p56\textsuperscript{kk} Interacts Via its src Homology 2 Domain with the ZAP-70 Kinase

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Summary

p56\textsuperscript{kk}, a member of the src family of protein tyrosine kinases, is an essential component in T cell receptor (TCR) signal transduction. p56\textsuperscript{kk} contains a src homology 2 (SH2) domain found in a number of proteins involved in intracellular signaling. SH2 domains have been implicated in protein–protein interactions by binding to sequences in target proteins containing phosphorylated tyrosine. Using an in vitro assay, we have studied specific binding of tyrosine-phosphorylated proteins to a recombinant p56\textsuperscript{kk} SH2 domain. In nonactivated Jurkat cells, two tyrosine-phosphorylated proteins were detected. Stimulation with anti-CD3 monoclonal antibodies induced the binding of seven additional tyrosine-phosphorylated proteins to the SH2 domain of p56\textsuperscript{kk}. We have identified the \(\zeta\)-associated tyrosine kinase, ZAP-70, as one of these proteins. Evidence suggests that binding of ZAP-70 to p56\textsuperscript{kk} SH2 is direct and not mediated by \(\zeta\). The significance of this interaction was further investigated in vivo. p56\textsuperscript{kk} could be coprecipitated with the \(\zeta\)/ZAP-70 complex and conversely, ZAP-70 was detected in p56\textsuperscript{kk} immunoprecipitates of activated Jurkat cells. The physical association of p56\textsuperscript{kk} and ZAP-70 during activation supports the recently proposed functional cooperation of these two tyrosine kinases in TCR signaling.

Stimulation of the TCR induces a signal transduction cascade leading to activation of the mature T cell. One of the earliest biochemical events after TCR stimulation is the activation of protein tyrosine kinases (PTKs)\textsuperscript{1} resulting in tyrosine phosphorylation of a number of cellular proteins (1, 2). Some of these proteins identified to date are \(\zeta\) (3) and CD3 \(\epsilon, \gamma,\) and \(\delta\) subunits (4, 5), phospholipase C (PLC) \(\gamma\) (6, 7), valosin-containing protein (VCP) (8), Vav (9, 10), CD5 (11, 12), CD6 (13), mitogen-activated protein kinase (MAPK) (14), and \(\zeta\)-associated protein (ZAP-70) (15). Accumulating evidence supports the involvement of two members of the src family of tyrosine kinases, p56\textsuperscript{kk} and p59\textsuperscript{ff}, in TCR signal transduction. p56\textsuperscript{kk} is stably associated with the cytoplasmic portions of CD4 and CD8 (16, 17), and this interaction is required for optimal antigen stimulation (18, 19). Expression of a constitutively active form of p56\textsuperscript{kk} enhances antigen-stimulated IL-2 production in a T cell hybridoma (20). In addition, TCR-CD3 stimulation results in an increase of p56\textsuperscript{kk} kinase activity (21). Moreover, mutants of Jurkat and CTLL-2 cell lines which lack p56\textsuperscript{kk} expression are defective in TCR-mediated signaling; transfection of the \(kk\) gene in these mutants restores a normal response (22, 23).

\textsuperscript{1}Abbreviations used in this paper: MBP, maltose binding fusion protein; PLC, phospholipase C; PTK, protein tyrosine kinase; SH2, src homology 2; ZAP-70, \(\zeta\)-associated protein.

p59\textsuperscript{ff} has been shown to coprecipitate with the TCR/CD3 complex (24, 25). Evidence that p59\textsuperscript{ff} is involved in TCR signaling is also provided by the correlation between the level of p59\textsuperscript{ff} expression and the amplitude of thymocyte response to TCR stimulation in transgenic mice (26–28). Recently, ZAP-70, a kinase belonging to a new family of cytosolic PTKs and associated with the \(\zeta\) chain after TCR stimulation, has also been implicated in TCR signal transduction (15, 29).

