The activation of protein kinases is one of the primary mechanisms whereby T cell receptors (TCR) propagate intracellular signals. To date, the majority of kinases known to be involved in the early stages of TCR signaling are protein-tyrosine kinases such as Lck, Fyn, and ZAP-70. Here we report a constitutive association between the TCR and a serine/threonine kinase, which was mediated through the membrane-proximal portion of CD3 e. Mass spectrometry analysis of CD3 e-associated proteins identified G protein-coupled receptor kinase 2 (GRK2) as a candidate Ser/Thr kinase. Transient transfection assays and Western blot analysis verified the ability of GRK2 to interact with the cytoplasmic domain of CD3 e within a cell. These findings are consistent with recent reports demonstrating the ability of certain G protein-coupled receptors (GPCR) and G proteins to physically associate with the α/β TCR. Because GRK2 is primarily involved in arresting GPCR signals, its interaction with CD3 e may provide a novel means whereby the TCR can negatively regulate signals generated through GPCRs.

The T cell receptor (TCR) is a multimeric complex composed of the polymorphic α and β subunits and the CD3 γ, δ, ε, and ζ invariant chains. Extracellular interactions between the α/β subunits of the TCR and peptide/major histocompatibility complexes initiate an intricate cascade of intracellular signals, which regulate T cell proliferation, effector function, and/or programmed cell death. Signaling events triggered through TCR interactions are controlled by a conserved amino acid motif, termed the immunoreceptor tyrosine-based activation motif (ITAM), which is found in the cytoplasmic domains of each of the CD3 invariant chains (reviewed in Refs. 2 and 3). Following TCR engagement, members of the Src family of protein-tyrosine kinases (PTK) phosphorylate tyrosine residues in the invariant chain ITAMs (4, 5). Bi-phosphorylated ITAMs subsequently associate with the Syk family PTK, ζ-associated protein of 70 kDa (ZAP-70), via its tandem Src homology 2 domains (6). Once associated, ZAP-70 is tyrosine-phosphorylated and catalytically activated. It then phosphorylates and/or interacts with additional signaling molecules, such as Vav, LAT, and SLP-76 (reviewed in Ref. 7). These initial tyrosine phosphorylation pathways are subsequently channeled into the activation of multiple serine/threonine (Ser/Thr) kinases, including MAPKs, IκB kinases, and PKC family members, which ultimately regulate transcription factor activity. In addition to influencing T cell effector function or proliferation, the association of certain PTKs with the TCR invariant chains has been demonstrated to induce cross-talk between the TCR and certain chemokine receptors, thus enhancing T cell migration (8, 9).

Tyrosine phosphorylation of the invariant chain ITAMs is one of the earliest detectable signaling events to occur upon TCR engagement (10). However, recent reports have identified a ligand-induced conformational change within the cytoplasmic tail of CD3 e that precedes ITAM phosphorylation and is independent of Src kinase activity (see Ref. 11 and reviewed in Ref. 12). Although the mechanisms resulting in this conformational change are still being elucidated, it is clear that engagement of the TCR results in the exposure of a proline-rich stretch (PRS) in the cytoplasmic tail of CD3 e, allowing it to complex the Src homology 3 domain-containing adaptor protein Nck (11, 13). In addition to this interaction, CD3 e is also capable of binding other signaling proteins, such as ZAP-70, CD3 e-associated signal transducer, nucleolin, and topoisomerase II β (14–16). Like CD3 e, the CD3 γ, δ, and ε subunits can also interact with a variety of effector/adaptor proteins. Examples include the ability of the cytoplasmic tail of CD3 ζ to bind UNC119, whereas the cytoplasmic domains of CD3 γ, δ, and ε can bind to Fyn and the regulatory subunit of phosphatidylinositol 3 kinase (p85) (17–19).

Given the complexity of these protein-protein interactions, as well as the importance of kinase cascades in many signaling pathways, we were interested in determining whether the CD3 invariant chains could differentially associate with kinases. Using a variety of biochemical assays, we found that CD3 e isolated from unstimulated T cells could constitutively associ-
ate with the Ser/Thr kinase, G protein-coupled receptor kinase 2 (GRK2). We also determined that TCR cross-linking resulted in interactions between CD3 ε and the Ser/Thr kinase hematopoietic progenitor kinase 1 (HPK1).

EXPERIMENTAL PROCEDURES

Mice—All mice, on a C57BL/6 background, were housed in the specific pathogen-free facility on the north campus at the University of Texas Southwestern Medical Center. Mouse procedures were undertaken with IACUC-approved protocols.

