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$p56^{Lck}$ and $p59^{Fyn}$ regulate CD28 binding to phosphatidylinositol 3-kinase, growth factor receptor-bound protein GRB-2, and T cell-specific protein-tyrosine kinase ITK: Implications for T-cell costimulation

(son of sevenless/T-cell anergy)

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ABSTRACT **T-cell activation requires cooperative signals generated by the T-cell antigen receptor ζ-chain complex (TCRζ–CD3) and the costimulatory antigen CD28. CD28 interacts with three intracellular proteins—phosphatidylinositol 3-kinase (PI 3-kinase), T cell-specific protein-tyrosine kinase ITK (formerly TSK or EMT), and the complex between growth factor receptor-bound protein 2 and son of sevenless guanine nucleotide exchange protein (GRB-2–SOS). PI 3-kinase and GRB-2 bind to the CD28 phosphotyrosine-based Tyr-Met-Asn-Met motif by means of intrinsic Src-homology 2 (SH2) domains. The requirement for tyrosine phosphorylation of the Tyr-Met-Asn-Met motif for SH2 domain binding implicates an intervening protein-tyrosine kinase in the recruitment of PI 3-kinase and GRB-2 by CD28. Candidate kinases include $p56^{Lck}$, $p59^{Fyn}$, ζ-chain-associated 70-kDa protein (ZAP-70), and ITK. In this study, we demonstrate in coexpression studies that $p56^{Lck}$ and $p59^{Fyn}$ phosphorylate CD28 primarily at Tyr-191 of the Tyr-Met-Asn-Met motif, inducing a 3- to 8-fold increase in p85 (subunit of PI 3-kinase) and GRB-2 SH2 binding to CD28. Phosphatase digestion of CD28 eliminated binding. In contrast to Src kinases, ZAP-70 and ITK failed to induce these events. Further, ITK binding to CD28 was dependent on the presence of $p56^{Lck}$ and is thus likely to act downstream of $p56^{Lck}$/$p59^{Fyn}$ in a signaling cascade. $p56^{Lck}$ is therefore likely to be a central switch in T-cell activation, with the dual function of regulating CD28-mediated costimulation as well as TCR–CD3-CD4 signaling.**

Optimal T-cell proliferation requires an antigen-specific signal generated by the T-cell antigen receptor (TCR)-CD3 and CD4–CD8-$p56^{Lck}$ complexes, followed by a second signal delivered by the CD28 antigen (1–4). The CD28 ligands CD80 (B7-1) and CD86 (B7-2/B70) are differentially expressed on presenting cells such as activated B cells, dendritic cells, and monocytes (5–7). In certain instances, antigen presentation without CD28 ligation induces nonresponsiveness to further challenge with antigen (1, 8–10). CD28 cosignals stabilize mRNA for interleukin 2 (IL-2) (11, 12) and induces the expression of cytotoxic T lymphocyte-associated protein 4 (CTLA-4) (2) and the CD40 ligand (13). CD28- and B7-1-deficient mice exhibit partial defects in immune function (14, 15). CD28 can regulate cytolytic responses against tumors (16–18) and induce autoimmune insulitis in transgenic models (19, 20).

Although the molecular basis of CD28 cosignaling is unclear, it binds to the intracellular proteins phosphatidylinositol 3-kinase (PI 3-kinase), T cell-specific protein-tyrosine kinase ITK (formerly EMT or TSK), and the complex between growth factor receptor-bound protein 2 and son of sevenless guanine nucleotide exchange protein (GRB-2–SOS) (21–25). PI 3-kinase and GRB-2 Src-homology 2 (SH2) domains bind to a phosphorylated version of the Tyr-Met-Asn-Met (YMNM) motif within CD28 (21, 22, 25). PI 3-kinase consists of a p85 adapter subunit coupled to a p110 catalytic subunit (26). The p85 C- and N-terminal SH2 domains bind the phosphorylated CD28 YMNM motif with an affinity comparable to the platelet-derived growth factor receptor. The C-terminal p85 SH2 domain bound to the CD28 phosphorylated YMNM motif with some 10-fold greater affinity than the N-terminal SH2 domain (21). Mutation of Tyr-191 in CD28 abrogates the induction of IL-2 by anti-CD28 antibody (27). The targets of PI 3-kinase may include protein-kinase Cζ (28), $p21^{ras}$ (29), and $p70^{S6K}$ (30).

