A number of protein-tyrosine kinases have been shown to be important in T cell activation. One such kinase, Lck, has been demonstrated genetically to be essential for T cell receptor (TcR) signaling, and the SH2 and SH3 (src homology 2 and 3) domains of Lck have been shown to be indispensable for T cell activation. We have sought substrates with which the SH2,3 domain would interact following T cell activation, using fusion proteins containing the Lck SH2 and SH3 domains linked to glutathione S-transferase. We demonstrate that the SH2,3 region interacts specifically and directly with numerous tyrosine-phosphorylated molecules following TcR cross-linking, including constitutively with mitogen-activated protein kinase (MAPK)/extracellular-regulated kinase and inductively with the ζ chain of the TcR. The interaction with MAPK/extracellular-regulated kinase was via the SH3 domain. The interaction with the tyrosine-phosphorylated ζ chain, while phosphotyrosine-dependent, required both the SH3 and SH2 domains. These interactions were specific as molecules known to be tyrosine-phosphorylated following TcR cross-linking, phospholipase C-γ1 and Fyn, were not bound. Thus, we suggest that during TcR signaling, Lck interacts with numerous molecules, including MAPK and TcR-ζ, via its SH2,3 domain. The interaction with MAPK would place Lck in a position to be involved in the complex resulting in the activation of MAPK. In addition, the binding of Lck to the tyrosine-phosphorylated ζ chain of the TcR would serve to strengthen the interaction of the associated CD4 and the TcR complex, leading to increased avidity for the antigen-major histocompatibility protein complex.

The antigen-specific T cell receptor serves to activate T cells and imparts antigen specificity. A number of investigators have demonstrated that following cross-linking of the TcR, a number of molecules become tyrosine-phosphorylated, including the activation of protein-tyrosine kinases such as p59Fyn and the ζ chain-associated kinase ZAP-70. These activated kinases presumably act on other substrates that result in the tyrosine phosphorylation of a number of other molecules, including the ζ chain of the TcR and the ε chains of CD3, CD5, CD6, phospholipase C-γ1, MAPK, and Vav, and the activation of Ras (reviewed Ref. 1). While the protein-tyrosine kinase Lck has been shown genetically to be essential for signaling via the TcR (2), whether this kinase actually becomes activated has been controversial, with some reports of it becoming activated following TcR cross-linking (3, 4) and some reports of no activation (1, 5).

The protein-tyrosine kinases Fyn, ZAP-70, and Lck all possess SH2 domains, examples of which have been demonstrated to bind to tyrosine-phosphorylated molecules in the context of surrounding amino acids (6). In addition, both Fyn and Lck have SH3 domains, examples of which have been shown to bind to proline-rich regions in a number of intracellular molecules, and both have the organization of these modules in the following order: N-terminal unique region, SH3 domain, SH2 domain. Recent work has shown that the kinase domain of Lck seems to be dispensable for mature T cell activation, at least in the context of activating T cell hybridomas in an antigen-specific manner with CD4 as a co-receptor (7, 8). The SH2 and SH3 domains, however, were found to be indispensable for the activation of these hybridomas, suggesting an important role for these domains of Lck (8). This suggests that molecules that interact with these domains of Lck may play important roles in T cell activation. Along these lines, several groups have recently demonstrated that the SH3 domain of Lck interacts with the lipid kinase phosphatidylinositol 3-kinase (9, 10).

The substrate specificity of some SH2-containing tyrosine kinases seems to reside in the specificity of their SH2 domains (11). To investigate the role of Lck in T cell activation and as a first step in finding substrates for this protein-tyrosine kinase, it would be of interest to determine the spectrum of molecules that would bind to the SH3 and SH2 domains of Lck, some of which would probably include substrates of this protein-tyrosine kinase. We demonstrate here that the SH2,3 domain of Lck binds the serine/threonine kinase MAPK and the tyrosine-phosphorylated ζ chains of the TcR. The binding of MAPK was via the SH3 domain of Lck and probably involved an indirect interaction. While the tyrosine-phosphorylated ζ chain did not bind the SH3 domain, it also did not bind the isolated SH2 domain, and binding required both the SH3 and SH2 domains, although this binding was phosphotyrosine-dependent. This supports the contention that the kinase specificity of these SH2-containing kinases is regulated by the specificities of their SH2 domains as the ζ chain has been reported to be a substrate of Lck (1, 11).