It has been proposed that a key event in T cell activation is the formation of a multimolecular TCR signaling complex (30–32). The recruitment of PTKs in this signaling complex is supported by the presence of a kinase activity within the CD3/TCR complex. The use of an in vitro kinase reaction in combination with mild detergent protein extraction has demonstrated the association of the CD3/TCR complex with p56\textsuperscript{kk} and p59\textsuperscript{ff} (11, 24, 25). An association of the ZAP-70 kinase with CD3\(\epsilon\) in addition to the \(\zeta\) chain after TCR stimulation, has also been demonstrated to occur after TCR or CD3 stimulation (33, 34). The functional interdependence of these kinases has been studied recently by cotransfection experiments in COS cells and by the analysis of the phosphorylation of a CD8/\(\zeta\) chimeric molecule (29). In that study, the association of ZAP-70 with the cytoplasmic tail of \(\zeta\) and its functional activity required the presence of p59\textsuperscript{ff} or p56\textsuperscript{kk}. However,
in T cells, the spatial and temporal course of interactions of these PTKs with each other and with other molecules of the signaling complex remain poorly understood.

Some of the molecular interactions taking place in the signaling complex may be mediated by src homology 2 (SH2) domains. These domains have been implicated in mediating protein–protein interactions by binding to tyrosine-phosphorylated proteins (35, 36). SH2 domains are found in a number of proteins involved in intracellular signaling, including the src family of tyrosine kinases and ZAP-70. The fact that ZAP-70 associates only with tyrosine-phosphorylated ζ chains supports a model involving an SH2–phosphotyrosine interaction of ZAP-70 with ζ (29, 37, 38). In the tyrosine kinases of the src family, SH2 domains are involved in both negative and positive regulation of the kinase activity. The mechanism of SH2-mediated negative regulation of p56lck kinase activity can be rationalized by the general model (39) proposed for src family kinases involving an intramolecular interaction between the phosphotyrosine 505 (Y505) and the SH2 domain of p56lck. Consistent with this model, deletion of the SH2 domain or substitution of Y505 by phenylalanine (P505) results in an increase of p56lck kinase activity in vivo when transfected in fibroblasts (40–42). In addition, an increased level of phosphorylation of Y505 in a CD45-negative Jurkat mutant diminishes p56lck binding to a p56lck COOH-terminal peptide containing phosphorylated tyrosine Y505 (43). The positive role of the SH2 domain has been studied using a constitutively active form of p56lck carrying phenylalanine at position 505 (p56lckF505). Deletion of the SH2 domain decreases the transforming activity of p56lckF505 in fibroblasts and abolishes the ability of p56lckF505 to enhance the response to antigenic stimulation or to induce antigen-independent IL-2 production in T cell hybridomas (44, 45). More recently, the role of the p56lck SH2 domain in the signaling complex was studied by comparing the capacity of several CD4/p56lck chimeras to mediate antigen response (46). Specific mutations in the p56lck SH2 domain impaired coreceptor activity of the CD4/p56lck chimera. Altogether, these results support the involvement of the p56lck SH2 domain in the interaction with specific substrates and/or regulatory proteins during T cell activation.

To better understand the mechanisms by which p56lck contributes to TCR signaling and, in particular, the role of the p56lck SH2 domain in this process, we examined the tyrosine-phosphorylated proteins that associate with the p56lck SH2 domain after CD3 stimulation of the Jurkat cell line with anti-CD3 mAbs. We show that one of the p56lck SH2-binding proteins is the PTK ZAP-70. Furthermore, our results indicate that, after activation, an interaction between ZAP-70 and p56lck takes place in vivo. We propose a model whereby the activated form of the TCR mediates the interaction of p56lck and ZAP-70 tyrosine kinases.

