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Tyrosine Phosphorylation of Grb2-Associated Proteins Correlates with Phospholipase Cγ1 Activation in T Cells

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Ligation of the T-cell antigen receptor (TCR) results in the rapid activation of several protein tyrosine kinases, with the subsequent phosphorylation of numerous cellular proteins. We investigated the requirement for tyrosine phosphorylation of proteins which bind the Grb2 SH2 domain in TCR-mediated signal transduction by transfecting the Jurkat T-cell line with a cDNA encoding a chimeric protein designed to dephosphorylate these molecules. Stimulation of the TCR on cells expressing this engineered enzyme fails to result in sustained tyrosine phosphorylation of a 36-kDa protein likely to be the recently cloned pp36/Lnk. Interestingly, TCR ligation of the transfected cells also fails to induce soluble inositol phosphate production and intracellular calcium mobilization, although receptor-mediated tyrosine phosphorylation of phospholipase Cγ1 still occurs. TCR-mediated Ras and mitogen-activated protein kinase activation remain intact in cells expressing the engineered phosphatase. These data demonstrate that tyrosine phosphorylation of a protein(s) which binds the SH2 domain of Grb2 correlates with phospholipase Cγ1 activation and suggest that such a phosphoprotein(s) plays a critical role in coupling the TCR with the phosphatidylinositol second-messenger pathway.

The earliest detectable biochemical event following ligation of the T-cell antigen receptor (TCR) is the activation of several protein tyrosine kinases (PTKs), with subsequent phosphorylation of numerous cellular proteins (18, 25, 28). Several of these PTK substrates have been characterized: components of the TCR itself (25, 28), phospholipase Cγ1 (PLCγ1) (23, 30, 38), Src and Syk family PTKs (Lck, Fyn, and ZAP-70) (reviewed in reference 36), GTPase-activated protein (16), ezrin (11), CD5 (4, 8), Shc (26), SLP-76 (17), Vav (5, 13), Cbl (10), and several cytoskeletal proteins (1). Although the importance of tyrosine phosphorylation of some of these molecules has been elucidated, the physiological relevance of many of these phosphorylation events remains to be determined. Additionally, many of the proteins which become tyrosine phosphorylated after TCR ligation remain to be identified.

One prominent substrate of the TCR-activated PTK(s) is a 36- to 38-kDa protein (pp36) that associates with the SH2 domains of numerous signaling molecules, including Grb2, PLCγ1, GTPase-activated protein, and Src family kinases (3, 22, 27, 31, 33, 35). Recently a molecule possessing several of these characteristics was identified and cloned from rat lymph node and has been termed Lnk (15). Although other phosphoproteins, such as the Shc adapter molecule and the Syp protein tyrosine phosphatase, have been shown to associate with the Grb2 SH2 domain, we find that pp36/Lnk is the predominant species that binds the SH2 domain of Grb2 in anti-TCR-stimulated T cells. Because of its interactions with other molecules known to participate in TCR signaling, it has been postulated that pp36/Lnk functions in the activation of both the phosphatidylinositol and Ras pathways (3, 31). To investigate the role of pp36/Lnk and other proteins which bind the SH2 domain of Grb2 in TCR signal transduction, we constructed a phosphatase that, when expressed in T cells, efficiently dephosphorylates molecules which associate with the SH2 domain of Grb2. This engineered enzyme consists of the extracellular and transmembrane domains of the A2 human major histocompatibility complex class I protein in frame with the SH2 domain of Grb2, followed by the cytoplasmic domain of the CD45 tyrosine phosphatase. Here we describe that expression of this engineered enzyme in the Jurkat human leukemic T-cell line results in markedly reduced TCR-mediated pp36/Lnk tyrosine phosphorylation, whereas phosphorylation of Shc does not appear to be affected. Interestingly, TCR stimulation of the transfected cells fails to result in appreciable soluble inositol phosphate production and intracellular calcium mobilization, although TCR-mediated PLCγ1 tyrosine phosphorylation remains intact and is comparable to that in the wild type. Receptor-mediated Ras GTP loading and mitogen-activated protein kinase (MAPK) activation remain intact in cells expressing the engineered phosphatase. These results demonstrate a correlation between tyrosine phosphorylation of pp36/Lnk and PLCγ1 activation and suggest that pp36/Lnk, and perhaps other proteins which bind the SH2 domain of Grb2, plays a critical role in coupling the TCR with the phosphatidylinositol second-messenger pathway.

MATERIALS AND METHODS

Cell culture and transfections. The human Jurkat T-cell line was maintained in RPMI 1640 supplemented with 10% fetal calf serum, penicillin (1,000 U/ml), streptomycin (1,000 U/ml), and glutamine (20 mM). Transfections of Jurkat cells were performed by electroporation with a Bio-Rad Gene Pulser set at 330 mV and 960 μF. After transfection, cells were selected and maintained in RPMI 1640 supplemented with G418 (2 mg/ml; Gibco). The cell lines described in this study are representative of several obtained from each transfection.

