SKAP55 Recruits to Lipid Rafts and Positively Mediates the MAPK Pathway upon T Cell Receptor Activation*

Received for publication, June 17, 2002
Published, JBC Papers in Press, August 8, 2002, DOI 10.1074/jbc.M206023200

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T cell receptor (TCR) engagement triggers a series of events including protein tyrosine kinase activation, tyrosine phosphorylation of adapter proteins, and multiple protein-protein interactions. We observed that adapter protein SKAP55, the Src kinase-associated phosphoprotein, formed homodimers through its SH3 domain and SK region. SKAP55 as a substrate interacted with Fyn kinase in vivo. In Jurkat cells, interaction between SKAP55 and Fyn kinase depended on TCR activation. Stable overexpression of SKAP55 in Jurkat cells caused mitogen-activated protein kinase activation following TCR engagement. Anti-CD3 stimulation also promoted the interaction of SKAP55 with Grb-2 in T cells. Mutational analysis revealed that tyrosine 271 in SKAP55 played a pivotal role for interaction with both Fyn kinase and adapter protein Grb-2, indicating that the Fyn-phosphorylated SKAP55 transiently associates with adapter Grb-2 to mediate mitogen-activated protein kinase activation. Intriguingly, T cell receptor engagement dramatically induced the translocation of endogenous SKAP55 to lipid rafts where SKAP55 was found to interact with Fyn kinase, suggesting that the positive function of SKAP55 via its association with Fyn and other signaling components may have been involved in raft-mediated T cell activation.

Engagement of the antigen-presenting cell (APC) leads to activation of the T cell receptor (TCR) and induces a series of signaling events including a variety of aspects of gene transcription, cell proliferation, and cell differentiation (1–3). The earliest signaling events in TCR activation involve the Src family kinases (Fyn and Lck), which are activated through CD45-mediated dephosphorylation on the C-terminal negative regulatory site of the kinases. The activated Src kinases are capable of phosphorylating the two immunoreceptor tyrosine-based activation motifs (ITAMs) within the intracellular tails of TCR. It has been established that the rapidly tyrosine-phosphorylated ITAMs provide docking sites for binding of the SH2 domain-containing protein tyrosine kinases, Syk and ZAP-70, which are subsequently tyrosine-phosphorylated and become fully activated. Hence, it is established that protein interactions through signaling binding motifs in vivo are regulated directly or indirectly by both protein tyrosine kinases and protein tyrosine phosphatases during T cell activation. Furthermore, recent data documents that lipid rafts, specialized membrane microdomains of the cell membrane, are involved in both TCR- and BCR-mediated signal transduction (4). Upon TCR engagement, various signaling molecules involved in TCR signal transduction, such as Vav, Grb-2, and TCRζ, are enriched in rafts, strongly suggesting that lipid rafts play an important functional role in TCR signaling. Presently, the mechanism responsible for recruitment of these TCR signaling molecules to rafts is largely unknown.

CD45, the most abundant membrane protein tyrosine phosphatase in T lymphocytes, has not been unequivocally demonstrated to localize to rafts. However, it is certain that CD45 plays a critical role in TCR-mediated signal transduction in T-cells in which this phosphatase is thought to positively regulate T cell activation. Conclusive evidence supporting the critical role of CD45 in TCR signaling comes from an analysis of a CD45-deficient cell line in which TCR signal transduction was totally abrogated (5, 6). Consistent with the positive function of CD45 in TCR signaling, it has been shown that in CD45-deficient cells the kinase activities of Src family kinases, such as Fyn and Lck, were largely suppressed by increased tyrosine phosphorylation at the negative regulatory site located on the C terminus. These data have led to the conclusion that Fyn and Lck interact with CD45 and are putative substrates for the phosphatase. However, there is no clear evidence to support that CD45 directly interacts with these two Src kinases. A recent study suggests that CD45 is unlikely to interact directly with Fyn for dephosphorylation. Rather, it transiently recruits adapter protein SKAP55 as a linker to access the negative regulatory tyrosine-phosphorylated site of Fyn for dephosphorylation, and thus positively regulates TCR signaling (7).