Materials and Methods

Cell Lines and Antibodies. Jurkat cells, clone 77-6.8 (a gift of Dr. K. A. Smith, Dartmouth Medical School, Hanover, NH) were grown in RPMI-1640 medium supplemented with 10% FCS, 2 mM L-glutamine, penicillin, and streptomycin. mAbs used included antibodies to: CD3 ε chain (2Ad2A2, IgM; kindly provided by E. Reinherz, Dana Farber Cancer Institute, Boston, MA), phosphotyrosine (4G10, IgG2b; UB1, Lake Placid, NY), and p56lck (N-ter, IgG1; kindly provided by S. Fischer, Institut Cochin de Génétique Moléculaire, Paris, France). Rabbit polyclonal antibodies used included: anti-p56lck serum directed against a synthetic peptide corresponding to amino acids 39–64 of p56lck sequence (kindly provided by S. Fischer); anti-p56lck serum raised against recombinant human p56lck (kindly provided by M. Guttering, M. Gassmann, and P. Burn, Hoffmann-La Roche Ltd., Basel, Switzerland); anti-ζ serum (a gift of J. Ravetch, Memorial Sloan-Kettering Institute, New York); and anti-ZAP-70 antiserum directed against a synthetic peptide corresponding to amino acids 485–499 of ZAP-70 sequence, which was produced in our laboratory using KLH as a carrier as described (29).

Construction and Expression of Recombinant Fusion Proteins. Oligonucleotides used to amplify the human p56lck cDNA by PCR were homologous to the boundaries of the SH2 or SH3 sequence and contained a BamHI site extension: SH2-N 5′GCG GAT CCG AGA TCT GGT TCT TCA AGA ACC TGA GC 3′ and SH2-C 5′GCG GAT CCT TAC TTC TGG CAG CAG GG 3′; SH2-N 5′GCG GAT CCG AGA TCT GGT TCT TCA AGA ACC TGA GC 3′ and SH2-C 5′GCG GAT CCG AGA TCT GGT TCT TCA AGA ACC TGA GC 3′; SH2-N 5′GCG GAT CCG AGA TCT GGT TCT TCA AGA ACC TGA GC 3′ and SH2-C 5′GCG GAT CCG AGA TCT GGT TCT TCA AGA ACC TGA GC 3′. These sequence data are available from EMBL Data Bank accession numbers: X14053, X14054, and X14055. PCR amplified fragments were cloned into the BamHI site of pDxA vector. This vector is a derivative of the vector pMAL cR1 (New England Biolabs Inc., Beverly, MA) where the cytoplasmic maltose binding fusion protein (MBP) is under the control of the maltose promoter. DNA sequencing of the constructs was performed to confirm that no mutations were introduced in the PCR amplified sequence. Fusion proteins were purified from cytoplasmic extracts by affinity chromatography on a cross-linked amylose column as described (47).

Immunoprecipitations. Jurkat cells were washed twice in RPMI-1640 medium and resuspended at 10⁶ cells/ml in RPMI-1640. Cells were preincubated for 10 min at 37°C and stimulated with anti-CD3 mAb (2Ad2A2 at 1/300 dilution of ascites) for the time indicated. Cells were harvested and solubilized at 10⁶ cells/ml for 30 min at 4°C in 1% Brij 96, in 1% NP-40, in 1 mM EGTA in the presence of inhibitors of proteases and phosphatases: 10 μg/ml leupeptin, 10 μg/ml aprotinin, 1 mM Pefabloc-sc, 50 mM NaF, 10 mM Na3P04, and 1 mM NaVO4. Postnuclear lysates were preclarified for 1 h with MBP-Sepharose beads and subjected to immunoprecipitation for 2 h with antibodies preadsorbed to protein A-Sepharose (Pharmacia, Upsala, Sweden) or goat anti-mouse-Sepharose (Zymed Laboratories, Inc., South San Francisco, CA) or covalently coupled to protein A-Sepharose (48). Immune complexes were washed twice in 1% detergent lysis buffer without inhibitors, and twice with 0.1% detergent lysis buffer, and boiled in sample buffer before electrophoresis.