Antibodies and flow cytometry. The following antibodies were used in this study: anti-TCR monoclonal antibody (MAb) C305 (39) (gift of A. Weiss, San Francisco, Calif.); anti-TCR MAb OKT3 (American Type Culture Collection); anti-A2 MAb CR11-351 (gift of C. Lutz, Iowa City, Iowa); antiphosphotyrosine MAb 4G10 (gift of B. Drucker, Portland, Oreg.); anti-PLCγ1 MAb (Upstate Biotechnology); anti-MAPK MAb (Zymed); anti-Shc antiserum (Transduction Laboratories); anti-Syp MAb (Transduction Laboratories); and negative control MAb MOPC-195 (Cappel). For flow cytometric analysis, the indicated cells were stained with either MOPC-195, OKT3, or CR11-351 and then with a fluorescein isothiocyanate-conjugated secondary MAb and analyzed with a FACScan (Becton Dickinson).

cDNA constructs and fusion proteins. The A2/SH2/CD45 chimeric cDNA was constructed as follows. The Grb2 SH2 domain was amplified by PCR using the

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Grb2 cDNA template, the SH2 domain sense primer described below, and the antisense primer 5′-CGCGGATCCCCATGGCTGCTGGACGTATGTCGGC TGCTGTGG-3′. The resulting product was digested with *Bam*HI and ligated into the *Bgl*II site of the vector pGEM A2/CD45 (14). After verification of the correct orientation, the entire A2/SH2/CD45 sequence was excised with *Hin*dIII and ligated into the pcDNA3 expression vector (Invitrogen). To construct the A2/SH2 cDNA, the Grb2 SH2 domain from the pGEX-2TK vector described below was excised with *Bam*HI and *Eco*RI and ligated into the vector pGEM A2/YP (21), previously digested with *Bgl*II and *Eco*RI to remove the YP sequence. The entire A2/SH2 sequence was then excised with *Hin*dIII and *Eco*RI and ligated into pcDNA3. The A2/R86K cDNA was constructed in an identical fashion except that the starting plasmid was pGEX-2TK containing Grb2 cDNA with the R86K (Arg-86→Lys) mutation described previously (22). The A2/CD45 cDNA in the pHβ-Apr-1-neo expression vector has been described elsewhere (14). The glutathione *S*-transferase (GST)–Grb2 fusion proteins were generated by PCR using Grb2 cDNA as the template and the following primers: full-length sense, 5′-GGCGGATCCGAAGCCATCGCCAAATATGACTTC-3′; full-length antisense, 5′-GGGAATTCACCATGGATGTGGTTTCATTTCTATGTAG-3′; SH2 domain sense, 5′-GGCGGATCCCCACATCCGTGGTTTTTTGGCAAA ATCCCC-3′; and SH2 domain antisense, 5′-GGGAATTCACTGGACGTATG TCGGCTGCTGTGG-3′. Resulting PCR products were digested with *Bam*HI and *Eco*RI and subsequently subcloned into the pGEX-2TK bacterial expression vector (gift of W. Kaelin, Boston, Mass.). GST fusion proteins were induced and affinity purified as described previously (32).

Protein precipitations and immunoprecipitations. For each experiment, the indicated cells were left unstimulated or were stimulated with an anti-TCR MAb for various lengths of time. For some experiments, cells were stimulated with pervanadate for 1 min (29). Cell lysates prepared with detergent lysis buffer (1% Nonidet P-40 [NP-40] or 1% digitonin, 150 mM NaCl, 10 mM Tris [pH 7.4]) including protease (50 μg of aprotinin per ml, 10 μg of leupeptin per ml, 50 μg of pepstatin A per ml, and 1 mM phenylmethylsulfonyl fluoride) and phosphatase (400 nM sodium vanadate, 10 mM sodium fluoride, and 10 mM sodium PP_i) inhibitors were then subjected to precipitation with a GST fusion protein or with an antibody conjugated to protein A (Sigma) for 2 h at 4°C. Resulting protein complexes were then washed extensively in high-salt lysis buffer (500 mM NaCl), resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), transferred to nitrocellulose, and immunoblotted with an MAb followed by a horseradish peroxidase-conjugated secondary antibody (Bio-Rad). Immunoreactive proteins were detected by enhanced chemiluminescence (Amersham).

Measurement of soluble inositol phosphate generation. Cells were loaded with [^{3}H]myoinositol (Amersham), cultured overnight, and stimulated with medium alone (time zero) or with medium plus anti-TCR MAb C305 (ascites fluid, 1:1,000). The cells were then lysed at various times in a 1:1 mixture of chloroform-methanol, and soluble inositol phosphate was isolated by anion-exchange chromatography as described previously (12, 19).

Assessment of Ras activation. GTP and GDP binding of Ras was determined as described previously (34). Briefly, cells were incubated for 3 h in phosphate-free medium supplemented with $^{32}P_i$, stimulated via their TCR or with phorbol myristate acetate (PMA) for 10 min, lysed, and immunoprecipitated with anti-Ras MAb. Immune complexes were subjected to thin-layer chromatography followed by autoradiography to assess amounts of GTP and GDP bound.

RESULTS

Construction and expression of an enzyme designed to dephosphorylate proteins which bind the SH2 domain of Grb2. Recently our laboratory and others have demonstrated that three tyrosine phosphoproteins present in lysates of TCR-stimulated T cells associate with Grb2 both in vitro and in vivo (Fig. 1 and references 3, 22, 27, and 37). These include c-Cbl (10), SLP-76 (17), and a protein that migrates at 36 to 38 kDa (3, 22, 27, 31), which recently has been identified potentially as Lnk (15). pp36/Lnk binds the SH2 domain of Grb2 as well as the SH2 domains of several other molecules, such as PLCγ1, thought to be important in T-cell activation (Fig. 1 and references 16 and 35). As the interactions between pp36/Lnk and these proteins are SH2 domain mediated, we reasoned that tyrosine phosphorylation of pp36/Lnk may be required for its function in TCR-mediated signal transduction.