A number of adapter proteins, including LAT, VAV, and SLP-76, have been identified in hematopoietic cells. Involvement of these adapters in signaling is strongly suggested by the fact that following their tyrosine phosphorylation, many adapter proteins are able to positively or negatively regulate T cell activation (8). Adapter proteins bearing protein-interacting modules act as signaling linkers to facilitate multiple protein-protein interactions required for intermolecular switches occurring in signaling events. For instance, adapter LAT, a substrate of protein tyrosine kinase Zap-70 (9), contains a long cytoplasmic tail rich in tyrosine residues that are highly phosphorylated upon T cell activation. The phosphorylated LAT recruits Grb2, PLC-γ1, and phosphatidylinositol 3-kinase to the membrane leading to the activation of several signaling events, such as Ras/MAPK pathway and calcium influx (9, 10). Likewise, adapter SLP-76, another substrate of Zap-70 kinase,
Localization of SKAP55 in Lipid Rafts

has been shown to interact with Grb2 and SLAP, and MAPK pathways (11). SKAP55, the Src kinase-associated phosphoprotein, was first identified as a Fyn SH2 domain binding protein. SKAP55 is exclusively expressed in T cells (12), but its biological function in T cells has been elusive since its identification. It was later reported that a novel proline-independent motif in SKAP55 could bind to SH3 domains. Very recently it was found that SKAP55, as a substrate, couples with CD45 and links CD45 with Fyn kinase for dephosphorylation, thus positively regulating TCR-mediated gene transcription (7). However, little is known in terms of the downstream effectors of SKAP55-mediated signaling during TCR activation. In the present report, we demonstrate that SKAP55, as a substrate, was phosphorylated by Fyn kinase. SKAP55 formed homodimers through its SH3 domain and SK region. Stable overexpression of SKAP55 significantly increased MAP kinase phosphorylation in response to anti-CD3 stimulation in vivo. Furthermore, in Jurkat cells, stimulation with anti-CD3 promoted the translocation of SKAP55 to lipid rafts, where it interacted with Fyn kinase. From these results, together with our previous findings, we conclude that SKAP55 in association with both CD45 and Fyn kinase plays a positive role in raft-mediated T cell activation.

EXPERIMENTAL PROCEDURES

Reagents and Cell Lines—Jurkat cells were obtained from American Type Culture Collection (ATCC) and cultured in RPMI 1640 medium with 10% fetal bovine serum, 2 mM L-glutamine (Invitrogen), and 50 mM Hepes. 293T cells (ATCC) were cultured in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum. Anti-CD3 antibody was purchased from BD Biosciences.

Epitope-tagged Constructs and Mutagenesis—SKAP55 was subcloned in-frame with the C-terminal Myc-His tag into pcDNA3.1 vector (Invitrogen). Fyn (p59) and its kinase-dead mutant Fyn-K299A in pRK5 were kindly provided by Dr. Korezkey (University of Pennsylvania, State College, PA). Anti-phospho MAPK (ERK1/ERK2) antibody was a kind gift from Dr. Zhao (Vanderbilt University, Nashville, TN). Anti-SKAP55 and anti-CD45 antibodies were from BD Biosciences.

Cell Culture and Cell Transfection—The cells were well maintained in log phase and fed the day before transfections. 50–70% confluent 293T cells were transfected overnight in a 37 °C incubator at 3% CO2 using highly efficient calcium phosphate transfection reagents. The transfection efficiency was measured using 1 µg of CMV-GFP plasmid as control and at least 30–50% green cells were observed under a fluorescent microscopy 18 h after transfection. Jurkat cells were cultured in RPMI 1640 medium with 10% fetal bovine serum and were fed with fresh complete culture medium 1 day before transfection. 10 × 10^5 Jurkat cells for each transfection were washed twice using RPMI 1640 medium containing 50 mM Hepes and mixed with 50 µg of appropriate plasmid. The mixture was reacted at 200 µl, 10 µs in 300–350 µl volume, using a BTX820 electroporator (BTX Corp., San Diego, CA). After incubation for 15 min at room temperature, the transfected cells were reconstituted with fresh full culture medium for an additional 48 h. For anti-CD3 stimulation, cells were treated with anti-CD3 antibody (5 µg/ml) and then treated with a rabbit anti-mouse secondary antibody (20 µg/ml) for 5 min. The reaction was stopped with 10 µl of ice-cold PBS before adding lysis buffer.