SH2-binding Assays. MBP, MBP-SH3, and MBP-SH2 proteins were coupled to CNBr-activated Sepharose beads (Pharmacia) according to the manufacturer’s recommendations. After coupling, beads were washed several times with 1% Brij 96 lysis buffer containing 1 M NaCl and 50 mM phenylphosphate and then extensively with 1% Brij 96 lysis buffer. To assay binding of proteins to MBP-SH3 or MBP-SH2 beads, preclarified lysates from 10⁶ cells were prepared as described above and incubated for 2 h with ~50 μg of MBP-SH3, MBP-SH2, or MBP-Sepharose. The protein complexes were washed in the same conditions as the immune com-
relevant peptide in 0.1% detergent lysis buffer under constant agitation. When indicated, by incubating 30 min at 4°C in presence of the detergent or, with SDS sample buffer or, with 1/1,000 dilution of goat anti-mouse biotinylated antibodies (Vector Laboratories, Inc., Burlingame, CA) for 1 h and with streptavidin-alkaline phosphatase (Boehringer Mannheim, Mannheim, FRG) for 1 h with a 1/2,000 dilution of goat anti-mouse biotinylated antibodies (Vector Laboratories, Inc., Burlingame, CA) for 1 h and with streptavidin-alkaline phosphatase for 1 h. All incubations were performed in TBST containing 0.2% gelatin and followed by several washes with TBST. Finally, filters were washed in alkaline phosphate buffer (100 mM Tris-HCl, pH 9.5, 100 mM NaCl, 5 mM MgCl₂) and were developed with nitroblue tetrazolium and 5-bromo-4-chloro-3-indoly phosphate according to manufacturer’s instructions (Promega, Madison, WI).

**Immunoblotting.** Gels were equilibrated in transfer buffer (25 mM Tris-base, 192 mM glycine, 20% ethanol) and transferred to nitrocellulose membranes at 40V overnight. Blots were blocked in PBST (10 mM phosphate buffer, pH 7.4, 1 mM KCl, 137 mM NaCl, 0.1% Tween 20) in the presence of 1% gelatin for at least 1 h and incubated overnight with antibodies in PBST plus 0.1% gelatin. Blots were washed, incubated with a 1/2,000 dilution of peroxidase goat anti-mouse (Amersham International, Amersham, Bucks, UK) or peroxidase goat anti-rabbit antibodies (Bioys, Compiègne, France) and developed with the enhanced chemiluminescence detection system (Amersham International). When the same blot was revealed with different probes, stripping of antibodies was performed according to the manufacturer’s recommendations (Amersham International).

**In Vitro Kinase Assay.** Immune complexes or SH2-binding proteins were prepared as described above except that an additional wash of the beads with kinase buffer (25 mM Heps, pH 7.3, 10 mM MnCl₂, 0.05% Brij 96) was performed. Reactions were started by the addition of 30 μl of kinase buffer containing 10 μCi of γ-[32P]ATP (3,000 Ci/mM) and stopped after 15 min by the addition of 2× sample buffer containing 10 mM EDTA. Samples were analyzed by SDS-PAGE. Gels were fixed, treated with 1 M KOH for 1 h at 55° C to remove the alkali-labile phosphate groups from Ser and Thr phosphorylated proteins, dried, and autoradiographed.

**V8 Protease Digestion.** Bands were excised from the non-KOH-treated dried gel and rehydrated for 30 min in V8 protease buffer (0.125 M Tris-HCl, pH 6.8, 0.1% SDS, 1 mM EDTA, 20 mM dithiothreitol, 10% glycerol). Gel slices were loaded on a 15% polyacrylamide gel, overlayed with 15 μl of 0.125 M Tris-HCl, pH 6.8, 0.1% SDS, 1 mM EDTA, 0.005% bromophenol blue, 20% glycerol, and 1.5 μg of V8 protease (Boehringer Mannheim) in V8 protease buffer. Once the bromophenol blue reached the end of the stacking gel, the electrophoresis was interrupted for 45 min to allow the protein to digest. At the end of the migration, gels were fixed, dried, and autoradiographed.