Reagents and GST Fusion Protein Preparations—Anti-CD3 ε (145-2C11 and OKT3) and anti-α were purified from culture supernatants using affinity chromatography (American Type Tissue Culture (ATCC)). The anti-CD28 (47.51) hybridoma was kindly provided by Dr. J. Allison (University of California, Berkeley). Anti-CD3 ε (6B10.2) and anti-ZAP-70 (IE7.2) were used as described (20). Anti-GRK2/3 and anti-Myc (9E10) were purchased from Upstate Biotechnology, Inc. Anti-PO4-GRK2 (Ser-670) was from BioSource (Camarillo, CA). Anti-HPK1 was purchased from Cell Signaling Technology, Inc. (Danvers, MA). Anti-GST was from BD Biosciences. Recombinant SDF-1α was purchased from R&D Systems (Minneapolis, MN).

Cell Lines—Jurkat T cells, P116 (ZAP-70−/−) Jurkat T cells, and HEK 293T cells were obtained from ATCC.

Plasmids—pGEX-2TK was from Amersham Biosciences; pcDNA3.1/myc-HIS was from Invitrogen. The GST-CD3 constructs were derived by PCR-based strategies. All sequences were verified by double-stranded DNA sequencing.

Immunoprecipitations, Western Blots, and In Vitro Kinase Reactions—Thymocytes were lysed at 2.0 × 10^8 cells/ml in a 1% Triton X-100-containing lysis buffer at pH 7.6 (20 mM Tris-HCl, 30 mM NaCl, 2 mM EDTA, 1 mM NaF, and protease inhibitors). Immunoprecipitations or GST pull-downs were undertaken with protein A/G Seize®-coated plates (Pierce) or glutathione-coated HS plates (Sigma), according to the manufacturer’s instructions. Kinase reactions were carried out for 30 min at 30 °C in a reaction mixture containing 2.5 μg of MBP, 50 μl of kinase buffer (50 mM Tris-HCl (pH 7.6), 2 mM MnCl2, 2 mM MgCl2, 1 mM benzamidine, 1 mM sodium fluoride, 0.05% Triton X-100, and 1 mM phenylmethylsulfonyl fluoride), 0.3 μM ATP, and 1 μl of radiophosphate (0.1 μCi of [γ-32P]ATP; Amersham Biosciences). Inhibitors were used at the following concentrations: SB203580 (10 μM), PD098059 (50 μM), piceatannol (25 μg/ml), wortmannin (100 μM), bisindolylmaleimide (5 μM), and Ly294002 (10 μM). Kinase reactions were quenched by adding 20 μl of pre-warmed 4X SDS sample buffer. Proteins were resolved by SDS-PAGE and transferred onto a polyvinylidene difluoride membrane (Millipore Corp.). Radiolabeled substrate was visualized using a Storm 820 PhosphorImager (Amersham Biosciences). Data are presented as relative light units. For Western blot analysis, proteins were resolved by SDS-PAGE, transferred onto polyvinylidene difluoride, and visualized using the Pierce ECL Western blotting substrate.

Stimulations—CD3 ε stimulations and recombinant SDF-1α treatments were performed as described previously (9, 21).

Phosphoamino Acid Analysis—Phosphoamino acid content was determined as described previously (22).

Chromatography Separations—Buffer A consisted of 50 mM Tris-HCl (pH 7.6), 2 mM 2-mercaptoethanol. Buffer B consisted of Buffer A plus 1 M NaCl. Cellular preparations were generated from 6.5 × 10^6 murine thymocytes. Cells were washed three times in phosphate-buffered saline. Pelleted cells were then resuspended in Buffer A supplemented with protease and phosphatase inhibitors, using a buffer volume 10 times the pellet size. The cells were Dounce-homogenized with 30 strokes and incubated on ice for an additional 15 min. Supernatants were obtained following a high speed spin (28,000 rpm for 1 h at 4 °C), filtered through a 0.22-μm syringe filter (Millex™, Millipore), and applied to a 2.5 × 30-cm anion-exchange column linked to an FPLC™ system (Q-Sepharose, Amersham Biosciences). Proteins retained on the column were eluted with a linear salt gradient (Buffer B) at a flow rate of 2.5 ml/min. Eighty 10-ml fractions were collected. An aliquot from each fraction was analyzed for the GST-BRS-associated kinase activity as described previously. Samples with the greatest kinase activity were pooled and diluted 1:2 in Buffer A. This was applied to a GST-BRS affinity column. Proteins retained on the affinity matrices were eluted with a linear salt gradient (Buffer B) at a flow rate of 0.5 ml/min. Forty five 2-ml fractions were collected. Fractions were assayed for kinase activity by GST-BRS pull-downs and kinase reactions as described.

Acetone precipitations were performed by adding 15 μl of 20% SDS, 6 μl of dithiothreitol (1 M), 2 μl of glycogen (20 mg/ml), and 1 ml of acetone (−80 °C) to 300 μl of sample. Samples were centrifuged for 10 min at 13,000–15,000 × g. The pellet was air-dried, and silver staining was performed using GelCode® SilverSNAP® Stain Kit II (Pierce) according to the manufacturer’s instructions. Silver-stained bands were submitted to the Protein Chemistry Technology Center at the University of Texas Southwestern Medical Center for trypsin digestion and mass spectrometry analysis.

