several proteins have been identified that act as substrates for the activated form of pp60$^{c\text{-}src}$ (28). To determine whether p120/130 was related to any of these proteins, reprecipitation analysis

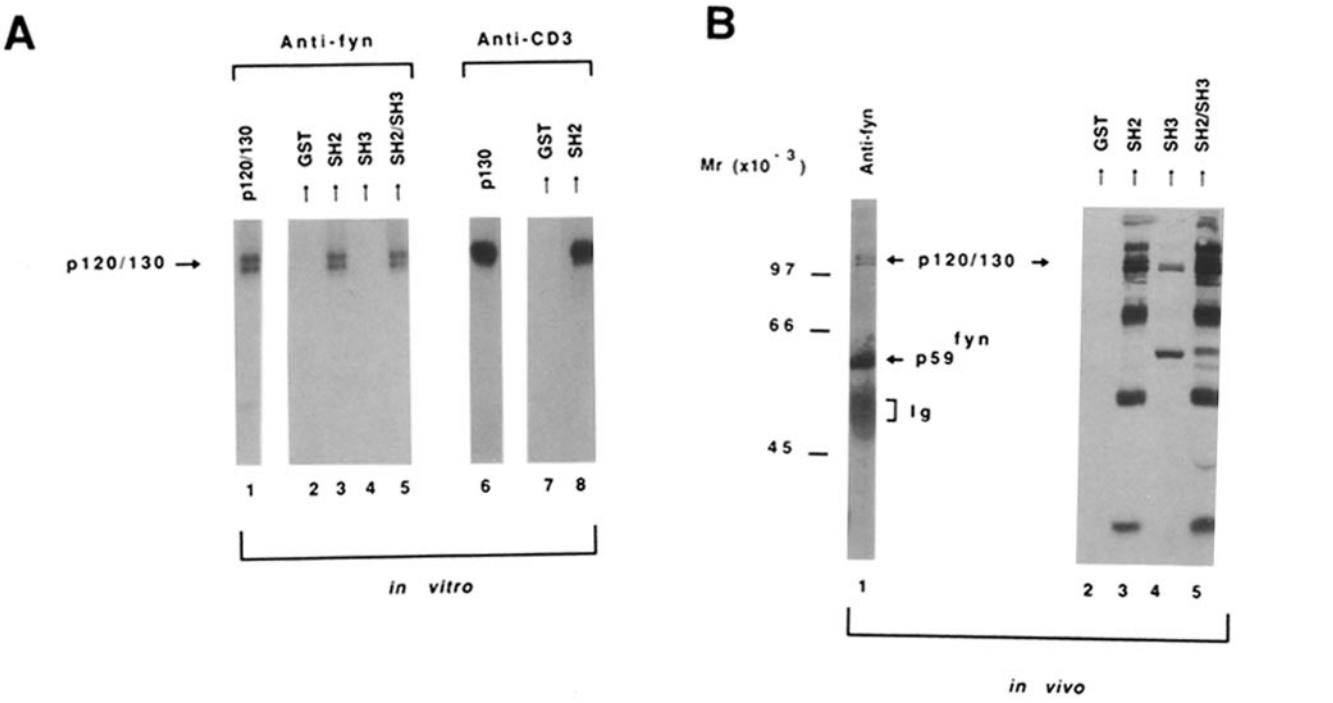

Figure 2. The SH2 domain of p59$^{fyn(T)}$ mediates binding of p120/130 to the TCR-ζ/CD3 complex. (*A*) Reprecipitation analysis of in vitro labeled p120/130. Phospholabeled anti-fyn (lanes *1–5*) or CD3 (lanes *6–8*) associated p120/130 material was eluted from polyacrylamide gels and equal aliquots subjected to precipitation with Glutathione *S*-Transferase (*GST*) fusion proteins expressing the SH2 and/or SH3 domains of fyn. Lanes *1* and *6* are control aliquots of the gel purified protein before reprecipitation. (*B*) Anti-phosphotyrosine immunoblotting. Lysates from 50 × 10^6 DC27.10 cells were prepared with 1% NP-40 lysis buffer and incubated with anti-fyn (lane *1*) and GST fusion proteins (lanes *2–5*).