**Results**

**Tyrosine-phosphorylated Proteins from CD3-activated Jurkat Cells that Bind to the p56k SH2 Domain.** Tyrosine-phosphorylated proteins that bind to the p56k SH2 domain of p56k were identified in lysates of 10⁷ Jurkat cells stimulated with anti-CD3 for 1 min and incubated with beads coupled to MBP alone, MBP-SH2, and MBP-SH3 fusion proteins as indicated. The beads were washed and the associated proteins were analyzed by 8% SDS-PAGE and immunoblotting with an antiphosphotyrosine antibody. (*) The lysate was boiled in the presence of 1% SDS, diluted to 0.1% SDS in lysis buffer, and precipitated by MBP-SH2 beads as for the nondenatured lysate. (Left) The nine major SH2-binding proteins detected by Western blotting (A) were identified by antiphosphotyrosine immunoblotting. B corresponds to a shorter exposure than that shown in A and therefore does not allow the detection of all the SH2-binding proteins. Note that slight differences in relative intensities of the bands occurred between the two experiments. Positions of molecular weight markers are shown in kilodaltons.

![Figure 1. Binding of phosphorylated proteins to the SH2 domain of p56k.](image-url)

(A) Lysates of 10⁷ Jurkat cells stimulated with anti-CD3 for 1 min were incubated with beads coupled to MBP alone, MBP-SH2, and MBP-SH3 fusion proteins as indicated. The beads were washed and the associated proteins were analyzed by 8% SDS-PAGE and immunoblotting with an antiphosphotyrosine antibody. (*) The lysate was boiled in the presence of 1% SDS, diluted to 0.1% SDS in lysis buffer, and precipitated by MBP-SH2 beads as for the nondenatured lysates. (Left) The nine major SH2-binding proteins detected by Western blotting (A) were identified by antiphosphotyrosine immunoblotting. B corresponds to a shorter exposure than that shown in A and therefore does not allow the detection of all the SH2-binding proteins. Note that slight differences in relative intensities of the bands occurred between the two experiments. Positions of molecular weight markers are shown in kilodaltons.
The specificity of the interaction between the p56kk SH2 domain and tyrosine-phosphorylated proteins was further investigated by using peptides to elute proteins from MBP-SH2 beads (Fig. 2). The phosphopeptide (EPQYEEIPI) containing the sequence predicted to be optimal for binding to the p56kk SH2 domain (35) was capable of eluting all of the proteins from MBP-SH2 beads at 5 μM. An ~100-fold higher concentration of the phosphopeptide corresponding to the COOH-terminal sequence of p56kk (EGQYQPQPG) was necessary to obtain complete elution. As previously reported (36), phosphorylation of tyrosine is essential for SH2 interaction since none of the proteins bound to the SH2 domain were released by a nonphosphorylated peptide containing the YEEI sequence.

The p56kk SH2 Domain Associates In Vitro with ZAP-70 upon CD3 Activation. To determine whether some of the proteins associating with p56kk SH2 domain had tyrosine kinase activity, in vitro kinase reactions were performed (Fig. 3 A). No specific kinase activity was detectable in MBP-SH2 precipitates from unstimulated cells. After CD3 cross-linking, proteins of 70, 72, and 74 kD were phosphorylated in MBP-SH2 precipitates. The phosphorylation of these proteins was resistant to KOH treatment, which suggests that they are phosphorylated primarily on tyrosine residues. These proteins might correspond to the SH2-binding proteins of identical molecular weights detected by antiphosphotyrosine immunoblots (Fig. 1).

A functional association of p56kk with the PTK ZAP-70 has been recently reported (29). We therefore tested whether the 70-kD SH2-binding protein detected in the in vitro kinase assay might correspond to ZAP-70 by performing an in vitro kinase reaction in anti-ZAP-70 immunoprecipitates from lysates of unstimulated or CD3-stimulated Jurkat cells (Fig. 3 A). In unstimulated cells, only a trace amount of ZAP-70 was present in precipitates. Its intensity increased dramatically after CD3 stimulation. ZAP-70 comigrated with the 70-kD phosphoprotein present in the in vitro kinase reaction of MBP-SH2 precipitates from stimulated cells. In addition, a broad band around 30 kD and bands at 56–60 kD were detected in ZAP-70 immunoprecipitates from stimulated cells.