Similarly, GRB-2 is an adapter protein with one SH2 domain that binds to the phosphorylated YMNM motif within CD28 (31). At the same time, GRB-2 binds to SOS through its SH3 domains (31). SOS converts $p21^{ras}$ into an active state by exchanging GDP for GTP (32). The GRB-2 SH2 domain binds to the CD28 phosphorylated YMNM motif with lower affinity than the PI 3-kinase SH2 domain. Nevertheless, both can be detected in association with CD28 in response to ligation. GRB-2–SOS binding to CD28 potentially implicates this surface receptor in the regulation of $p21^{ras}$ and downstream targets such as Raf-1, mitogen-activated protein kinase (ERK 1,2), and Jun kinase (33).

Nonreceptor tyrosine kinases can interact with surface receptors, as initially shown with CD4-$p56^{Lck}$ (4). Similarly, the T cell-specific protein-tyrosine kinase ITK has been reported to bind to CD28 (24). While the general structure of ITK is similar to members of the Src family (with a unique N terminus, a SH3 and a SH2 domain, and a kinase domain), it shares greatest homology with members of the Tec family (34–36). It is restricted to T cells, being expressed at its highest levels in the thymus (36). CD28 crosslinking has been reported to both activate and recruit ITK from the cytoplasm (24). The mechanism underlying the interaction has yet to be established.

The requirement for tyrosine phosphorylation of the YMNM motif for SH2 domain binding implicates an inter-

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Abbreviations: PI 3-kinase, phosphatidylinositol 3-kinase; GRB-2, growth factor receptor-bound protein 2; SOS, son of sevenless; TCR, T-cell antigen receptor; ZAP-70, 70-kDa ζ chain-associated protein; YMNM, Tyr-Met-Asn-Met motif; SH2 and SH3, Src-homology domains 2 and 3; HA, hemagglutinin; Tyr(*P*), phosphotyrosine; mAb, monoclonal antibody.

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vening protein-tyrosine kinase in the recruitment of PI 3-kinase and GRB-2 to CD28. Various kinases in T cells ($p56^{Lck}$, $p59^{Fyn}$, ZAP-70, ITK) could fulfill this function. Here, we demonstrate that the kinases $p56^{Lck}$ and $p59^{Fyn}$ phosphorylate CD28 at Tyr-191 and induce PI 3-kinase, GRB-2, and ITK binding to the receptor. ZAP-70 and ITK did not mediate this event. $p56^{Lck}$ and $p59^{Fyn}$ have the dual function of regulating TCRζ–CD3-CD4 signaling, and CD28 induced costimulation.