was conducted on ^{32}P-labeled p120/130 eluted from SDS-PAGE. As shown in Fig. 3 *A*, the p120/130 was directly recognized by a mAb (termed 4F4) raised against the p130 molecule (lane *5*), but not by 2B12 (against a distinct p120) (lane *3*) or by 2A7 (anti-p125^{fak}) (lane *4*). The specificity of this recognition was further established by the failure of two isotype-matched (IgM) control Abs, anti-CD6 (lane *6*), and anti-class I (lane 7), to recognize purified p120/130. Similar results were obtained with anti-CD3–associated material (lanes *8–10*). Identical results were also obtained from fyn-associated p120/130 material purified from human T lymphocytes and Jurkat cells (data not shown). The 4F4 Ab also recognized the SH2-*fyn*–associated p120/130 from cell lysates (Fig. 3 *B*, lane *5*). Similarly, antiphosphotyrosine Abs and GST-*fyn*-SH2 domains precipitated the protein (lanes *8* and 7, respectively). The negative controls GST and RaM failed to precipitate material (lanes *6* and *4*, respectively). In a reciprocal experiment, p120/130 material immunoprecipitated by the 4F4 Ab could be reprecipitated by the SH2-*fyn* but not by SH3-*fyn* (data not shown).

Having established p120/130 as a phosphotyrosine containing SH2 binding protein, we next attempted to determine whether there was an increase in *fyn*-SH2 recognition of the phosphotyrosine labeled p120/130 due to TCR-ζ/CD3 ligation. As shown in Fig. 3 *C*, anti-CD3 stimulation induced an increase in the levels of tyrosine phosphorylation of p120/130 as detected by antiphosphotyrosine immunoblotting against whole cell lysates (lanes *1* and *2*) and in precipitates with anti-CD3 (lanes *3* and *4*), anti-fyn (lanes *5* and *6*), and anti-p130 (lanes 7 and *8*). Significantly, TCR-ζ/CD3 stimulation also resulted in increased detection of tyrosine phosphorylated p120/130 in SH2-*fyn* precipitates (lanes *9* and *10*). These observations are consistent with the results obtained with the in vitro labeled p120/130 (Fig. 1). Whether increased detection of tyrosine phosphorylated p120/130 results from increased recruitment to the TCR-ζ/CD3:p59$^{fyn(T)}$ complex and/or increased phosphorylation of pre-bound p120/130 remains to be determined.

Discussion

In this study, we have defined a potential downstream target bound to p59$^{fyn(T)}$ and the associated TCR-ζ/CD3 complex. We had previously shown that p120/130 associates directly with p59^{fyn} in fibroblasts (14). In this study, we have mapped the site of p120/130 binding to the SH2 domain of p59$^{fyn(T)}$. p59$^{fyn(T)}$ is therefore organized into functionally discrete motifs that can mediate binding to both its receptor and downstream intracellular proteins. p59$^{fyn(T)}$ associates with the TCR ζ and CD3 γ, δ, and ε chains via its NH_2-terminal 10 amino acids (11) and to the phosphatidylinositol (PI) 3-kinase via its SH3 domain (32). The *Fyn*-SH2 domain is still available to bind and recruit p120/130. This was demonstrated by the fact that receptor-bound p59$^{fyn(T)}$ is capable of