To investigate this hypothesis, we constructed a chimeric phosphatase designed to associate with and dephosphorylate proteins which bind the SH2 domain of Grb2. This engineered enzyme consists of the extracellular and transmembrane domains of the A2 human major histocompatibility complex class I protein in frame with the SH2 domain of Grb2, followed by

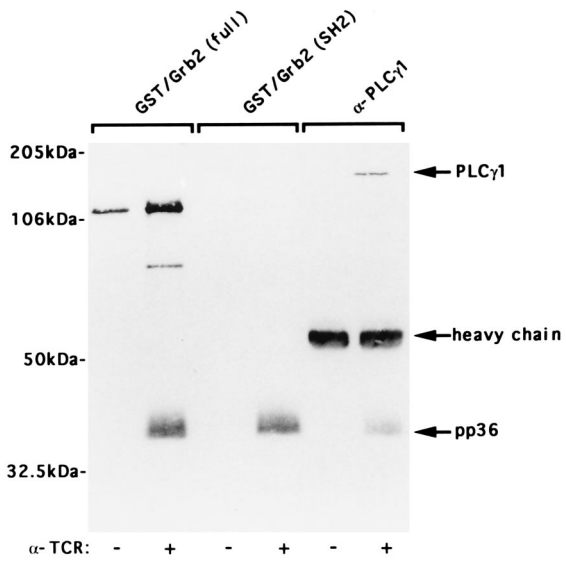

FIG. 1. pp36/Lnk associates with the SH2 domain of Grb2 and with PLCγ1. NP-40 lysates of resting or TCR-stimulated Jurkat T cells were precipitated with the indicated GST-Grb2 fusion proteins (5 μg of fusion protein, 20×10^6 cell equivalents per condition) or with anti-PLCγ1 MAb (α-PLCγ1) (50×10^6 cell equivalents per condition), and the resulting protein complexes were resolved by SDS-PAGE, transferred to nitrocellulose, and immunoblotted with antiphosphotyrosine MAb 4G10. The migrations of pp36/Lnk and phospho-PLCγ1 are as indicated. α-TCR, anti-TCR antibody.

the cytoplasmic domain of the CD45 tyrosine phosphatase (A2/SH2/CD45 [Fig. 2A]). We chose the cytoplasmic domain of CD45 as the phosphatase sequence for our chimeric protein because we reasoned that as wild-type CD45 is a transmembrane molecule, expression of its cytoplasmic domain at the plasma membrane would not be relocating it from its native context. Additionally, work from other laboratories has demonstrated that one potential substrate for our engineered enzyme, pp36/Lnk, is found exclusively in the plasma membrane fraction of stimulated T cells (3, 31); thus, through its extracellular and transmembrane sequences, the A2/SH2/CD45 chimeric molecule will be targeted to the site of pp36/Lnk localization. For experimental controls, we also created cDNAs encoding the following molecules, which are shown schematically in Fig. 2A: A2/SH2 (A2 linked only to the SH2 domain of Grb2), A2/R86K (A2 linked to the SH2 domain of Grb2 possessing a loss-of-function mutation [6]), and A2/CD45 (A2 linked only to the cytoplasmic domain of CD45). Transfection of each of these cDNAs into the Jurkat human leukemic T-cell line gave rise to several lines (designated "J" [for Jurkat] followed by the cDNA construct designation [from Fig. 2A] followed by the individual clone number) expressing high levels of both A2 and TCR, as analyzed by flow cytometry; representative cell lines from each transfection are shown in Fig. 2B.

Expression of the A2/SH2/CD45 chimeric phosphatase results in reduced TCR-mediated pp36/Lnk tyrosine phosphorylation. To determine if the A2/SH2/CD45 and A2/SH2 molecules are able to bind pp36/Lnk, we subjected anti-A2 immunoprecipitates from stimulated JA2/SH2/CD45.7 and JA2/SH2.3 cells to antiphosphotyrosine Western blotting (immunoblotting) (Fig. 3A). As shown, pp36/Lnk is detected as the predominant phosphotyrosine-containing protein in anti-A2 precipitates from JA2/SH2.3 cells stimulated either with anti-TCR MAb or with the tyrosine phosphatase inhibitor pervanadate. In contrast, pp36/Lnk is detected only in anti-A2 immu-