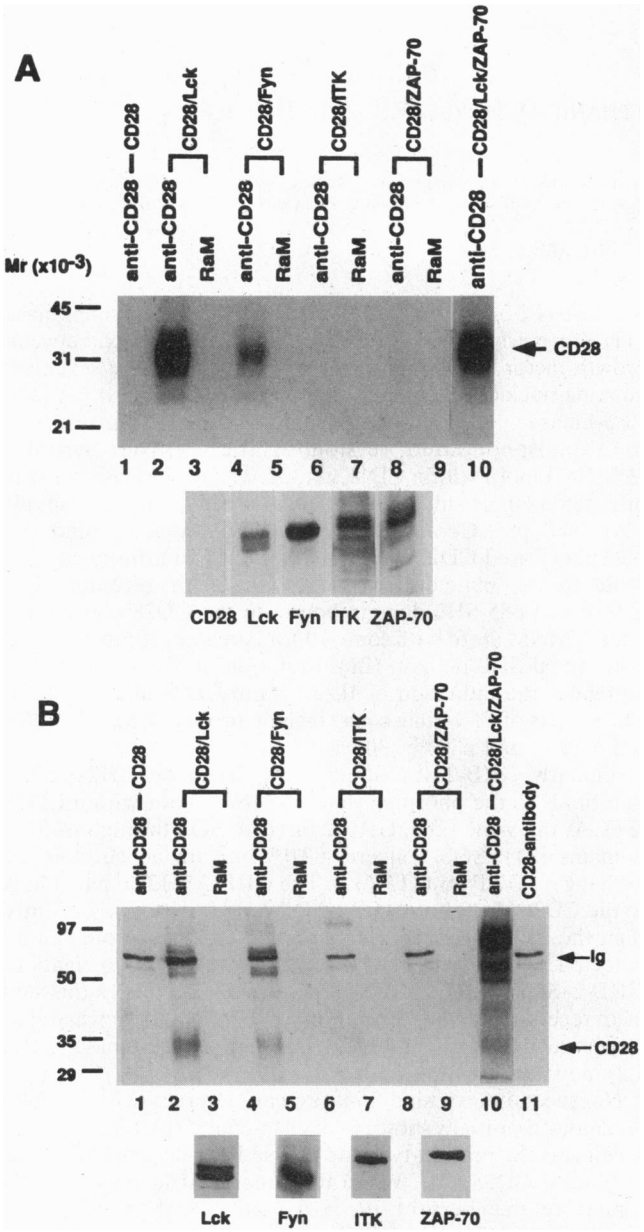

FIG. 1. (*A* and *B Upper*) CD28 phosphorylation *in vitro* (*B*) and *in vivo* (*A*) by the Src-related protein-tyrosine kinases $p56^{Lck}$ and $p59^{Fyn}$. Sf21 cells infected with virus carrying cDNAs encoding CD28, Lck, ITK, Fyn, ZAP-70, or combinations thereof were lysed in 1% Triton X-100 lysis buffer, immunoprecipitated with anti-CD28 antibody (lanes 1, 2, 4, 6, and 8) or rabbit anti-mouse antibody (RaM) (lanes 3, 5, 7, and 9) and labeled in a phosphotransferase assay (*A*), or immunoblotted with an anti-Tyr (P) antibody (*B*). Lanes show cells expressing CD28 (lane 1), CD28/Lck (lanes 2 and 3), CD28/Fyn (lanes 4 and 5), CD28/ITK (lanes 6 and 7), CD28/ZAP-70 (lanes 8 and 9), and CD28/Lck/ZAP-70 (lane 10). Positions of molecular mass markers (kDa) are indicated. (*A Lower*) *In vitro* autokinase assay of anti-CD28, anti-Lck, anti-Fyn, anti-ITK, and anti-ZAP-70 precipitates from cells infected with virus containing CD28, Lck, Fyn, ITK, and ZAP-70 cDNAs, respectively. (*B Lower*) Lysates prepared from 1.5×10^5 cells transfected with the indicated kinases were immunoblotted with anti-kinase-specific antibodies.

MATERIALS AND METHODS

Monoclonal Antibodies (mAbs), Antisera, and Cells. Various antibodies were used: anti-CD28 (L293) (Becton Dickinson), anti-Tyr(*P*) mAb (4G10) (B. Druker and T. Roberts, Dana–Farber Cancer Institute), rabbit anti-mouse antibody (Dako), and anti-hemagglutinin (anti-HA) (12CA5) (Harvard Cell Culture Facility). *Spodoptera frugiperda* (Sf) cells (IPLB-SF21) were propagated as described (37).

cDNA Cloning. cDNAs encoding CD28, p85, (α isoform) and GRB-2 were prepared by PCR with specific oligonucleotides that included restriction sites for subcloning into the transfer vector pVL1393. To obtain recombinant viruses, transfection of *Spodoptera frugiperda* cells was performed as described (37). Infection of Sf21 cells for protein production was performed as described (37). For alkaline phosphatase treatment, lysates were incubated with 50 units of phosphatase (Boehringer Mannheim) in 0.5 M Tris·HCl/1 mM EDTA, pH 8.5, for 1 hr at 37°C.

Immunoprecipitation and Immunoblotting. Immunoprecipitation and immunoblotting were carried out as described (37). For blotting, membranes were blocked with 5% (wt/vol) milk in TBS (10 mM Tris, pH 7.6/150 mM NaCl) and probed with mAb [anti-Tyr(*P*), anti-p85, anti-GRB-2, or anti-HA antiserum] followed by horseradish peroxidase (HRP)-