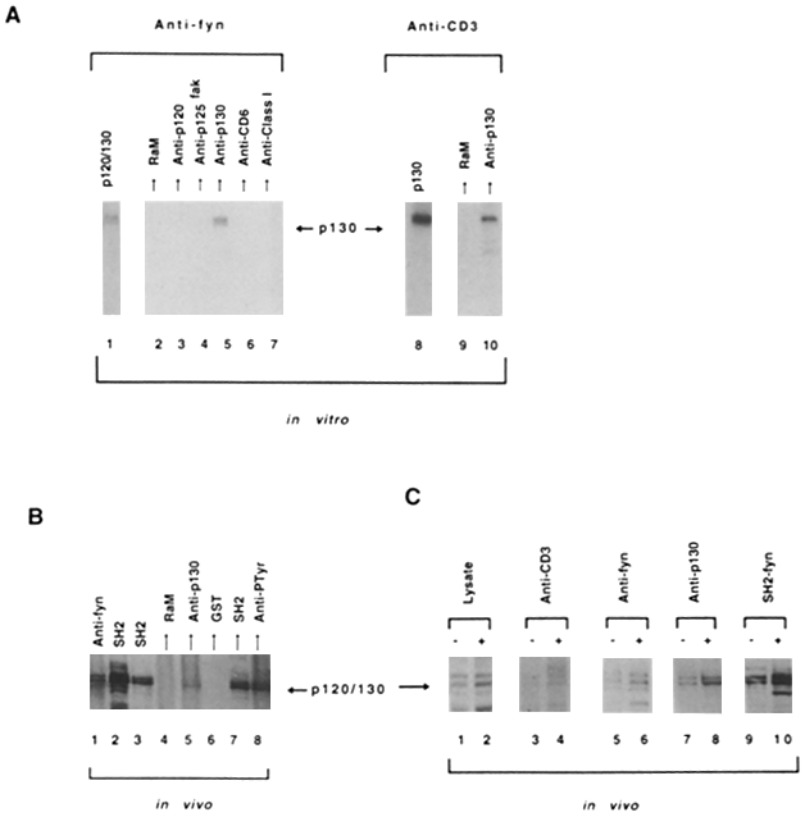

Figure 3. p120/130 corresponds to a v-src substrate from chicken fibroblasts and is tyrosine phosphorylated in response to TCR-ζ/CD3 ligation. (*A*) Reprecipitation analysis of in vitro labeled p120/130. Phospholabeled anti-fyn (lanes *1–7*) or CD3 (lanes *8–10*) associated p120/130 material was eluted from polyacrylamide gels and equal aliquots subjected to precipitation with rabbit anti-mouse (lanes *2* and *9*), anti-p120 (lane *3*), anti-p125^{fak} (lane *4*), anti-p130 (lanes *5* and *10*), anti-CD6 (lane *6*), and anti-class I (lane 7). Lanes *1* and *8* are control aliquots of the gel-purified protein before reprecipitation. (*B*) Anti-p130 recognition of SH2-associated p120/130. Cell lysates were precipitated with anti-fyn (lane *1*) or GST-SH2(*fyn*) fusion protein (lanes *2–8*). (Lanes *3–8*) After precipitation with GST-SH2(*fyn*), complexes were dissociated by boiling for 5 min in 3% SDS and equal aliquots reprecipitated with rabbit anti-mouse (lane *4*), anti-p130 (lane *5*), GST (lane *6*), GST-SH2(*fyn*) (lane 7), and antiphosphotyrosine (lane *8*). Lane *3* is a control aliquot of the solubilized p120/130 before reprecipitation. Samples were separated by SDS-PAGE, transferred to nitrocellulose, and probed with antiphosphotyrosine. (*C*) TCR-ζ/CD3 complex was cross-linked with anti-CD3 (+ samples) for 10 min and the cells solubilized in 1% NP-40 (Brij 96 for *anti-CD3*) lysis buffer. After incubation of lysates (lanes *1* and *2*) with anti-CD3 (lanes *3* and *4*), anti-fyn (lanes *5* and *6*), anti-p130 (lanes 7 and *8*) or GST-SH2(fyn) (lanes *9* and *10*), the samples were separated by SDS-PAGE, transferred onto nitrocellulose, and probed with antiphosphotyrosine Ab.

coprecipitating p120/130 (Fig. 3 *C*, lanes *3* and *4*). $p59^{fyn}$ association with p120/130 in fibroblasts showed that the $p59^{fyn}$-p120/130 association can occur independent of TCR-ζ/CD3 receptor complex expression (14). Furthermore, p120/130 was found associated with $p59^{fyn(T)}$ from cell lysates depleted by anti-CD3 Ab (data not shown). Preassembly of a portion of $p59^{fyn(T)}$-p120/130 in a complex, before its association with the TCR-ζ/CD3 complex is therefore likely to occur. Whether the NH_2-terminal binding to TCR-ζ/CD3 further influences the ability of the *fyn*-SH2 to bind p120/130 is unclear.

The fyn-SH2:p120/130 interaction may serve as a model in understanding mechanisms by which receptor-associated src-related kinases use SH2 domains in the recruitment of downstream signaling molecules. Limited SH2 mediated binding to PI 3-kinase has also been noted; however, it occurs only subsequent to TCR-ζ/CD3 ligation (32). By contrast, p120/130 binding to fyn-SH2 was detected both before and after receptor ligation. The mechanism of receptor-substrate binding is clearly different from that used by receptor tyrosine kinases such as the PDGF-R. Whereas binding to both types of receptor is regulated by tyrosine phosphorylation, the association with receptor tyrosine kinases such as the PDGF-R is regulated by autophosphorylation between homologous receptors. Distinct autophosphorylation sites serve to bind SH2 domains of multiple molecules such as the PI-3 kinase, *ras*GAP, PLCγ, NCK, SHC, and $p59^{fyn}$ itself (22, 23, 36). By contrast, in the case of TCR-ζ/CD3-$p59^{fyn(T)}$, the receptor-associated *src* kinase complex defines the specificity of binding by virtue of its own SH2 domain. Thus, since a given *fyn*-SH2 domain can bind only a single phosphotyrosine carrying polypeptide at a time, the nature of the SH2-mediated intracellular signal from a given TCR-ζ/CD3-$p59^{fyn(T)}$ receptor is likely to be more restricted and specific.