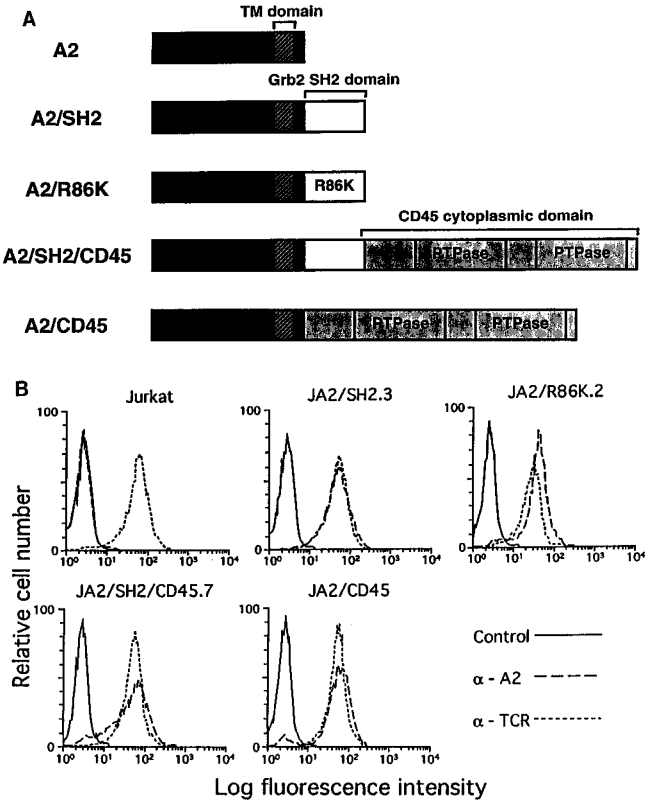

FIG. 2. (A) Schematic representation of the cDNA constructs used in this study. TM, transmembrane; PTPase, protein tyrosine phosphatase. (B) TCR and A2 surface expression on cell lines Jurkat, JA2/SH2/CD45.7 (Jurkat transfected with the A2/SH2/CD45 cDNA), JA2/SH2.3 (Jurkat transfected with the A2/SH2 cDNA), JA2/R86K.2 (Jurkat transfected with the A2/R86K cDNA), and JA2/CD45 (Jurkat transfected with the A2/CD45 cDNA), as analyzed by flow cytometry. α-A2 and α-TCR, anti-A2 and anti-TCR antibodies.

noprecipitates from pervanadate-stimulated JA2/SH2/CD45.7 cells, suggesting that in the absence of a phosphatase inhibitor, pp36/Lnk which associates with the A2/SH2/CD45 molecule becomes dephosphorylated. As expected, pp36/Lnk is not detected in anti-A2 precipitates from JA2/R86K.2 (the cell line expressing the A2/Grb2 SH2 domain loss-of-function mutant) or JA2/CD45 (the cell line expressing the A2/CD45 chimera with no Grb2 sequences [Fig. 3A]).

We next addressed whether expression of the engineered enzyme affects phosphorylation of the entire cellular pool of pp36/Lnk by using a GST-Grb2 fusion protein to precipitate pp36/Lnk from lysates of wild-type Jurkat or JA2/SH2/CD45.7 cells stimulated with anti-TCR MAb (Fig. 3B). As shown, the GST-Grb2 fusion protein precipitates very little pp36/Lnk from stimulated JA2/SH2/CD45.7 cells, while pp36/Lnk is readily detected in GST-Grb2 precipitates from stimulated Jurkat cells. Importantly, as shown also in Fig. 3B, stimulation of the TCR on JA2/SH2/CD45.7 cells still results in tyrosine phosphorylation of c-Cbl and SLP-76, two other Grb2-binding proteins. It should be noted that we are able to overcome the effects of the transfected phosphatase by subjecting JA2/SH2/CD45.7 cells to more potent TCR stimulation, but the amount of tyrosine-phosphorylated pp36/Lnk detected is always considerably less than that observed in wild-type cells.

It was important to determine if expression of the A2/SH2/CD45 chimeric molecule affects tyrosine phosphorylation of other substrates of the TCR-activated PTKs, including those