Immuno precipitation and Western Blot Analysis—Cells were washed once with cold PBS and lysed with 1% Triton X-100 lysis buffer containing 50 mM Hepes, 150 mM NaCl, 1% Triton, 1.5 mM MgCl2, 10 mM NaF, 1 mM phenylmethylsulfonyl fluoride, 2 mM EDTA, 10 µg/ml aprotinin, and 10 µg/ml leupeptin for 30 min on ice. The samples were transferred to Eppendorf tubes and centrifugated at 12,000 rpm for 15 min. The supernatants were transferred to clean tubes and subjected to immunoprecipitation for 4 h at 4 °C with appropriate antibodies conjugated to protein A Sepharose (Sigma). The immunocomplexes were washed three times with lysis buffer and once with PBS, and then subjected to 7% SDS-PAGE and Western blot analysis with appropriate antibodies. The membranes were incubated with 5% non-fat milk in Tris-buffered saline for 1 h, incubated with the first antibody, and washed for 30 min, followed by the second antibody incubation. The membranes then were washed for 15 min and rinsed with PBS once before for Western blot analysis.

MAP Kinase Assay—SKAP55 and its mutants were transfected into Jurkat cells, and stable cell lines were selected with 200 µg/ml G418 in RPMI 1640 medium. The selected clones were poured together and divided equally into two portions. One portion was treated with anti-CD3 antibody, while the other portion was left untreated. For each treatment, 1.5 × 10^6 cells were collected by centrifugation and lysed in 200 µl of 1% Triton X-100 lysis buffer for 15 min on ice. The total cell lysates (4%) were subjected to Western blot analysis with anti-phospho MAPK antibody, anti-ERK1/ERK2 antibody, and anti-Myc antibody, respectively.

Sucrose Gradient Centrifugation Experiment and Lipid Rafts Fractionation—Jurkat cells (5 × 10^6) in log phase were cultured in RPMI 1640 medium with 10% fetal bovine serum. Cells were washed once with PBS and resuspended in a full culture medium and divided into two portions. One portion was left untreated, and the other portion was treated with anti-CD3 (10 µg/ml) plus goat anti-mouse antibody (20 µg/ml) for 5 min. Both the treated and untreated cells were immediately washed once with ice-cold PBS before lysis with 1% Triton X-100 lysis buffer on ice for 30 min. The lysates were homogenized by 10 strokes with a Dounce homogenizer. One ml of cell lysates was mixed with 1 ml of 80% sucrose dissolved in 1% Triton X-100 lysis buffer and added to the bottom of a centrifuge tube. The tube was overlaid with 2 ml of 30% sucrose and 1 ml of 5% sucrose on the top. The sucrose gradient samples were centrifuged at 38,400 rpm with a Beckman SW55Ti rotor at 4 °C for 10 h. After centrifugation, each fraction (1 ml of sample) was carefully collected. In some experiments, aliquots from the raft fractions (fractions 3–5) were used for immunoprecipitation and Western blotting.