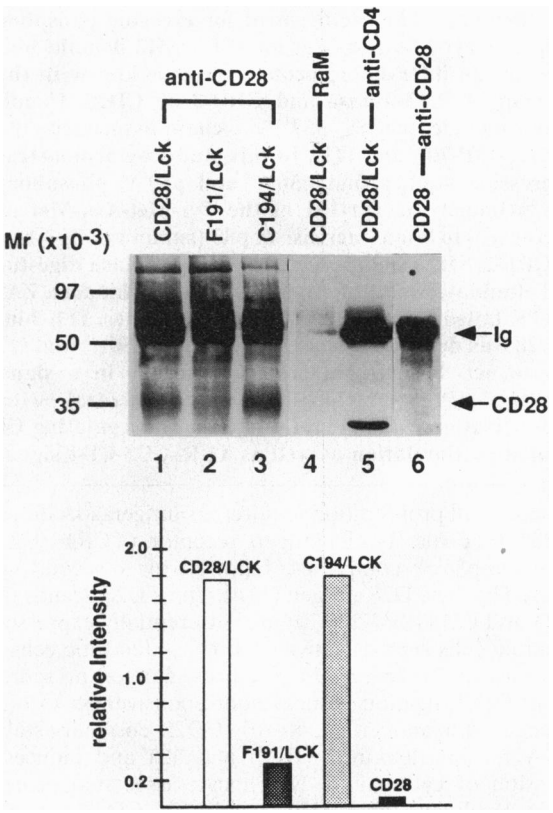

FIG. 2. CD28 is phosphorylated at Tyr-191 within the YMNM motif. (*Upper*) Wild-type CD28 or mutant CD28 with Tyr-191 → Phe or Met-194 → Cys changes were coexpressed with Lck in insect cells, immunoprecipitated with anti-CD28 antibody (lanes 1 and 3) and immunoblotted with an anti-Tyr(*P*) antibody. Lanes show cells expressing CD28/Lck (lanes 1, 4, and 5), CD28 Phe-191 mutant with Lck (lane 2); CD28 Cys-194 mutant with Lck (lane 3), and CD28 alone (lane 6). Rabbit anti-mouse antibody (RaM) (lane 4) and anti-CD4 precipitation (lane 5) are negative controls. (*Lower*) Densitometric analysis using the Scantjet laser scanner (Hewlett–Packard) of anti-Tyr(*P*) binding to CD28 in different cells.