**EXPERIMENTAL PROCEDURES**

CD4s and Reagents—The Jurkat leukemic cell line clone E6-1 was obtained from the American Type Culture Collection and maintained in RPMI 1640 medium (fetal calf serum (10%) with antibiotics at 37 °C. Rabbit antisera to p59Fyn, Ras GTPase-activating protein, and rat

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1 The abbreviations used are: TcR, T cell receptor; MAPK, mitogen-activated protein kinase; GST, glutathione S-transferase; PAGE, polyacrylamide gel electrophoresis; PVDF, polyvinylidene difluoride; MOPS, 4-morpholinepropanesulfonic acid.
SH2,3 Domain of Lck Binds MAPK and γ Chain of TcR

MAPK were from Upstate Biotechnology, Inc. (Lake Placid, NY). Rabbit antiserum 387 to the γ chain of the TcR was from Dr. L. E. Samelson (National Institutes of Health, Bethesda, MD). Mouse monoclonal antibody to phospholipase C-γ1 was from Zymed Laboratories, Inc. (South San Francisco, CA). The recombinant anti-phosphotyrosine antibody RC20 was obtained from Transduction Laboratories (Lexington, KY), and a monoclonal antibody (PT66) against phosphotyrosine was from Sigma. Both antibodies gave the same results and are collectively referred to as anti-pY. The OKT3 monoclonal antibody (anti-CD3) was grown in this laboratory. The MAPK substrate was a region from myelin basic protein (residues 95–98) and was purchased from Upstate Biotechnology, Inc. [γ-32P]ATP and ECL were from DuPont NEN. All other reagents were from Sigma.

Recombinant Techniques—The SH3 and SH2, SH3, or SH2 domain of Lck was amplified by polymerase chain reaction from the cDNA of Lck (12) and subcloned into the pGEX3X vector (Pharmacia LKB Biotechnology, Uppsala). SH2,3 fusions included two extra amino acids at the junction between GST and the Lck domain(s), proline and threonine. The SH3-only and SH2-only constructs were constructed similarly. Fusion proteins and GST were generated and purified as described (13). The purified proteins were kept on agarose beads and stored in phosphate-buffered saline at 4 °C. The protein-bead combination was used directly for precipitates. Other fusion proteins were kind gifts of Drs. B. Knudsen, S. Feller, and R. Birge (The Rockefeller University).

Cell Manipulations—Jurkat cells were stimulated with the OKT3 antibody and lysed exactly as described (14) for the indicated periods of time, and if not stated, for 2 min. Following clarification, the lysates were precleared with GST-agarose beads, followed by treatment with fusion proteins or GST. Precipitates were washed with lysis buffer, boiled in 2 × SDS-PAGE buffer, and loaded onto 10% polyacrylamide gels.

Immunological Procedures—Following separation on a 10% polyacrylamide gel, the proteins were transferred to PVDF membrane and blocked overnight in Tris-buffered saline containing 5% nonfat milk in Tris-buffered saline as described previously (14). Some blots were stripped in 62 mM Tris (pH 6.8), 100 mM 2-mercaptoethanol, 2% SDS for 1 h at 50 °C and washed and blocked overnight in Tris-buffered saline containing 5% nonfat milk. Other antibodies used were as follows: MAPK (1 μg/ml), (1:200), phospholipase C-γ1 (1:5000), Fyn (1:10000), and Ras GTPase-activating protein (1:1000), with detection of rabbit antibodies with protein A-horseradish peroxidase and of the mouse antibody with horseradish peroxidase-conjugated goat antimouse Fc. These were all detected by ECL. In Vitro Kinase Assay—MAPK assay was done as described previously (15). Briefly, the washed precipitates were washed once in MAPK assay buffer (12.5 mM MOPS (pH 7.2), 12.5 mM b-glycerol phosphate, 7.5 mM MgCl2, 0.5 mM EGTA, 2 mM dithiothreitol, 2 mM NaVO3, 25 μM b-glycine bovine serum albumin) and then incubated in 50 μl of assay buffer containing 20 μM of [γ-32P]ATP, 20 μM unlabelled ATP, and 1 mM myelin basic protein peptide substrate for 15 min at 30 °C. The pellet was spun out, and the supernatants were spotted onto P-81 paper squares (2 × 2 cm; Whatman International Ltd., Maidstone, United Kingdom). These were washed with 1% phosphoric acid, dried, and counted. In vitro tyrosine kinase assays were performed as described previously (14).