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ZAP-70 is recognized directly by the p56k SH2 domain. (A) Lysates were prepared from 10^7 unstimulated Jurkat cells (−) or cells stimulated with anti-CD3 for 1.5 min (+). Immunoprecipitates with anti-z' and control (anti-B-galactosidase) antisera were analyzed on a 12% SDS polyacrylamide gel, transferred to nitrocellulose, and revealed with the indicated probes. (Arrows) The position of the ZAP-70 protein. (B) Lysates were prepared as in (A). Proteins associated with the MBP-SH2 fusion protein and z' and ZAP-70 immunoprecipitates were run on a 12% SDS polyacrylamide gel, blotted, and detected by an antiphosphotyrosine antibody. Lysates of 10^7, 5 × 10^6, and 10^5 activated cells were used for preparation of the MBP-SH2, anti-ZAP-70, and anti-z' precipitates, respectively. On a shorter exposure, ZAP-70 present in the MBP-SH2 precipitate is clearly distinguishable from the other SH2-binding proteins of similar molecular weight, and its intensity is comparable to that in the other two lanes (data not shown). (Arrow) The position of the ZAP-70 protein. (C) Lysates of 3 × 10^6 activated cells were precleared by three successive rounds of immunodepletions (ID) using either control (C, anti-B-gal) or anti-z' antibodies, and half of each sample was precipitated with MBP-SH2 or anti-z' antibodies as indicated. Samples were analyzed on an 8% SDS-polyacrylamide gel by antiphosphotyrosine immunoblotting. (Double arrow) The position of the ZAP-70 doublet. Positions of molecular weight markers are shown in kilodaltons.

To establish more directly whether the 70-kD SH2-binding protein corresponds to ZAP-70, V8 protease mapping and reimmunoprecipitation studies were performed. Identical peptide maps were obtained for the 70-kD bands comigrating in z' immunoprecipitates and in MBP-SH2 precipitates (Fig. 3 B). In activated cells, a 70-kD protein (migrating as a doublet in Fig. 3 C) present among the SH2-binding proteins comigrates with ZAP-70 and can be reprecipitated by anti-ZAP-70 antibodies as detected by antiphosphotyrosine immunoblotting (Fig. 3 D). Altogether, these results demonstrate that the p56k SH2 domain associates with ZAP-70 in vitro.

ZAP-70 Binds Directly to the p56k SH2 Domain In Vitro

Since ZAP-70 binds to the chain after TCR stimulation, the interaction of ZAP-70 with the p56k SH2 domain described above could be mediated by z'. To determine whether the p56k SH2 domain interacts directly with ZAP-70 or z', a biotinylated MBP-SH2 fusion protein was used as a probe to detect direct interaction with proteins immobilized on nitrocellulose (Fig. 4 A). In z' immunoprecipitates of CD3-activated cells, ZAP-70 was readily detectable by the SH2 probe whereas z' was barely detectable, even though a large amount of phosphorylated z' was present, as shown by antiphosphotyrosine staining of the same blot. The direct binding of the SH2 probe to ZAP-70 was also detectable in ZAP-70 immunoprecipitates of activated cells (data not shown).

The ratio of tyrosine-phosphorylated ZAP-70 to tyrosine-phosphorylated z' in the MBP-SH2 and anti-ZAP-70 precipitates was similar and much greater than in the anti-z' precipitate (Fig. 4 B). This result further supports the direct binding of phosphorylated ZAP-70 to the p56k SH2 domain.

To determine whether the population of phosphorylated ZAP-70 bound to the p56k SH2 domain represents the z'-associated ZAP-70 population, immunodepletion experiments were performed. After three rounds of immunodepletion with anti-z' antibodies, no z'-associated phosphorylated ZAP-70 could be detected, whereas the amount of phosphorylated ZAP-70 was only slightly reduced in MBP-SH2 precipitates (Fig. 4 C). Taken together, these results show that in vitro the p56k SH2 domain binds directly to the entire population of ZAP-70 (i.e., both associated and not associated with z').

p56k Interacts In Vivo with ZAP-70/z'

To test if an interaction between p56k and ZAP-70 occurs in vivo, im-
firmed that a small fraction of total p56<sup>lek</sup> was present in ± immunoprecipitates (Fig. 5 B).