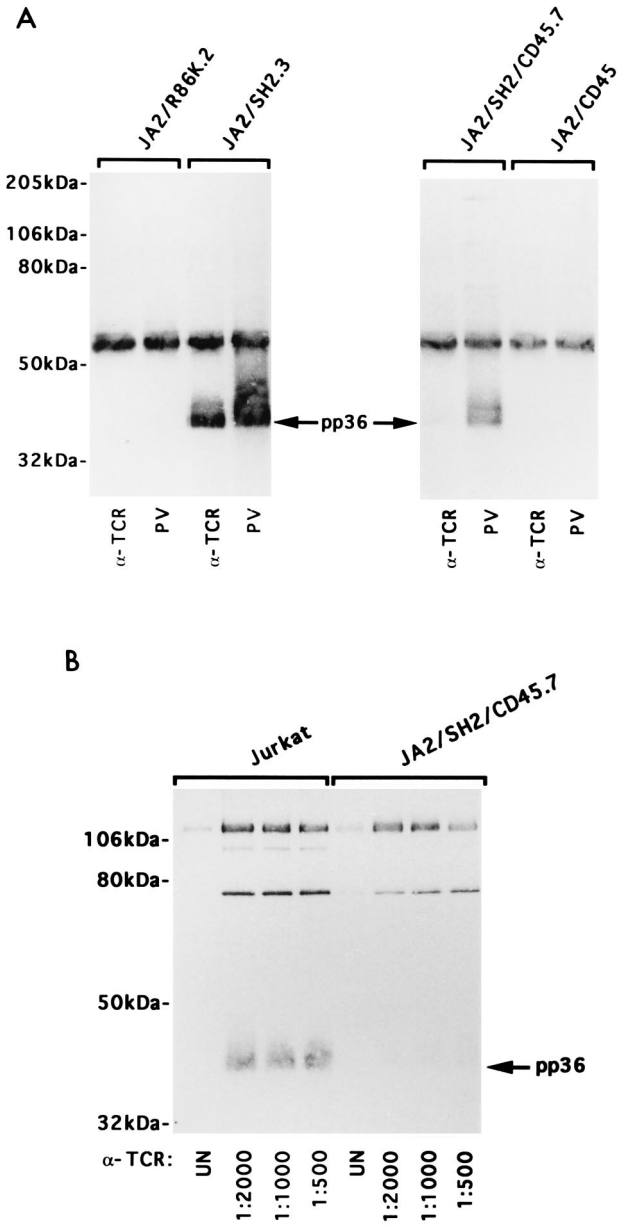

FIG. 3. Expression of the A2/SH2/CD45 chimeric molecule results in markedly reduced TCR-mediated pp36/Lnk tyrosine phosphorylation. (A) Antiphosphotyrosine Western blot of anti-A2 immunoprecipitates from cell lines JA2/R86K.2, JA2/SH2.3, JA2/SH2/CD45.7, and JA2/CD45 stimulated either with anti-TCR MAb (α-TCR) or with the tyrosine phosphatase inhibitor pervanadate (PV). The indicated cells (50×10^6 per condition) were stimulated either with anti-TCR MAb C305 or with pervanadate for 1 min, and NP-40 lysates were subjected to immunoprecipitation with the anti-A2 MAb CR11.351. Resulting protein complexes were resolved by SDS-PAGE (10% gel), transferred to nitrocellulose, and immunoblotted with 4G10. The migration of pp36/Lnk is as indicated. (B) Antiphosphotyrosine Western blot of GST-Grb2 fusion protein precipitations from anti-TCR-stimulated Jurkat and JA2/SH2/CD45.7 cells. Cells (20×10^6 per condition) were left unstimulated (UN) or were stimulated with the indicated dilutions of anti-TCR MAb OKT3 for 1 min, and digitonin lysates were subjected to precipitation with full-length GST-Grb2 fusion protein (10 μg per condition). Resulting protein complexes were resolved by SDS-PAGE (10% gel), transferred to nitrocellulose, and immunoblotted with 4G10. The migration of pp36/Lnk is as indicated.

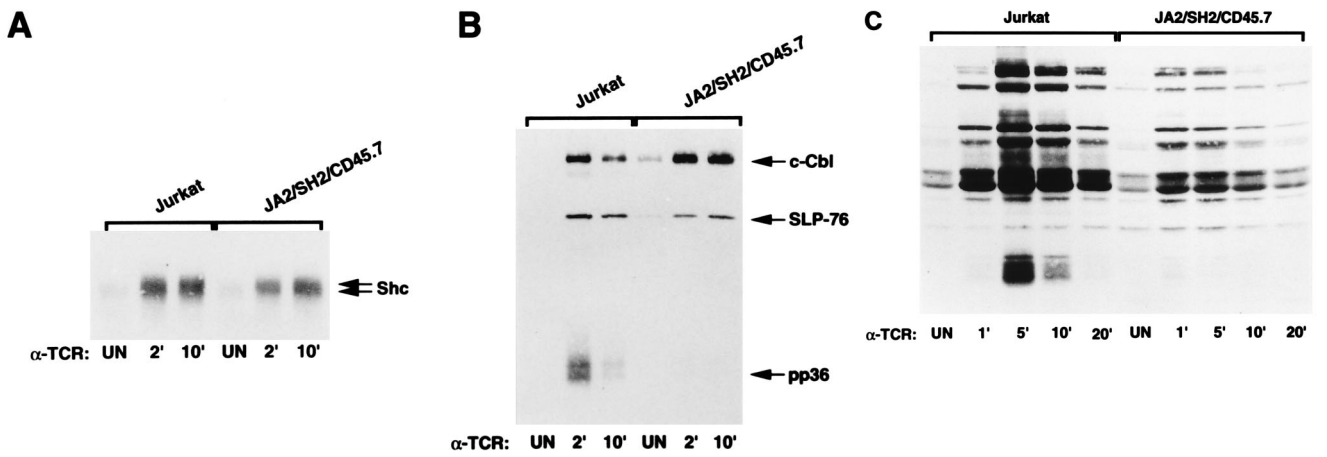

FIG. 4. Expression of the A2/SH2/CD45 molecule does not seem to affect significantly other substrates of the TCR-activated PTKs. (A and B) TCR-mediated tyrosine phosphorylation of Shc in Jurkat and JA2/SH2/CD45.7 cells. The indicated cells (50×10^6 per condition) were left unstimulated (UN) or were stimulated with anti-TCR MAb OKT3 (α-TCR) for the indicated lengths of time (minutes), and NP-40 lysates were split equally (25×10^6 cell equivalents each) and subjected to precipitation with anti-Shc antiserum (A) or with GST-Grb2 as a control (B). Resulting protein complexes were resolved by SDS-PAGE (10% gel), transferred to nitrocellulose, and immunoblotted with 4G10. The migrations of Shc, pp36, c-Cbl, and SLP-76 are as indicated. Subsequent stripping and reprobing of the nitrocellulose membrane in panel A with anti-Shc antiserum demonstrated equal Shc immunoprecipitation in each lane (not shown). (C) Global TCR-mediated induction of PTK activity in Jurkat and JA2/SH2/CD45.7 cells. The indicated cells were left unstimulated (UN) or were stimulated with anti-TCR MAb OKT3 for the indicated lengths of time (minutes), and NP-40 lysates (1.25×10^6 cell equivalents per lane) were resolved by SDS-PAGE (10% gel), transferred to nitrocellulose, and immunoblotted with 4G10.

which potentially also bind the Grb2 SH2 domain. Therefore, we investigated TCR-induced tyrosine phosphorylation of Shc, an adapter protein which has been shown to be a substrate of the TCR-stimulated PTKs and is thought to be involved in coupling the TCR with the Ras signaling pathway (Fig. 4A). As shown, Shc becomes tyrosine phosphorylated in JA2/SH2/CD45.7 cells similarly as in wild-type Jurkat cells. In this same experiment, however, pp36 tyrosine phosphorylation was considerably greater in Jurkat cells than in JA2/SH2/CD45.7 cells (Fig. 4B). A similar set of experiments was performed to examine tyrosine phosphorylation of Syp, a protein tyrosine phosphatase that has been shown to bind the SH2 domain of Grb2 in other systems (20). However, although expression was found in both Jurkat and JA2/SH2/CD45.7 cells, we detected very little TCR-induced tyrosine phosphorylation of Syp in either cell line (not shown).