RESULTS

The SH3 Domain of SKAP55 Mediates Self-interaction—It is known that SH3 domains bind to proline-rich motifs. Because the SH3 region of SKAP55 contains proline-rich sequences, it is likely that self-association of SKAP55 may occur in vivo. To test this possibility, we made two SKAP55 constructs, one with a HA tag at the N terminus (HA-SKAP55) and the other with a Myc tag at the C terminus (SKAP55-Myc). Both constructs were cotransfected into Jurkat cells, and the transfected cells were treated with anti-CD3 stimulation or left untreated. Co-immunoprecipitations were performed with anti-HA or anti-Myc antibodies and precipitants were analyzed by Western blot. As shown in Fig 1A, in the anti-Myc immunoprecipitants, SKAP55 was detected with anti-HA antibody, indicating that the HA-tagged SKAP55 interacted with Myc-tagged SKAP55. Anti-CD3 stimulation enhanced the formation of SKAP55 homodimers (Fig. 1A, right panel). Similarly, in the reciprocal experiment, the Myc-tagged SKAP55 was detectable in the anti-HA immunoprecipitants (data not shown). The conserved tryptophan residue in the SH3 domain is known to be critical for the binding of proline-rich motifs. To determine whether the SH3 domain of SKAP55 is involved in self-association, a mutant, SKAP55-W333R, with the replacement of arginine for tryptophan 333 at the SH3 domain was made. Both the Myc-tagged mutant SKAP55-W333R (SKAP55-W333R-Myc) and the Myc-tagged wild type SKAP55 (SKAP55-Myc) were cotransfected with HA-SKAP55 into 293T cells. As shown in Fig. 1B, the amount of mutant SKAP55-W333R-Myc precipitated with HA-SKAP55 was dramatically decreased in comparison with that of SKAP55-Myc wild type. This mutant also suppressed the SKAP55-mediated MAPK activation (see Fig. 6 A)
SKAP55 is self-associated. A, homodimerization of SKAP55 is enhanced with anti-CD3 stimulation in Jurkat cells. The N-terminal HA-tagged SKAP55 and the C-terminal Myc-tagged SKAP55 were transiently cotransfected into Jurkat cells for 48 h. The transfected cells were treated with anti-CD3 stimulation for 5 min or left untreated. The cell lysates were prepared, and immunoprecipitations were performed either with anti-HA or with anti-Myc antibody. The immunocomplexes were subjected to Western blot analysis with anti-HA antibody. B, the SH3 domain of SKAP55 is involved in self-interaction. The N-terminal HA-tagged SKAP55 was co-transfected either with wild-type C-terminal Myc-tagged SKAP55, or with mutant SKAP55-W333R-Myc into 293T cells. The cell lysates were prepared and subjected to immunoprecipitation and Western blot analysis with antibodies as indicated. These results suggest that the SH3 domain of SKAP55 contributes to the self-interaction in vivo. (Fig. 2).

Interaction of SKAP55 with Fyn Is Kinase Activity-dependent—SKAP55 was first identified as a Fyn kinase-interacting protein (12). Later, it was reported that SKAP55 was tyrosine-phosphorylated when cotransfected with Fyn kinase, but not with Lck kinase or with ZAP-70 kinase (13), indicating that SKAP55 is a potential substrate for Fyn kinase. To investigate the substrate nature of SKAP55 for Fyn, we compared the interaction of SKAP55 with the wild-type Fyn and with the Fyn kinase-dead mutant Fyn-K299A (14) in vivo in conjunction with its tyrosine phosphorylation. SKAP55 tagged with Myc at the C terminus was transfected either with wild-type Fyn or with the kinase-dead mutant Fyn-K299A into 293-T cells. The interactions of SKAP55 with Fyn and its mutant were characterized by immunoprecipitation. As shown in Fig. 2A, left panel, whereas SKAP55 was largely communoprecipitated with wild-type Fyn kinase, it was barely detectable in immunoprecipitates produced by the kinase-dead mutant Fyn-K299A. The status of the tyrosine phosphorylation of SKAP55 in the immunoprecipitants was examined by anti-phosphotyrosine antibody. As shown in the right panel of Fig. 2A, SKAP55 was highly tyrosine-phosphorylated in immunocomplex with wild-type Fyn kinase, whereas its tyrosine phosphorylation was not detectable in the immunoprecipitants generated by kinase-dead mutant Fyn-K299A. The interaction of SKAP55 with Fyn kinase was further examined in T lymphocytes where both SKAP55 and Fyn are predominantly expressed. SKAP55 was transiently transfected with Fyn kinase into Jurkat cells. After transfections, the cells were treated with anti-CD3 antibody for 5 min or left untreated. Immunoprecipitations were performed with anti-Fyn antibody and anti-Myc antibody, respectively. SKAP55 was readily detected in the immunocomplex prepared from anti-CD3-stimulated cells but not from non-treated cells (Fig. 2B). Taken together, these data show that SKAP55 interacts with Fyn kinase in response to TCR activation in Jurkat cells and that Fyn kinase activity is required for this interaction and for the tyrosine phosphorylation of SKAP55 in vivo.