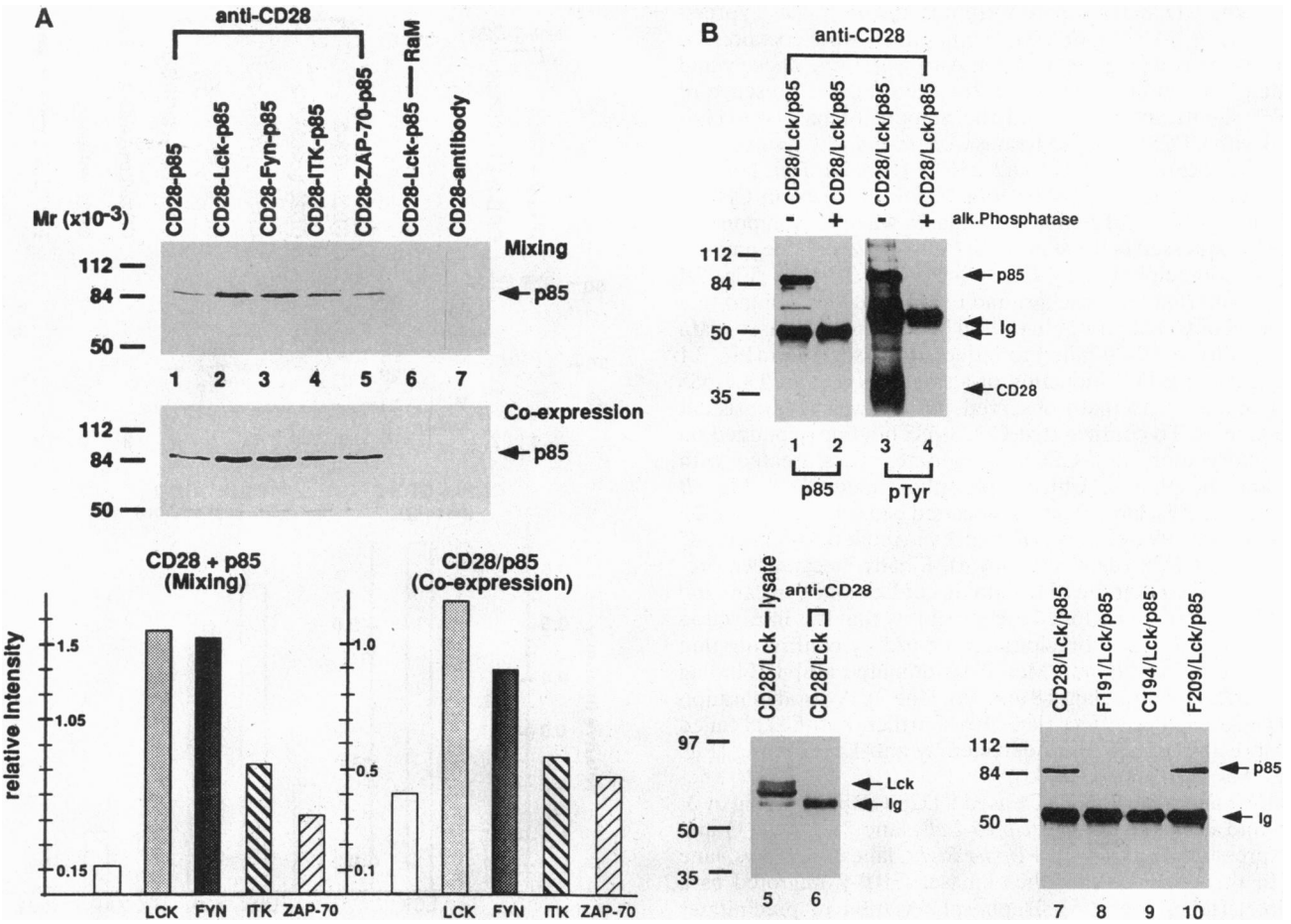

FIG. 3. $p56^{Lck}$ and $p59^{Fyn}$ induce CD28 recruitment of PI 3-kinase. (*A*) Lysates from Sf21 cells infected with virus carrying cDNAs encoding CD28 (lane 1), CD28/Lck (lane 2), CD28/Fyn (lane 3), CD28/ITK (lane 4), and CD28/ZAP-70 (lane 5) were combined with the lysate of Sf21 cells expressing p85 alone (mixing) (*A Top*) or the Sf21 cells were also infected with the virus carrying the cDNA encoding p85 (coexpression) (*A Middle*). After mixing or cell lysis, respectively, immunoprecipitates with anti-CD28 antibody (lanes 1–5) or rabbit anti-mouse antibody (RaM) (lane 6) were prepared and subjected to immunoblotting with anti-p85 antiserum. Lane 7 shows the anti-CD28 antibody control. (*A Bottom*) Densitometric analysis of the mixing and coexpression experiments (*Top* and *Middle*). (*B Upper*) Sf21 cells coinfected with viruses carrying CD28, Lck, and p85 cDNAs (lanes 1–4) were lysed and treated with or without 50 units of alkaline phosphatase (Boehringer Mannheim) (lanes 2 and 4) for 1 hr at 37°C, followed by immunoprecipitation and immunoblotting with anti-p85 antibody (lanes 1 and 2) or anti-Tyr(*P*) antibody (lanes 3 and 4). (Different immunoglobulin positions are due to different gels.) Alternatively, lysates from cells expressing CD28 and Lck were lysed and blotted with anti-Lck (lane 5) or were first immunoprecipitated with anti-CD28 and then blotted with anti-Lck (lane 6). (*B Lower Right*) Finally, Lck and p85 were coexpressed with wild-type CD28 (lane 7) or with CD28 mutants containing Phe-191 (lane 8), Cys-194 (lane 9), or Phe-209 (lane 10) in Sf21 cells; precipitated with anti-CD28; and subjected to anti-p85 blotting (lanes 7–10).

conjugated rabbit anti-mouse or donkey anti-rabbit antibodies for detection by enhanced chemiluminescence (Amersham).

RESULTS

A major question concerns the nature of the protein-tyrosine kinase responsible for phosphorylating CD28 and regulating the recruitment of downstream proteins. To assess this, $p56^{Lck}$, $p59^{Fyn}$, ITK, and ZAP-70 were individually expressed with CD28 in insect cells and assessed for an ability to phosphorylate CD28 *in vitro*. $p56^{Lck}$ and $p59^{Fyn}$ were the only kinases found to phosphorylate CD28 (Fig. 1*A Upper*, lanes 2 and 4). ITK and ZAP-70 failed to phosphorylate the antigen (lanes 6 and 8), despite the fact that the kinases were active as assessed by *in vitro* autophosphorylation (Fig. 1*A Lower*). Overexposure of gels failed to show phosphorylated CD28 (data not shown). $p56^{Lck}$ was also coexpressed with ZAP-70, but this failed to show further enhancement of CD28 phosphorylation beyond that due to $p56^{lck}$ alone (lane 10). *In vivo* phosphorylation was conducted by immunoblotting with an anti-Tyr(*P*) mAb. Again, CD28 became tyrosine-phosphorylated when coexpressed with $p56^{Lck}$ or $p59^{Fyn}$ (Fig. 1*B Upper*, lanes 2 and 4) but not with ITK or ZAP-70 (lanes 6 and 8). Occasionally, ITK induced weak CD28 phosphorylation, but this was not consistent (lane 6). Immunoblotting of cell lysates with antisera specific for these kinases showed that $p56^{Lck}$, $p59^{Fyn}$, ITK, and ZAP-70 were comparably expressed (Fig. 1*B Lower*).