In Brij 96 lysates, CD3 and ± chains remain associated. Using NP-40 as a detergent to disrupt the CD3/± chain association, p56<sup>lek</sup> was equally well detected in ± immunoprecipitates from NP-40 and Brij 96 lysates (data not shown), indicating that p56<sup>lek</sup> associates directly with the ±/ZAP-70 complex.

In p56<sup>lek</sup> immunoprecipitation, two phosphoproteins of 70 and 74 kD could be coprecipitated with p56<sup>lek</sup> after CD3 stimulation (Fig. 6). The 74 kD protein may correspond to the tyrosine-phosphorylated protein of the same molecular weight detected in MBP-SH2 precipitates (Fig. 1). The 70-kD doublet comigrated exactly with the ZAP-70 doublet precipitated with a specific antiserum (Fig. 6). Because of the weakness of the antiserum used in this experiment, the anti-ZAP-70 immunoprecipitate contains only a small fraction of total phosphorylated ZAP-70. Under similar conditions, very small amounts of phosphorylated ± chains in p56<sup>lek</sup> immunoprecipitates were detected (data not shown).

These results suggest that p56<sup>lek</sup> associates in vivo with the ZAP-70/± complex during T cell activation.

**Discussion**

In this paper, we provide direct evidence that p56<sup>lek</sup> physically associates in vivo with the ±/ZAP-70 complex shortly after stimulation of the TCR. This interaction appears to

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be mediated by a specific binding of the SH2 domain of p56k to tyrosine-phosphorylated ZAP-70. The direct association of ZAP-70 with p56k SH2 domain was demonstrated using the p56k SH2 domain as a probe to detect proteins immobilized on a nitrocellulose membrane. In a SH2 immunoprecipitate, phosphorylated ZAP-70 was clearly recognized by the SH2 domain, whereas the chain was barely detectable. Thus, if a direct interaction of the p56k SH2 domain with takes place, it must concern only a very small fraction of total phosphorylated or may be of very low affinity.

The optimal binding sequence of the p56k SH2 domain determined by using a random library of tyrosine-phosphorylated peptides is (p)YEEI (35). Indeed, a phosphorylated peptide containing this sequence was able to elute proteins bound to p56k SH2 about 100-fold more efficiently than a phosphopeptide corresponding to the COOH-terminal sequence of p56k. Inspection of the ZAP-70 sequence did not reveal the presence of this exact SH2 binding motif. However, it cannot be excluded that sequences of ZAP-70 different from the optimal one described above may act as an acceptor site for the p56k SH2 domain. In this regard, it is worth noting that a physiological binding site of the p56k SH2 domain on the platelet-derived growth factor (PDGF) receptor was reported to be (p)YI(p)YV (52) which is only distantly related to (p)YEEI.

Previous reports have suggested an association of p56k with the CD3/TCR complex using a sensitive in vitro kinase labeling assay. Osman et al. (53) have shown that CD3 immunoprecipitates from stimulated Jurkat cells lyses in Brij 96 contained proteins of 70, 21, and 18 kD in addition to phosphorylated bands of 59 and 56 kD. However, the question of whether the latter two were p56k was not investigated. In addition, Burgess et al. (11) found that the CD3/TCR complex isolated from unstimulated Jurkat cells contained a p56k kinase activity. By anti-p56k immunoblotting, we have shown that p56k coprecipitates with the /CD3/TCR complex and that the amount of associated p56k increases after CD3 stimulation. In addition, we showed that p56k coprecipitates with a polypeptide of 70 kD that comigrates, as a doublet, with ZAP-70 after CD3 stimulation. Our in vitro data demonstrate that the p56k SH2 domain binds directly to the entire population of tyrosine-phosphorylated ZAP-70 (i.e., both associated and not associated with ). It strongly favors the existence of a similar interaction of p56k with ZAP-70 in vivo. Because of the low affinity and/or titer of our anti-ZAP-70 antibodies, we have not been able to quantify the percentage of ZAP-70 bound to p56k by immunoblotting. The weakness of the anti-ZAP-70 antiserum may also explain why we were not able to detect p56k in ZAP-70 immunoprecipitates (data not shown). Alternatively, it is possible that the peptide epitope recognized by the antiserum is not accessible in the p56k/ZAP-70 complex. After TCR stimulation, ZAP-70 associates with the CD3 chain in addition to the chain (33, 34). Experiments are in progress to determine whether p56k also associates with the ZAP-70/e complex in vivo.