Tyrosine 271 of SKAP55 Is Critical for Interaction with Fyn Kinase—The requirement of tyrosine phosphorylation for interaction of SKAP55 with Fyn kinase prompted us to define Fyn kinase-binding sites in SKAP55. There are three potential tyrosine residues in SKAP55 for binding to the SH2 domain of Fyn, namely, tyrosine 219, tyrosine 232, and tyrosine 271 (15). Using site-directed mutagenesis, a tyrosine (Y) to phenylalanine (F) mutant was made for those tyrosine residues in SKAP55 that was tagged with Myc at the C terminus. A Fyn kinase expression vector was cotransfected with a vector alone, or containing SKAP55 or one of its mutants (Y219F, Y232F, and Y271F), into 293T cells. Cell lysates were prepared and subjected to immunodetections following immunoprecipitations with anti-Fyn antibody and anti-Myc antibody, respectively. Fig. 3A shows that Fyn was detected in the anti-Myc immunoprecipitants from cells transfected with wild-type SKAP55 and SKAP55-Y219F. In the latter case, a large amount of SKAP55-Y219F was observed, probably due to its very high level of expression in the transfected cells as shown in Fig. 3B, lower panel. Fyn was also detectable in the SKAP55-Y232F-transfected cells, although a reduced precipitant was observed with this mutant when compared with wild type SKAP55. In contrast, Fyn was not detected from the cells transfected with SKAP55-Y271F. The reciprocal experiments also confirmed that SKAP55-Y271F was not able to interact with Fyn kinase (Fig. 3B). Taken together, these data show that the tyrosine at position 271 within the EDIYXX motif of SKAP55 contributes to its interaction with the SH2 domain of Fyn kinase.

Endogenous SKAP55 Recruits to Lipid Rafts upon Anti-CD3 Stimulation—The components of lipid rafts are disassembled in resting T cells but are rapidly assembled when T cells are triggered by anti-CD3 stimulation (16). Previously, we have shown that SKAP55 is translocated from the cytoplasm to the membrane in response to anti-CD3 stimulation, and CD45 is required for such dynamic translocation of SKAP55 in Jurkat cells (7). Because rafts are well correlated with TCR activation, we were interested in further examining whether the observed translocation of SKAP55 in Jurkat cells is related to the localization of lipid rafts. Jurkat cells were either left unstimulated or stimulated with anti-CD3, and the cell lysates were ultracentrifuged over a sucrose step gradient. The distribution of endogenous SKAP55 in the sucrose gradient was examined by
immunoblotting. Without anti-CD3 stimulation, SKAP55 was hardly detectable in the raft fractions. However, after TCR cross-linking a significant amount of SKAP55 was distributed in the raft fractions (Fig. 4, upper panel in fractions 3, 4 and 5).

Consistent with other reports (16, 17), the endogenous Fyn kinase was constitutively present in the raft fractions, although its amount slightly increased with anti-CD3 stimulation (Fig. 4, lower panel). These results suggest that SKAP55 is recruited to lipid rafts upon TCR activation whereas Src kinases and other characterized TCR signaling molecules are pre-targeted into membrane rafts without TCR stimulation (Fig. 4).