CD28 possesses a phosphorylated binding motif YMNM [Tyr(*P*)-Met-Asn-Met; residues 191–194 from the start codon and 173–176 in the mature protein] for the SH2 domains of PI 3-kinase and GRB-2 (21, 25). Mutation of Tyr-191 interferes with PI 3-kinase and GRB-2 binding (21, 25). The CD28 Tyr-191 → Phe mutant when coexpressed with $p56^{Lck}$ showed greatly reduced phosphorylation (by about 85%) when compared with wild-type CD28 (Fig. 2, lane 2 vs. lane 1). By contrast, the CD28 Met-194 → Cys mutant became similarly phosphorylated relative to wild type (lane 3). CD28 without coexpressed $p56^{Lck}$ underwent little if any tyrosine phosphorylation (lane 6). Hence, $p56^{Lck}$ phosphorylates CD28 at Tyr-191 and, to a lesser extent, other sites. Rabbit anti-mouse and anti-CD4 antibodies served as negative controls (lanes 4 and 5).

$p56^{Lck}$ and $p59^{Fyn}$ phosphorylation at Tyr-191 suggested that they might regulate CD28 binding to intracellular proteins.

Two approaches were taken: (*i*) either lysates from cells expressing CD28 and kinase were mixed with lysates expressing p85 (Fig. 3*A Top*), or (*ii*) all components were coexpressed in the same cell (Fig. 3*A Middle*). Although some background binding was observed (Fig. 3*A Top*, lane 1), the presence of $p56^{Lck}$ significantly increased the amount of p85 coprecipitated with CD28 when p85 lysates were mixed with lysates from cells that expressed CD28 and $p56^{Lck}$ (Fig. 3*A Top*, lane 2). Densitometric readings showed a 10-fold increase in CD28–p85 binding (Fig. 3*A Bottom*). Similarly, when all components were coexpressed in the same cell (Fig. 3*A Middle*), the amount of p85 coprecipitated by CD28 increased 3.5-fold (Fig. 3*A Middle* and *Bottom*). Background binding may be related to a low level of CD28 phosphorylation by insect cell kinases (data not shown). ZAP-70 failed to induce the association (Fig. 3*A Upper*, lane 5); ITK induction of a small degree of CD28-p85 binding was occasionally observed, but this was not consistent (see lane 4). To confirm that CD28-p85 binding depended on phosphorylation, anti-CD28 precipitates were treated with alkaline phosphatase, which dephosphorylated CD28 (Fig. 3*B Upper*, lane 3 vs. lane 4) and dissociated p85 (lane 1 vs. lane 2). A kinase-inactivated form of $p56^{Lck}$ also failed to induce p85 binding to CD28 (data not shown). Finally, because we previously showed that the SH3 domains of Lck and Fyn can bind to p85 (38, 39), we excluded the possibility that this interaction contributed to the coprecipitation of p85 by confirming that mutation at Tyr-191 and Met-194 eliminated all p85 binding (Fig. 3*B Lower Right*, lanes 8 and 9 vs. lane 7). A distal mutation at Tyr-209 had no effect (lane 10). Further, anti-CD28 failed to coprecipitate $p56^{Lck}$, as detected by anti-Lck blotting (Fig. 3*B Lower Left*, lanes 5 and 6).

$p56^{Lck}$ and $p59^{Fyn}$ also increased CD28-GRB-2 binding by 3- to 6-fold in mixing (Fig. 4 *Upper Left*, lane 2 vs. lane 1) and coexpression studies (Fig. 4 *Upper Right*, lanes 4 and 5 vs. lane 3). In the presence of either kinase, GRB-2 migrated as a doublet (lanes 4 and 5). Dephosphorylation of precipitates with alkaline phosphatase eliminated GRB-2 binding (data not shown). As a control, GRB-2 could not be detected in anti-Lck precipitates from cells coexpressing Lck and GRB-2 (lane 10). Neither ITK nor ZAP-70 were capable of inducing the association (lanes 6 and 7).

ITK has recently been reported to associate with CD28, although the basis of this interaction is not known (24). ITK was expressed with an influenza HA tag and recognized by an anti-HA mAb in immunoblots (Fig. 5, lane 1). In coexpression studies, its ability to bind to CD28 depended entirely on the presence of $p56^{Lck}$ (lane 3). Expression of CD28 and ITK alone has failed to induce its own association (lane 2). As a control, ITK was not detected in anti-Lck precipitates from cells coexpressing Lck and ITK (lane 5).