The structure and function of p120/130 remain to be determined. Proteins within a similar molecular weight range have been found to associate with activated forms of other *src* kinases (37), and with the EGF-R via a transforming isoform of *v-crk* (38). *V-crk* is a SH2 carrying adaptor protein derived from CT10 avian sarcoma virus. Both v-crk and its NH_2-terminal SH2 domain bind to a denatured fibroblast protein at 130 kD (38). Reprecipitation analysis (Fig. 3) indicated that p120/130 corresponds to a 130-kD substrate of transforming form of $pp60^{c-src}$ in v-src transfected chicken embryo fibroblasts (28). In other studies (39) the same authors further revealed that SH2 deletion mutants of v-src failed to bind to p130. Our studies show that the fyn-SH2 domain alone is sufficient to mediate the interaction. Interestingly, in nontransfected fibroblasts, p130 was primarily detected in the nucleus, whereas transformation by v-src resulted in an apparent translocation of p130 to the plasma membrane (39). We have further shown that p120/130 is coupled to a surface receptor, and as such is likely to play a role in receptor mediated signaling. Consistent with this, p120/130 undergoes rapid tyrosine phosphorylation upon TCR-ζ/CD3 ligation (Figs. 1 and 3). The structure of the 130-kD polypeptide recognized by this Ab has yet to be established. Antibodies to PLCγ and GTP'ase-activating protein have failed to precipitate the p120/130 molecules (data not shown). The presence of fyn-associated p120/130 in fibroblasts (14) suggests a signaling function for this molecule that is common to mammalian cells. Other studies have shown that in transfected NIH-3T3 fibroblasts expressing human muscarinic receptors, stimulation with carbachol but not PDGF, leads to increased tyrosine phosphorylation of p130 and $p125^{fak}$ (40), suggesting that p120/130 may also be involved in a signaling from G-protein coupled receptors. We are presently attempting to determine the structure of p120/130.

Given the fact that several receptor-associated *src*-related kinases ($p56^{lck}$, $p59^{fyn}$, $p62^{yes}$) are expressed in T cells, it will be important to identify the downstream targets that are specifically linked to one kinase relative to another. In this sense, it is interesting that p120/130 was found to be preferentially associated with $p59^{fyn(T)}$, as detected by in vitro labeling (Fig. 1). Whereas accepting the unlikely possibility that p120/130 may be resistant to labeling by $p56^{lck}$, these data argue that p120/130 preferentially associates with $p59^{fyn(T)}$ in T cells. p120/130 may therefore serve as a marker for signals derived specifically from $p59^{fyn(T)}$. The mechanism for this preferential association is still unclear. Preliminary data have shown that GST *lck*-SH2 fusion proteins are capable of reacting with p120/130 (data not shown). Other regions of the kinase such as the NH_2-terminal region or SH3 domain may influence the ability of the SH2 domains to interact with substrate. Indeed, we have recently demonstrated that SH3 mediated binding to PI-3 kinase is altered by the presence of an adjacent SH2 domain (32).

Current evidence suggests that TCR-ζ/CD3 complex is a multimeric complex with the capacity to generate distinct signals emanating from the TCR ζ chains and various CD3 chains (41, 42). Nevertheless, in both cases, receptor ligation induced the tyrosine phosphorylation of a prominent 120-kD protein. This is consistent with the report that $p59^{fyn(T)}$ can associate with the TCR-ζ and various CD3 chains (11), and introduces the possibility that p120/130 may be used in both pathways of activation. In addition, $p59^{fyn(T)}$ negative transgenic mice showed a marked reduction in the phosphorylation of a 130-kD band in TCR-ζ/CD3 stimulated thymocytes, providing further suggestive evidence for the involvement of p120/130 in the *fyn*-generated signals (18).

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