**Endogenous SKAP55 Associates with Fyn in Rafts upon TCR Engagement**—We have shown that SKAP55 was phosphorylated by its interaction with Fyn in vivo (Fig. 2). To investigate the molecular mechanism responsible for the recruitment of endogenous SKAP55 to lipid rafts, we tested whether there is a physiological association between SKAP55 and Fyn in rafts following anti-CD3 stimulation in vivo. To this end, lipid raft fractions 3, 4, and 5 (see Fig. 4) were combined from Jurkat cells and before and after CD3 stimulation. The association of SKAP55 with Fyn was examined by coimmunoprecipitation and immunodetection. As shown in Fig. 5, SKAP55 was readily detected in the coimmunoprecipitants produced from the CD3-stimulated cells, but not in the immunocomplexes obtained from the unstimulated cells in which, as shown in Fig. 4, little SKAP55 was localized in the raft fractions. Taken together, these results suggest that upon TCR engagement, endogenous SKAP55 recruits to rafts probably through its association with Fyn, and it may thereby exert its regulatory function in raft-mediated T cell activation (Fig. 5).

**Overexpression of SKAP55 Leads to Activation of MAP Kinase in T Cells**—It has been well established that there is a cross-talking between the TCR signaling pathway and the MAPK signaling pathway through lymphocyte-specific adapter proteins such as SLP-76 and LAT. These two adapters, the two common effectors downstream of T cell signaling (18), bind to Grb-2, which in turn leads to Ras activation. Because SKAP55 plays a positive role in TCR-mediated gene transcription (7), we examined whether SKAP55 has any effects on the MAPK pathway. For this purpose, Jurkat cells were stably transfected with SKAP55 and its mutants, SKAP55-Y219F, SKAP55-Y232F, and SKAP55-Y271F. The cell lysates were prepared from cells either treated with anti-CD3 or left untreated. The prepared cell lysates were subjected to Western blot analysis with anti-phospho-MAPK antibody. Fig. 6 shows that in comparison with control vector alone, overexpression of wild-type SKAP55 dramatically induced MAPK phosphorylation in the CD3-stimulated cells. Mutant SKAP55-Y219F exhibited a moderate increase in MAPK phosphorylation in response to CD3 stimulation when compared with vector alone. In contrast, the other two mutations, SKAP55-Y271F and SKAP55-Y232F, nearly completely abolished the observed enhancing effect of wild-type SKAP55 on MAPK activation after TCR cross-linking. In the rest Jurkat cells, the inactive MAPK remained unphosphorylated regardless of the expression of wild-type SKAP55. The endogenous SKAP55 recruits to rafts probably through its association with Fyn, and it may thereby exert its regulatory function in raft-mediated T cell activation (Fig. 5).
SKAP55 or its mutants. In addition, we also transfected mutant SKAP55-W333R into Jurkat cells and found that the activation of MAPK phosphorylation was significantly reduced when compared with the transfectants of wild-type SKAP55 (Fig. 6, upper panel), indicating that dimerization of SKAP55 may have contributed to its regulatory functions in the signaling. Taken together, these results strongly suggest that SKAP55 positively mediates the MAPK pathway in conjunction with TCR activation (Fig. 6).

SKAP55 Interacts with Grb-2, but Not with SLP-76 and LAT—It has been demonstrated that several adapter proteins, such as Grb-2 (18), SLP-76 (11), and LAT (9), are involved in the MAPK pathway in conjunction with TCR activation. Following TCR cross-linking, these adapter proteins are rapidly tyrosine phosphorylated (19). To study the mechanism by which SKAP55 mediates the activation of MAP kinase, we examined whether these adapter proteins associate with SKAP55 during TCR activation. In this regard, SKAP55 tagged with Myc at the C terminus was cotransfected with Grb-2 into Jurkat cells. The transfected cells were either treated with anti-CD3 antibody or left untreated. Cell lysates were prepared, immunoprecipitated by anti-Myc antibody, and immunodetected by anti-Fyn antibody (A), or reciprocally, the cell lysates were immunoprecipitated with anti-Fyn antibody and blotted with anti-Myc antibody (B).