DISCUSSION

$p56^{Lck}$ and $p59^{Fyn}$ are closely linked to the initiation of signaling from the TCRζ–CD3 and CD4–CD8 complexes (4, 40–43). The baculovirus expression system has proven to be a powerful tool in dissecting signaling pathways (44). In this study, by reconstituting CD28 and interactive components, we demonstrate that $p56^{Lck}$ and $p59^{Fyn}$ phosphorylate CD28, leading to the recruitment of p85, GRB-2, and ITK. Increased CD28-p85, CD28-GRB-2, and CD28-ITK binding was observed by coexpressing CD28 with $p56^{Lck}$ prior to mixing with ligand or by expressing all components in the same cell (Figs. 2–4). $p56^{Lck}$ and $p59^{Fyn}$ increased binding by 3- to 10-fold. Tyr-191 of pYMNM was identified as the major site of $p56^{Lck}$ phosphorylation (Fig. 2), a result concordant with the use of this site for p85 and GRB-2 SH2 domain binding (21, 25). Phosphatase digestion confirmed that the interaction was phosphorylation-dependent (Fig. 3*B*). $p56^{Lck}$ and $p59^{Fyn}$ regulation of p85 and GRB-2 binding implicates these kinases in

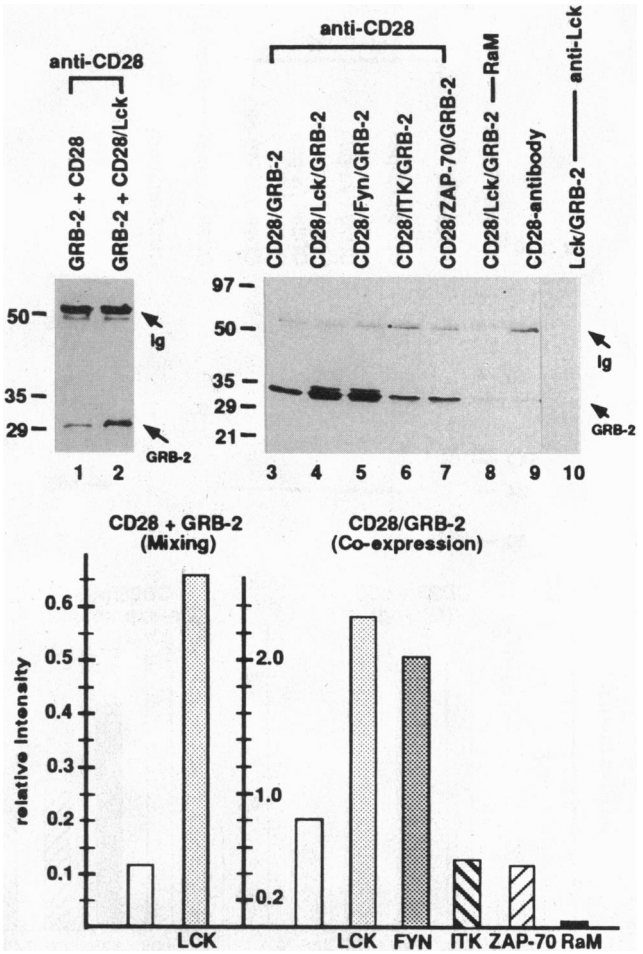

FIG. 4. $p59^{Lck}$ and $p59^{Fyn}$ phosphorylation of CD28 induces recruitment of GRB-2. (*Upper Left*) Lysates from cells infected with virus containing cDNAs encoding GRB-2 and CD28 (lane 1) or GRB-2 and CD28/Lck (lane 2) were combined and precipitated with anti-CD28 and subjected to anti-GRB-2 immunoblotting. (*Upper Right*) Alternatively, cells expressing CD28/GRB-2 (lane 3), CD28/Lck/GRB-2 (lanes 4 and 8), CD28/Fyn/GRB-2 (lane 5), CD28/ITK/GRB-2 (lane 6), CD28/ZAP-70/GRB-2 (lane 7) and Lck/GRB-2 (lane 10) were precipitated with anti-CD28 (lanes 3–7), rabbit anti-mouse antibody (RaM) (lane 8), or anti-Lck (lane 10) and blotted with anti-GRB-2. Lane 9 shows the anti-CD28 antibody control. (*Lower*) Densitometric profile of GRB-2 in anti-CD28 precipitates.

regulating CD28-mediated costimulation. These kinases act upstream of CD28 signaling in the T-cell activation cascade. Therefore, $p56^{Lck}$ acts as a central switch in T-cell activation, regulating TCRζ–CD3-CD4 signaling as well as costimulation via CD28.