RESULTS

The protein-tyrosine kinase Lck has been shown to be essential for TcR signaling (2), and the SH2 and SH3 domains were deemed to be essential in this process (8). We reasoned that part of this may represent the binding of the Lck SH2 and/or SH3 domain to important effector molecules required for the efficient activation of the T cell. To this end, a SH2,3lckgst fusion protein was generated and used as a probe for molecules binding to the SH2,3 domain of Lck. These fusion proteins were used as probes using lysates of the J urkat T cell line E6-1 following cross-linking of the TcR. As previously reported, cross-linking of the TcR complex resulted in tyrosine phosphorylation of a number of proteins (Fig. 1a). A subset of these proteins bound to the SH2,3lckgst fusion protein (Fig. 1, b and c), with none interacting with GST alone (see also Fig. 5b, lane 2 A. August, and B. Dupont, unpublished data.)

FIG. 1. Binding of tyrosine-phosphorylated molecules to the SH2,3 domain of Lck. J urkat cells were stimulated for the indicated periods of time using anti-CD3 antibodies, and either total cell protein (a) or SH2,3lckgst fusion protein precipitates (b and c) were separated by PAGE, blotted onto PVDF membrane, and probed with anti-phosphotyrosine antibodies (a,pY). In b, the arrowheads point to the 42-kDa MAPK. In c, the arrowheads point to the γ chain of the TcR. 

3) or when J urkat cells were stimulated with pervanadate.2 Two prominent tyrosine-phosphorylated molecules were observed (arrowheads in Fig. 1, b and c), one of 42 kDa and one of ~20 kDa. The one at 42 kDa had a molecular mass similar to that of the serine/threonine kinase MAPK (16). Cross-linking the TcR results in the tyrosine phosphorylation and activation of MAPK (1), suggesting that this protein may be MAPK. We therefore tested if MAPK would bind to the SH2,3lckgst fusion protein. The CD3 complex of the TcR was cross-linked, and the resultant lysates were precipitated with the SH2,3lckgst fusion protein. The resultant proteins were then probed for the presence of MAPK. Fig. 2 demonstrates that MAPK binds the SH2,3 domain in both resting and TcR-cross-linked cell lysates. The lysates were also denatured to determine if the SH2,3 domain of Lck could interact directly with MAPK. Fig. 2 demonstrates that the amount of MAPK bound to the SH2,3 domain after denaturation of the lysates was less than that seen when the lysates were in the native form. It is possible that the reductive residual binding that we observed is due to incomplete denaturation. The results observed do, however, clearly demonstrate reduced binding of MAPK to the Lck SH2,3 domain under denaturing conditions and support an indirect interaction. MAPK bound in a manner that was independent of TcR stimulation, binding both before and after TcR cross-linking. This binding was also largely phosphotyrosine-independent, as illustrated in Fig. 3, where phenyl phosphate, a phosphotyrosine mimic, competed very poorly to elute the bound MAPK since only minimal MAPK was recovered in the eluate. Other tyrosine-phosphorylated proteins, including ZAP-70, were, however, eluted using this treatment with phenyl phosphate, as seen in anti-phosphotyrosine immunoblots of these.
immunoprecipitate. The precipitates were then blotted for MAPK. The membranes. In addition, a similar amount of MAPK equivalent protein. Jurkat cells were stimulated with anti-CD3 antibodies for 2 min, and half of the lysates were boiled (lanes 3 and 4) or not (lanes 1 and 2). The lysates were then precipitated with the SH2,3 fusion protein. The precipitates were washed and split in half; one-half was incubated with phenyl phosphate, and the supernatant was collected. The other half was not treated. The proteins from the supernatant (lanes 3 and 4) and the untreated beads (lanes 1 and 2) were separated by 10% SDS-PAGE, blotted onto PVDF membrane, and probed for MAPK. Lanes 1 and 3 are control cells; lanes 2 and 4 are CD3-stimulated cells. The precipitates were then blotted for MAPK. The isoforms of MAPK are bracketed. CD3X, crosslinked CD3; Ip, immunoprecipitate.