Fig. 3. Mutant SKAP55-Y271F abolishes its interaction with Fyn kinase. The C-terminal Myc-tagged SKAP55 and its mutants, SKAP55-Y271F, SKAP55-Y219F, and SKAP55-Y232F were co-transfected with Fyn into 293T cells. Forty-eight hours after transfections, cell lysates were prepared, immunoprecipitated by anti-Myc antibody, and immunodetected by anti-Fyn antibody (A), or reciprocally, the cell lysates were immunoprecipitated with anti-Fyn antibody and blotted with anti-Myc antibody (B).

Fig. 4. Endogenous SKAP55 was translocated to lipid rafts in response to TCR activation. Jurkat cells (5 × 10^6) in log phase were treated with anti-CD3 antibody for 5 min or left untreated. Cells were lysed with lysis buffer containing 1% Triton X-100, and the lysates were subjected to equilibrium sucrose gradient centrifugation. An aliquot of each fraction was electrophoresed under reducing conditions, and immunoblotted with anti-SKAP55 antibody (upper), or immunoblotted with anti-Fyn antibody (lower).
undetectable in the immunoprecipitants obtained from the unstimulated Jurkat cells (Fig. 7A, left panel). In parallel, the Myc-tagged SKAP55 was cotransfected either with LAT tagged with FLAG at the N terminus or with SLP-76 tagged with FLAG at the N terminus, into Jurkat cells. After the transfections, the cells were either treated with anti-CD3 or left untreated. Total cell lysates were immunoprecipitated with appropriate antibodies. As shown in Fig. 7, B and C, both FLAG-LAT and FLAG-SLP-76 proteins failed to coimmunoprecipitate with Myc-tagged SKAP55 protein, irrespective of the treatment with anti-CD3 antibody; hence, the interaction between SKAP55 and Grb-2 is of a relatively specific nature.

Because the SH3 domain of Grb-2 failed to interact with SKAP55 (20), we suspect that the interaction between Grb-2 and SKAP55 probably occurs through the binding of the tyrosine-phosphorylated SKAP55 to the SH2 domain of Grb-2. To reveal the nature of the interaction between the two proteins, mutants of SKAP55, designated SKAP55-Y271F, SKAP55-Y219F, and SKAP55-Y232F, were tested for their interaction with Grb-2 after stimulation with anti-CD3 in Jurkat cells. Whereas mutants SKAP55-Y219F and SKAP55-Y232F displayed their interactions with Grb-2 similar to that of wild-type SKAP55, the interaction of mutant SKAP55-Y271F with Grb-2 was significantly reduced (Fig. 7D). These results clearly show that SKAP55 associates with Grb-2 through its phosphorylated residue 271 following TCR engagement.

**DISCUSSION**

Previously, we have shown that CD45 recruits adapter protein SKAP55 as a linker to access the negative regulatory site in Fyn kinase for dephosphorylation, leading to activation of TCR signaling. Thus, SKAP55 positively regulates TCR-mediated gene transcription (7). In this report, we further demonstrated that SKAP55 itself, while acting as a linker between CD45 and Fyn, was tyrosine-phosphorylated by Fyn kinase, and the phosphorylated SKAP55 subsequently interacted with Fyn. The interaction between SKAP55 and Fyn requires the kinase activity of Fyn because the kinase-dead mutant Fyn-K299A failed to interact with SKAP55. In Jurkat cells, anti-CD3 stimulation greatly promoted interaction between SKAP55 and Fyn kinase. It is very likely that such CD3 stimulation-dependent association of SKAP55 with Fyn kinase results in localization of the adapter protein to the membrane, thereby facilitating exertion of its positive regulatory function in TCR signaling (see below). Such tyrosine phosphorylation-dependent association of SKAP55 with Fyn was somehow unnoticed in a previous report (21). Studies using Tyr→Phe site-directed mutants of SKAP55 proved that tyrosine residue 271, which resides within EDIYXXL motif, is the only tyrosine residue critical for binding to the SH2 domain of Fyn. Together with the previous reports (12, 13), our results indicate that Fyn is the main kinase responsible for tyrosine phosphorylation of SKAP55 in vivo, although the possibility of phosphorylation by other tyrosine kinases in the TCR complex cannot be excluded.