Of course, it is possible that expression of the chimeric phosphatase affects tyrosine phosphorylation of other proteins which have not been identified as binders of the Grb2 SH2 domain and against which we do not have specific reagents. To address this possibility, we next compared the global pattern of tyrosine phosphorylation induced by TCR stimulation in Jurkat cells with that demonstrated by JA2/SH2/CD45.7 cells (Fig. 4C). As can be appreciated, although the intensity of phosphorylation of most proteins in JA2/SH2/CD45.7 cells is less than that seen in Jurkat cells, the most striking difference between phosphorylated substrates in the two cell lines is the marked decrease in phosphorylation of a protein migrating at 36 to 38 kDa in JA2/SH2/CD45.7 cells. It should be noted that we find considerable variability in tyrosine phosphorylation of specific substrates between clones of wild-type Jurkat cells and clones expressing the A2/SH2/CD45 chimera; however, in each of the Jurkat clones, we are always able to detect tyrosine phosphorylation of the 36- to 38-kDa species. Conversely, in each of the clones expressing the engineered phosphatase, we fail to detect phosphorylation of this protein. Thus, although it is formally possible that expression of the A2/SH2/CD45 protein affects other molecules of which we are unaware or cannot measure, our results suggest that one major substrate of the engineered phosphatase is pp36/Lnk.

Expression of the A2/SH2/CD45 protein uncouples the TCR from activation of the phosphatidylinositol pathway. Previous work has demonstrated that pp36/Lnk associates with the phosphoinositide-specific enzyme PLCγ1 (16, 31, 35), another substrate of the TCR-activated PTK (23, 30, 38). We therefore investigated whether PLCγ1 activity is affected by expression of the A2/SH2/CD45 molecule by measuring TCR-mediated soluble inositol phosphate generation in JA2/SH2/CD45.7 cells (Fig. 5A). As shown, TCR stimulation of JA2/SH2/CD45.7 cells results in a marked reduction of total inositol phosphate production compared with both Jurkat and JA2/CD45 cells. Because in wild-type cells, production of inositol trisphosphate results in a rapid and transient increase of intracellular free calcium (2, 37), we investigated whether the defect in soluble inositol phosphate generation exhibited by JA2/SH2/CD45.7 cells results in the inability of these cells to mobilize intracellular calcium in response to TCR ligation (Fig. 5B). As shown, the JA2/SH2/CD45.7 cells demonstrate a profound inability to increase intracellular free calcium compared with anti-TCR responses generated by Jurkat, JA2/SH2.3, JA2/R86K.2, and JA2/CD45 cells. These data demonstrate that expression of the A2/SH2/CD45 chimeric molecule uncouples the TCR from activation of the phosphatidylinositol pathway and suggest that tyrosine phosphorylation of pp36/Lnk is required for TCR-mediated PLCγ1 activation. Interestingly, the inositol phosphate and calcium defects exhibited by JA2/SH2/CD45.7 cells are not due to the inability of these cells to phosphorylate PLCγ1, because TCR stimulation of JA2/SH2/CD45.7 cells results in PLCγ1 phosphorylation comparable to that seen in wild-type Jurkat cells (Fig. 5C).

Expression of the A2/SH2/CD45 molecule does not affect TCR-mediated activation of the Ras pathway. It has been demonstrated recently that both Grb2 and pp36/Lnk can be detected in immunoprecipitates of the SOS guanine nucleotide exchange factor from lysates of stimulated T cells (3, 31), giving rise to the hypothesis that pp36/Lnk, through its associ-