In NK cells, an association of p56k with the chain of the FcRIII has been reported (54). The same study showed, using cotransfections of various src-related kinases with in COS cells, that associates specifically and directly with p56k. However, in similar cotransfection experiments in HeLa cells, no interaction of p56k with could be detected (55). The reason for this discrepancy is unclear but might be partially explained by the use of different detection systems in these two studies. If a p56k direct interaction exists in T cells, it might account for the presence of a small amount of p56k in immunoprecipitates from unstimulated cells. This result might also be explained by the presence of low but detectable amounts of phosphorylated ZAP-70 associated with in unstimulated Jurkat cells (29, and our unpublished observations). In view of our present results and the fact that ZAP-70 is expressed and associated with in NK cells upon activation (56), it is possible that ZAP-70 is required for optimal p56k- interaction in NK cells.

A functional interaction of p56k with ZAP-70 has been previously suggested by cotransfection experiments in COS cells expressing a CD8- chimera (29). In that report, a dramatic increase in tyrosine phosphorylation of cellular proteins including CD8- was observed only when ZAP-70 was coexpressed with either p56k or p59fm. More recently, Kolman et al. (57) have provided additional evidence for such an interaction. They showed that cross-linking of chimeric constructs composed of the extracellular domain of CD16 fused to p56k or ZAP-70, coexpressed in a human CTL line, results in enhanced cellular activation.

Our data suggest that this functional interaction is mediated by a direct physical interaction of the p56k and ZAP-70 kinases. What are the consequences of the physical interaction between these two kinases in terms of signaling? At least two potential signaling effects can be considered: altered subcellular localization of p56k and ZAP-70 and/or modulation of their respective kinase activities. Examples for both effects have been documented in the literature. Recent analysis of the role of p56k in the coreceptor activity of CD4 using CD4/p56k chimeric molecules suggests that the SH2 domain of p56k may induce the association of CD4 with the TCR, possibly by interactions with phosphorylated components of the activated TCR complex (46). In view of our results, tyrosine-phosphorylated ZAP-70 may mediate the recruitment of CD4-associated p56k to the activated TCR. The increased activity of the src family tyrosine kinases after association with the PDGF or the CSF-1 receptor tyrosine kinases is an example of modulation of kinase activity after SH2-mediated interaction between two kinases (58-60).

The interaction of the p56k SH2 domain with ZAP-70 described here is likely to represent an essential step in the course of antigen-mediated T cell activation. Models have been proposed in which one of the earliest activation events is the phosphorylation of on tyrosine by p56k or p59fm in T cells (61, 62). This phosphorylation event may allow the recruitment of ZAP-70 to the TCR complex through an SH2 phosphotyrosine interaction. This may lead to the initial phosphorylation of ZAP-70 either by autophosphorylation or by transphosphorylation of two ZAP-70 molecules.
bound to a ζ dimer. We propose that after these initiating events, the SH2 domain of p56kk binds to tyrosine-phosphorylated ZAP-70. The physical interaction between these two kinases may regulate their respective kinase activities, allowing them to interact with and/or further phosphorylate ζ and CD3 chains and other effector molecules. Studies of tyrosine kinase activity of the different cellular populations of ZAP-70 and p56kk should give further insight into their functional interaction.

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