TCR activation triggered by engagement of the antigen-presenting cell ultimately leads to transcriptional activation through a series of signaling events in which the MAP kinase pathway is thought to be involved. We demonstrated that in Jurkat cells, overexpression of SKAP55 resulted in activation of MAP kinase following anti-CD3 stimulation. Both Y271F and Y232F mutations in SKAP55 did not exhibit any enhanced

**FIG. 5.** Endogenous SKAP55 associates with Fyn kinase in lipid rafts. As with Fig. 4, Jurkat cells (5 × 10⁸) were treated with anti-CD3 antibody for 5 min or left untreated. The cell lysates were subjected to sucrose gradient centrifugation. Aliquots from the raft fractions (fractions 3–5) were combined and immunoprecipitated with anti-SKAP55 and anti-Fyn antibodies, respectively. The immunocomplexes were subjected to Western blot analysis with anti-SKAP55 antibody. The expression of Fyn protein in Jurkat cells was monitored with anti-Fyn antibody (lower panel).

**FIG. 6.** SKAP55 functions as a positive regulator for the MAPK pathway in Jurkat cells. Jurkat cells were transfected with vector alone, SKAP55, or the mutants SKAP55-Y271F, SKAP55-Y219F, SKAP55-Y232F, or SKAP55-W333R. The stable transfected Jurkat cells were poured together and treated with anti-CD3 for 5 min or left untreated. Total cell lysates were subjected to Western blot analysis with anti-phospho-MAPK antibody (upper), anti-ERK1/ERK2 antibody (middle), or anti-SKAP55 antibody (lower).
MAP kinase activation observed with wild-type SAKP55 (Fig. 6). The fact that the interaction of CD45 with SAKP55 is via residue Tyr 232 and that Tyr 271 is critical for interaction of SAKP55 with Fyn supports the notion that the positive regulatory function of SAKP55 in TCR signaling is exerted through its interaction with both CD45 and Fyn kinase. The SAKP55-mediated downstream effectors in TCR signaling were further targeted to an adapter protein, Grb-2, which is known to be involved in activation of the MAPK pathway. In Jurkat cells, interaction of SAKP55 with Grb-2 was induced by anti-CD3 stimulation. Interestingly, the interaction between SAKP55 and Grb-2 depends on Tyr 271, the same residue that is tar-
Dimerization of SKAP55 may also increase its stability. Itsmultiple functional domains for intermolecular interactions amplify its effects on signaling cascades in T cells by providing transcriptional activation. For SKAP55, dimerization may dimerization of STAT family proteins promotes their function in phosphorylation-dependent CD45 activity. However, the recruitment of CD45 itself to rafts is still a controversial issue for this key positive regulator in TCR signaling. It remains unclear exactly how SKAP55 is translocated into rafts, and the question of which component(s) of the TCR signaling cascade is directly responsible for the recruitment of SKAP55 to lipid rafts demands further investigation. Nevertheless, it has been well established that under resting conditions TCRs reside outside rafts but are recruited into rafts upon activation, indicating that membrane compartmentation is a prerequisite for TCR signal transduction. Thus, the TCR-activated translocation of SKAP55 to rafts and its association with Fyn in rafts strongly suggest that the positive function of SKAP55 in TCR signaling may have been involved in raft-mediated T cell activation.

Acknowledgments—We thank Mark Slater and Jason Boyd for very useful comments on the manuscript, Drs. Denis Banville and Hui-Fen Zhao for valuable discussion and advice, D. L’Abbé for technical assistance, and N. Jolicoeur for preparation of the figures.

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