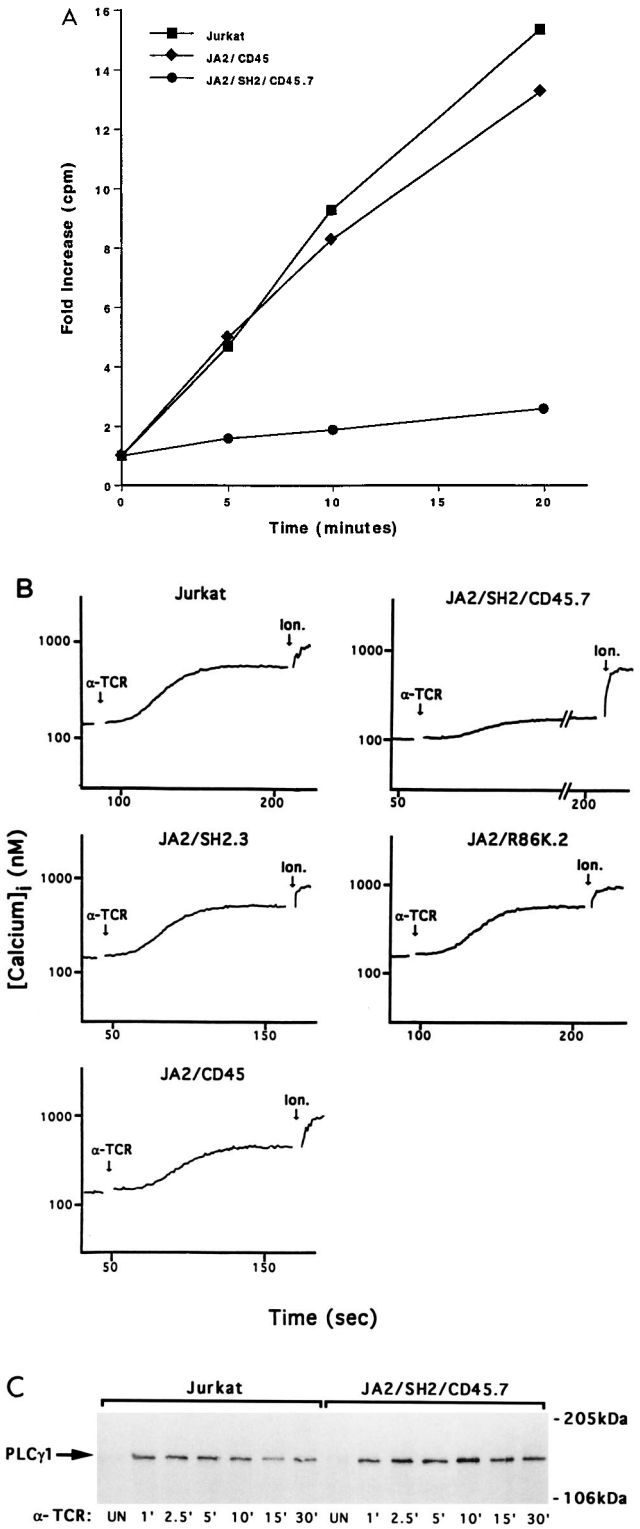

FIG. 5. Expression of the A2/SH2/CD45 protein uncouples the TCR from activation of the PLCγ1 pathway. (A) TCR-mediated soluble inositol phosphate generation in Jurkat, JA2/SH2/CD45.7, and JA2/CD45 cells. (B) TCR-mediated intracellular calcium ($[Calcium]_i$) mobilization in Jurkat, JA2/SH2/CD45.7, JA2/SH2.3, JA2/R86K.2, and JA2/CD45 cells, as measured by fluorimetry. Cells were loaded with the calcium-sensitive dye Indo-1, and real-time fluorescence was measured by fluorimetry as described previously (18). The additions of anti-TCR MAb OKT3 (α-TCR) and ionomycin (Ion.) are indicated. (C) Antiphosphotyrosine Western blot of anti-PLCγ1 immunoprecipitates from resting and TCR-stimulated Jurkat and JA2/SH2/CD45.7 cells. Cells were left unstimulated (UN) or were stimulated with anti-TCR MAb OKT3 (α-TCR) for the lengths of time (minutes) indicated, and NP-40 lysates (25×10^6 cell equivalents per condition) were then subjected to immunoprecipitation with anti-PLCγ1 MAb. Resulting protein complexes were then resolved by SDS-PAGE (8% gel), transferred to nitrocellulose, and immunoblotted with 4G10. The migration of phospho-PLCγ1 is indicated.

ation with Grb2, may play a role in TCR-mediated Ras activation. We therefore investigated whether expression of the A2/SH2/CD45 chimera affects Ras activation by examining the electrophoretic mobility of MAPK (9) before and after TCR stimulation (Fig. 6A). As shown, stimulation of both wild-type Jurkat and JA2/SH2/CD45.7 cells with either anti-TCR MAb or the phorbol ester PMA results in the appearance of a doublet on MAPK immunoblots, indicating Ras-mediated activation of this molecule. Further experiments demonstrated that TCR stimulation results in an increase in GTP loading of Ras in both cell lines (Fig. 6B). Phosphorimage analysis revealed ratios of Ras/GTP to Ras/GDP after TCR stimulation equal to 0.45 in Jurkat cells and 0.41 in JA2/SH2/CD45 cells. Additionally, TCR stimulation of JA2/SH2/CD45.7 cells results in expression of the CD69 activation antigen (not shown), which in T cells has been reported to be a Ras-mediated event (7). Thus, expression of the A2/SH2/CD45 molecule does not seem to affect significantly TCR-mediated Ras activation.

DISCUSSION

In this study, we investigated the role of phosphoproteins which bind the SH2 domain of Grb2 in TCR-mediated signal transduction. We focused on these proteins because such molecules have been hypothesized to function in the activation of both the phosphatidylinositol and Ras pathways. One Grb2-binding protein which has received considerable recent attention is pp36/Lnk, a prominent substrate of the TCR-activated PTKs which has been shown to associate with both PLCγ1 and Grb2 after TCR ligation. As the associations between pp36/Lnk and these signaling molecules are SH2 domain mediated, we hypothesized that tyrosine phosphorylation of pp36/Lnk is required for the protein's function. We reasoned further that if it were possible to disrupt the tyrosine phosphorylation of pp36/Lnk and perhaps other phosphoproteins which bind the SH2 domain of Grb2 in vivo, we might gain insight into the function of these molecules.

Therefore, we constructed and expressed in Jurkat T cells a chimeric phosphatase designed to associate with and dephosphorylate proteins which associate with the SH2 domain of Grb2. In this report, we demonstrate first that expression of this engineered molecule indeed results in significantly reduced TCR-mediated tyrosine phosphorylation of pp36/Lnk but has much less of an impact on tyrosine phosphorylation of the adapter protein, Shc. We demonstrate further that TCR stimulation of cells expressing the chimeric phosphatase fails to result in the activation of the phosphatidylinositol pathway, as assessed by measurement of soluble inositol phosphate generation and mobilization of intracellular calcium. These results demonstrate that tyrosine phosphorylation of pp36/Lnk correlates with the ability of the TCR to couple with PLCγ1.