Fig. 2. MAPK can bind constitutively to the SH2,3ckgst fusion protein. Jurkat cells were stimulated with anti-CD3 antibodies for 2 min, and half of the lysates were boiled (lanes 3 and 4) or not (lanes 1 and 2). The precipitates containing the full-length Grb2-GST and Nck-GST fusion proteins (Fig. 6). MAPK was also not bound by the SH2,3 domain of Csk or by GST (Fig. 5) or to the SH2 domains of Lck and Src (Fig. 6). Indeed, MAPK bound to a fusion protein consisting of only the SH3 domain of Lck, but not to GST (Fig. 5) or to the SH2 domains of Lck and Src (Fig. 6). MAPK was also not bound by the SH2,3 domain of Csk or by the full-length Grb2-GST and Nck-GST fusion proteins (Fig. 6). Thus, the data indicate that MAPK can bind constitutively to the SH2,3 domain of Lck.

It is shown in Fig. 1c that a tyrosine-phosphorylated protein with a molecular mass similar to that of the z chain of the TcR was precipitated by the SH2,3ckgst fusion protein following TcR cross-linking. This protein could be recognized by antibodies to the z chain of the TcR, suggesting that the binding is inducible. To investigate this further, precipitates of the fusion protein either before or after TcR cross-linking were blotted with antibodies to the z chain of the TcR. Fig. 7 shows that the z chain can be detected only after TcR cross-linking, and GST alone does not bind the z chain (data not shown and Fig. 5b). This suggests that tyrosine phosphorylation of the z chain could serve as a signal for the binding of the SH2,3 domain of Lck. This is unlike the results seen with MAPK, where the binding was constitutive and via the SH3 domain. The binding of the z chain to the SH2,3 domain of Lck is largely phosphotyrosine-dependent as a phosphotyrosine mimic, phenyl phosphate, can compete for the binding and can elute the z chain from the domains. In addition to the z chain, we also detected a protein of ~70 kDa, which represents the z chain-associated kinase ZAP-70 (Fig. 8a) (very little z immunoreactivity was left on the beads following elution with phenyl phosphate). Since the z chain becomes associated with ZAP-70 following TcR cross-linking (1), one would expect to see this molecule in precipitates containing the z chain. In accord with the phosphotyrosine-dependent binding of the z chain to the SH2,3
Fig. 5. The SH3 domain of Lck binds MAPK, but not the ζ chain of the TcR. Jurkat cells were either stimulated or not with anti-CD3 antibodies, and the lysates were precipitated with an SH3ckgst fusion protein (lanes 1 and 2) or with GST alone (lane 3). Lane 4 is the whole cell lysate of Jurkat E6-1 cells. The bound proteins were separated by SDS-PAGE, blotted, and probed for MAPK (a) or the ζ chain of the TcR (b). The bracket indicates MAPK in a, and the arrow indicates the ζ chain in b. CD3X, cross-linked CD3; Ip, immunoprecipitate.

Fig. 6. Other SH2 and SH3 domains do not bind MAPK. Jurkat lysates were incubated with the indicated GST fusion proteins, and the precipitates were separated by 10% SDS-PAGE. The gel was blotted onto PVDF membranes and probed with an antibody to MAPK. Lane 1, glutathione-agarose beads alone; lane 2, Lck SH2 domain; lane 3, Src SH3 domain; lane 4, Abl SH3 domain; lane 5, Src SH2 domain; lane 6, Grb2; lane 7, Nck; lane 8, Csk SH2,3 domain; lane 9, whole cell (W/C) lysate of J urkat cells. The band seen in lane 2 is a nonspecific band seen with secondary reagents alone. The MAPK isoforms are bracketed. Ip, immunoprecipitate.