In contrast to Src-related kinases, catalytically active ZAP-70 and ITK failed to reproducibly phosphorylate CD28 and induce intracellular ligand binding. Optimal ZAP-70 kinase activation has been shown to require $p56^{Lck}$ (45). Attempts to augment ZAP-70 activity by coexpression with $p56^{Lck}$ resulted in a CD28 phosphorylation equal to that of $p56^{Lck}$ alone (Figs. 1 and 2). Receptor chimeras of ZAP-70 phosphorylate numerous other proteins (46). In terms of ITK, although we had difficulty detecting CD28-associated ITK in T-cells (data not shown), we confirmed binding in insect cells (Fig. 5). Occasionally, ITK induced weak but nonreproducible CD28 phosphorylation. In fact, the binding of ITK itself to CD28 was entirely dependent on $p56^{Lck}$ (Fig. 5). The site of ITK binding is unknown; however, $p56^{Lck}$ phosphorylated the Tyr-191 mutant (albeit much reduced compared with wild type), denoting the presence of other possible phosphorylation

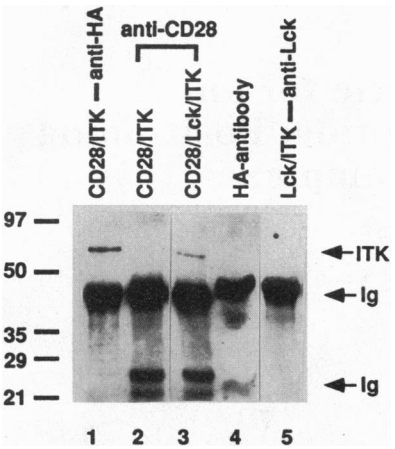

FIG. 5. $p56^{Lck}$ phosphorylation of CD28 induces recruitment of ITK. Sf21 cells were coinfected with viruses containing cDNAs for CD28/ITK (lanes 1 and 2), CD28/Lck/ITK (lane 3), or Lck/ITK (lane 5). After cell lysis, immunoprecipitates were prepared with anti-HA (lane 1), anti-CD28 (lanes 2 and 3) or anti-Lck (lane 5) and subjected to immunoblotting with anti-HA antiserum. Lane 4 shows the HA-antibody control.

sites. ITK binding to CD28 is likely to act downstream of $p56^{Lck}$ and $p59^{Fyn}$ in a manner analogous to the binding of ZAP-70 to TCRζ (40). After being recruited by CD28, ITK is likely to phosphorylate other targets. Consistent with an involvement of tyrosine kinases, inhibitors block CD28-mediated costimulation (47).

$p56^{Lck}$ and $p59^{Fyn}$ are predicted to indirectly regulate costimulation and CD28 generation of D-3 lipids by associated PI 3-kinase (48). This pathway may operate in parallel with $p56^{Lck}$ and $p59^{Fyn}$ SH3 domain recruitment of PI 3-kinase (38, 39). Src kinase regulation of CD28 binding to GRB-2–SOS or PI 3-kinase may indirectly influence the activation of $p21^{ras}$, mitogen-activated protein kinase (49), and Jun kinase (50). $p56^{Lck}$ and $p59^{Fyn}$ binding to CD4 and CD8 (41–43) and to the TCRζ–CD3 complex (44) raises the issue as to whether these kinases regulate CD28 signaling as receptor-bound or free kinases. CD5-CD28 activation of T cells (51) could be mediated by CD5-$p56^{Lck}$ (37). However, current evidence suggests that CD28 acts as an independent signaling unit. Ligation of CD28 in the absence of TCR ligation or TCR expression induces phosphorylation and PI 3-kinase recruitment (21, 22, 25, 52). Direct CD28-$p56^{Lck}$ binding was not detected in Triton X-100 by blotting (Fig. 3), although CD28 coprecipitates $p56^{Lck}$ activity (ref. 52; Fig. 1*A*) and weakly stimulates $p56^{Lck}$ activity (53).

$p56^{Lck}$ regulation of CD28 signaling may account for reports implicating Src kinases in T-cell anergy. Mizoguchi and colleagues have reported that nonresponsiveness of T cells to tumors is accompanied by reduced $p56^{Lck}$ expression (54). This defect could impair CD28 induction of antitumor responses observed in mice (16–18).

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