Although our data are consistent with this role for pp36/Lnk in TCR signal transduction, it is formally possible that the transfected chimeric molecule affects other substrates of the TCR-stimulated PTKs. We believe, however, that pp36/Lnk is at least one of the relevant targets for the engineered phosphatase for several reasons: (i) pp36/Lnk is the predominant protein species that we and others observe associated with

Grb2 SH2 domain constructs with in vitro fusion protein assays, (ii) pp36/Lnk is the only phosphoprotein that we detect in immunoprecipitates of our A2/SH2 and A2/SH2/CD45 chimeric molecules by antiphosphotyrosine immunoblot analysis, (iii) pp36/Lnk associates with PLCγ1 in vivo, and (iv) expression of the A2/SH2/CD45 chimera does not appear to influence tyrosine phosphorylation of other proteins (such as Shc) reported by others to associate with the Grb2 SH2 domain.

Interestingly, our data demonstrate also that under conditions in which TCR-mediated activation of the phosphatidylinositol pathway is severely compromised, receptor stimulation still results in PLCγ1 tyrosine phosphorylation. Thus, TCR-mediated activation of PLCγ1 seems to require one or more biochemical events in addition to its tyrosine phosphorylation. Our results suggest that two of these additional events may be the tyrosine phosphorylation of pp36/Lnk and its subsequent association with PLCγ1.

Although our data suggest that pp36/Lnk may play a role in coupling the TCR with the phosphatidylinositol signaling pathway, we can only speculate on the mechanism by which this may occur. Among the possibilities are the following. (i) Plasma membrane-associated pp36/Lnk may serve as a binding site for PLCγ1, allowing it to interact with its appropriate substrate. This hypothesis is supported by the observation by others (3, 31) that tyrosine phosphorylated pp36/Lnk localizes exclusively to the particulate fraction of cell lysates. (ii) Similarly, binding of tyrosine phosphorylated pp36/Lnk may allow PLCγ1 to associate with the plasma membrane, possibly by promoting a conformation of PLCγ1 that allows its pleckstrin homology domain better access to the plasma membrane (24). (iii) Alternatively, binding of pp36/Lnk to PLCγ1 may directly stimulate its enzymatic activity or (iv) displace an inhibitory molecule. A definitive role for pp36/Lnk in coupling the TCR with the phosphatidylinositol signaling pathway and whether any of the above models are correct will be testable once reagents become available for the biochemical characterization of this molecule.

Recently it has been hypothesized that pp36/Lnk, through its association with the Grb2-SOS complex, may play a role in Ras activation mediated via the TCR. Our results demonstrate that expression of the A2/SH2/CD45 chimeric molecule, which results in the reduction of TCR-mediated pp36/Lnk tyrosine phosphorylation, does not affect significantly TCR-mediated activation of the Ras pathway. Thus, tyrosine phosphorylation of pp36/Lnk does not seem to correlate with the ability of the TCR to couple with Ras. However, this lack of phenotype does not necessarily preclude a role for pp36/Lnk in the activation of Ras, as it remains possible that the function of pp36/Lnk in other pathways is less dependent on the absolute level and/or duration of its tyrosine phosphorylation compared with its role in PLCγ1 activation. Alternatively, as is the case with growth factor receptors, it is possible that there are multiple pathways which function to couple the TCR with Ras. Thus, abrogation of one pathway may not be expected to result in a detectable phenotype.

Finally, in addition to addressing the role of phosphoproteins which bind the Grb2 SH2 domain in T-cell activation, our results demonstrate further that it is possible to investigate the functions of cellular proteins through the use of engineered molecules which target enzymatic function to a protein of interest. Similar techniques may prove valuable in addressing the role of posttranslational modifications of other molecules, especially when specific reagents for those molecules are not yet available. Experiments addressing whether SH3 domains can serve also as targeting sequences in similar chimeric enzymes are in progress.

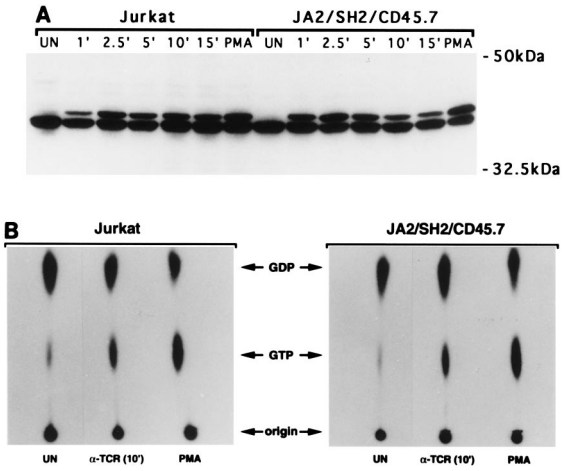

FIG. 6. Expression of the A2/SH2/CD45 chimeric molecule does not affect TCR-mediated activation of the Ras pathway. (A) Lysates of resting and TCR-stimulated Jurkat and JA2/SH2/CD45.7 cells were immunoblotted with anti-MAPK MAb, demonstrating the electrophoretic mobility shift that indicates activation of this enzyme. Cells were left unstimulated (UN) or were stimulated with either anti-TCR MAb OKT3 for the lengths of time (minutes) indicated or with PMA (50 ng/ml) for 10 min. NP-40 lysates (10^6 cell equivalents per condition) were then subjected to SDS-PAGE (12% gel; 75:1 acrylamide/bisacrylamide), transferred to nitrocellulose, and immunoblotted with anti-MAPK MAb. (B) Increase of Ras-GTP complexes in Jurkat and JA2/SH2/CD45.7 cells following TCR stimulation. Cells (50×10^6 per condition) were loaded for 3 h with $^{32}P_i$ and subsequently were left resting or stimulated with either OKT3 (α-TCR; 1:2,000) or PMA (50 ng/ml) for 10 min. GTP and GDP loading of Ras was then assessed by immunoprecipitation followed by thin-layer chromatography as described previously (34). The migrations of GTP and GDP are as indicated.

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