Fig. 7. Regulation of binding of ζ with SH2,3ckgst by the TcR. Jurkat cells were stimulated with anti-CD3 antibodies, and the lysates were precipitated with the fusion protein. The gel was then blotted for ζ using antisera to the ζ chain of the TcR. Lane 1 is control lysates; lane 2 is CD3-stimulated lysates. The arrow denotes the position of the ζ chain. CD3X, cross-linked CD3; Ip, immunoprecipitate.

The SH2,3 domain of Lck binds MAPK and ζ Chain of TcR

The SH2,3 domain of Lck binds to the ζ chain in a phosphotyrosine-dependent manner. Jurkat cells were stimulated or not with anti-CD3 antibodies, and the lysates were precipitated with the SH2,3 domain fusion protein. The precipitated proteins were incubated with phenyl phosphate, and the supernatants were collected and separated by SDS-PAGE, blotted, and probed for anti-phosphotyrosine antibodies (αpY) (a) and the ζ chain (b). CD3X, cross-linked CD3; Ip, immunoprecipitate.

Since a number of molecules become tyrosine-phosphorylated following TcR cross-linking, the SH2,3 domain of Lck may not bind the tyrosine-phosphorylated ζ chain (Fig. 5b).

To determine if the binding to the tyrosine-phosphorylated ζ chain was determined by the SH2 domain of Lck, similar experiments were performed using the isolated SH2 domain of Lck. Fig. 9 (a and b) demonstrates that the SH2 domain of Lck does not bind to the tyrosine-phosphorylated ζ chain of the TcR, although it does bind to a tyrosine-phosphorylated protein of ~70 kDa (which represents ZAP-70) (17). The SH2,3 domain of Lck, on the other hand, does bind to the tyrosine-phosphorylated ζ chain. Thus, the SH2 domain alone is insufficient for binding to the tyrosine-phosphorylated ζ chain, the binding of which requires the SH3 domain along with the SH2 domain as the binding is phosphotyrosine-dependent. This binding was direct as it was also observed to the same level under denaturing conditions. The SH2 domain of Src, however, does bind to the tyrosine-phosphorylated ζ chain (Fig. 9, a and b), suggesting that the Src SH2 domain has higher affinity for the tyrosine-phosphorylated ζ chain. The SH2,3 domains of Csk and Emt/ITK (Fig. 9b) did not bind to the tyrosine-phosphorylated ζ chain. Fusion proteins of full-length Grb2 and Nck, molecules with both SH2 and SH3 domains, both bound to the tyrosine-phosphorylated ζ chain. While the SH2 domain of Grb2 has been reported previously not to bind the tyrosine-phosphorylated ζ chain (18), we have used full-length Grb2, and the presence of the SH3 domains may increase the binding affinity for the tyrosine-phosphorylated ζ chain, similar to our findings with Lck. Thus, the data with the Lck fusion proteins fit well with the recently derived crystal structure of Lck and a tyrosine-phosphorylated peptide modeled after the C-terminal tail of Lck, suggesting that this phosphopeptide fits in the groove between the SH3 and SH2 domains (19). Therefore, the SH3 domain may be important in determining binding to some tyrosine-phosphorylated proteins, as demonstrated here for binding to the tyrosine-phosphorylated ζ chain.
bind to these molecules nondiscriminately. Arguing against this is the fact that only a subset of the molecules that become tyrosine-phosphorylated following TcR cross-linking are bound by the fusion protein. In addition, Fyn, phospholipase C-γ1, and Ras GTPase-activating protein could not be detected in the precipitates before or after TcR cross-linking.

To determine if these interactions occur in vivo, we stimulated Jurkat cells with pervanadate. Immunoprecipitates were formed using antibodies to the ζ chain, and in vitro kinase reactions were performed. The precipitates were then washed and boiled to release the precipitated proteins. The resultant proteins were re-immunoprecipitated using antibodies against either Lck or the anti-ζ chain. Fig. 10a shows that Lck can be re-immunoprecipitated from the anti-ζ precipitates. In similar experiments in which cells were stimulated with anti-CD3 antibodies, anti-Lck immunoprecipitates were probed with anti-MAPK antibodies (Fig. 10b), demonstrating that MAPK is present both before and after stimulation. Finally, when Jurkat cells were stimulated with anti-CD3 antibodies and Lck was immunoprecipitated, an increase in MAPK activity against a MAPK substrate could be detected (Fig. 10c).

**DISCUSSION**

The TcR signals given to T cells result in the tyrosine phosphorylation of numerous molecules. Some of these molecules are activated as a consequence of this phosphorylation. Another consequence of tyrosine phosphorylation can be relocation within the cell, such as from the cytoplasm to the membrane, or association with other molecules. We suggest that some of the molecules that are tyrosine-phosphorylated as a consequence of TcR signaling bind to the SH2,3 domain of Lck and localize themselves to this kinase. Lck would then be able to act on these molecules. Peri et al. (21) and Amrein et al. (20) have recently reported that a GST fusion protein of the SH2 domain of Lck can interact with numerous tyrosine-phosphorylated molecules in T lymphocytes following CD3 cross-linking and in fibroblasts, respectively. We demonstrated herein that the SH2,3 domain of Lck can interact with both MAPK and the ζ chain of the TcR, with the interaction with MAPK occurring in a phosphotyrosine-independent manner via the SH3 domain and the interaction with the ζ chain occurring in a phosphotyrosine-dependent manner via the SH2 and SH3 domains. Furthermore, we demonstrated that the pool of MAPK that can associate with the SH3 domain of Lck does so both before as well as after TcR cross-linking. Of interest are findings that Lck may be associated with the kinase Raf1 (22) or Raf1-related kinases (23). The Raf1 kinase is upstream of MAPK (24) and may be found in complexes with MAPK/extracellular-regulated kinase and Ras (25). As MAPK/extracellular-regulated kinase can interact with MAPK, one may expect that MAPK also associates with Lck. Finally, Lck is a substrate for activated MAPK, with MAPK phosphorylating Lck on Ser^59 following TcR and B cell receptor cross-linking (26). Recently, Chou and Hanafusa (27) reported a serine/threonine kinase Nck-associated kinase that interacts with the SH3 domain of Nck. This kinase was different from the known MAPKs. In addition, Weng et al. (28) have reported a serine/threonine kinase different from MAPK that interacts with the Src SH3 domain.
results add credence to the results obtained in this report that serine/threonine kinases can interact with SH3 domains. We also demonstrated that the tyrosine-phosphorylated ζ chain only associates with the SH2 domain of Lck when the SH3 domain is present. The fact that the binding is inducible following TcR cross-linking, when the ζ chain becomes tyrosine-phosphorylated, suggests that the binding is via a phosphotyrosine moiety. This was confirmed by the phenyl phosphate competition experiments. Thus, the binding of the SH2,3 domain of Lck to the tyrosine-phosphorylated ζ chain is unlike the binding of the SH2 domain of Lck to the ZAP-70 tyrosine kinase (17). This result also rules out an indirect binding effect since we can detect binding of the SH2 domain only to ZAP-70, but not to the tyrosine-phosphorylated ζ chain. These results fit the crystallographic model of Lck by Eck et al. (19); the two domains (SH2 and SH3) complexed with the Lck C-terminal phosphopeptide, with the peptide bound in a phosphotyrosine-dependent manner, fitted in a groove running between the two domains.

The association of the tyrosine-phosphorylated ζ chain with Lck would seem to follow as ζ is a substrate for Lck (1) and becomes tyrosine-phosphorylated both after activation of Lck as well as after cross-linking of the TcR (29). Thus, one can envision a scenario in which Lck tyrosine-phosphorylates the ζ chain and then associates with it, leading to the localization of kinase activity at the signaling complex. Since phospholipase C-1 has been shown to associate with the TcR complex (30) as well as with Lck, in this case via the SH2 domain of phospholipase C-1 (31), this would place Lck in an optimal position for its action. This localization of tyrosine kinases may serve to increase the potency of activation of the T cell. This is underscored in T cell lines that lack the ζ chain, the increase in activation when CD4-Lck is co-transfected in T cell lines that lack the ζ chain, as seen in our studies and as described by guest on April 29, 2019

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