Transfections and siRNA Knockdown—HEK 293T cells were transfected using a standard CaPO4 method as described (23). RNA interference was carried out using a GRK2 human siRNA (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) as described (24). All statistical comparisons were performed using a paired, two-tailed Student’s t test.

RESULTS

CD3 ε Associates with a Kinase Activity Independent of ZAP-70—Several signaling motifs are located in the cytoplasmic domains of the CD3 subunits, including the ITAMs and the PRS (Fig. 1A). These motifs, as well as other less well defined sequences, mediate protein-protein interactions with at least 10 distinct intracellular signaling molecules (2). We were interested in determining whether the cytoplasmic tails of the CD3 chains could associate with protein kinases other than ZAP-70 (a PTK known to interact with the bi-phosphorylated ITAMs). Accordingly, GST fusion proteins containing the cytoplasmic regions of CD3 γ, δ, ε, or ζ were used as affinity ligands to pull down associating proteins from unstimulated thymocyte lysates. After washing, the pulldowns were subjected to in vitro kinase reactions in the presence of [γ-32P]ATP. Associating proteins were then resolved by SDS-PAGE, and phosphoproteins were visualized by autoradiography. GST-ε associated
CD3 ε Association with GRK2

A. Structure of the TCR complex showing different signaling domains. B. GST fusion proteins containing the cytoplasmic portion of CD3 γ, δ, ε, or ζ were prepared and quantified by Coomassie Brilliant Blue (CBB) staining (lower panel). Equivalent amounts of the GST fusion proteins were incubated with thymocyte WCLs, followed by in vitro kinase (i.v.k) reactions in the presence of [γ-32P]ATP. Proteins were resolved by SDS-PAGE, and phosphoproteins were visualized by autoradiography (upper panel). C. Thymocyte WCLs were prepared from C57BL/6 mice, followed by immunoprecipitations (IP) of the indicated molecules. In vitro kinase reactions, SDS-PAGE, and autoradiography were used to visualize phosphoproteins. D. Equivalent numbers of Jurkat T cells (lanes 1–3) or P116 Jurkat T cells (lanes 4–6) were lysed, and the indicated molecules were immunoprecipitated. Precipitates were incubated in a kinase reaction mix containing both [γ-32P]ATP and MBP. Phosphoproteins were visualized by autoradiography. These results are representative of over a dozen independent assays. NMS = normal mouse sera.

with a kinase as indicated by the presence and intensity of a number of 32P-labeled proteins (Fig. 1B, lane 3). Only a modest amount of kinase activity was detected following GST-γ, -δ, or -ζ pulldowns (Fig. 1B, lanes 1, 2, and 4).

To address whether full-length CD3 ε could associate with a kinase activity in primary cells, CD3 ε was directly immunoprecipitated from murine thymocyte lysates and analyzed by kinase reactions. As seen, full-length CD3 ε also associated with a kinase activity (Fig. 1C, lane 1). A small amount of activity was detected in CD3 ζ immunoprecipitates, likely because CD3 ε and CD3 ζ can co-precipitate (Fig. 1C, lane 2). Importantly, the banding pattern of the 32P-labeled proteins detected in both the CD3 ε and CD3 ζ precipitates were distinct from those observed following ZAP-70 immunoprecipitations, suggesting the CD3 ε-associated kinase was not ZAP-70 (Fig. 1C, lane 3). No substantial kinase activity was observed in either the CD28 immunoprecipitations or in the normal mouse sera controls (Fig. 1C, lanes 4 and 5).

To confirm that ZAP-70 was not responsible for the CD3 ε-associated kinase activity, a Jurkat cell line deficient in ZAP-70 expression (P116) was utilized. ZAP-70, CD3 ε, and CD28 were immunoprecipitated from the lysates of unstimulated Jurkat or P116 Jurkat T cells, followed by in vitro kinase reactions in the presence of the exogenous substrate MBP (Fig. 1D). CD28 immunoprecipitations served as controls for background kinase activity. Kinase reactions performed using ZAP-70 immunoprecipitates from Jurkat T cells revealed a substantial level of kinase activity (Fig. 1D, lanes 1 and 3). This activity was reduced to background levels in the ZAP-70-deficient cells (Fig. 1D, lanes 1 and 4). In contrast, an equivalent level of CD3 ε-associated kinase activity was detected in both the wild type and ZAP-70-deficient Jurkat T cells (Fig. 1D, lanes 2 and 5). These results indicated the CD3 ε-associated kinase was distinct and independent of ZAP-70.

CD3 ε Constitutively Associates with a Serine/Threonine Kinase Distinct from the PKC, Phosphatidylinositol 3-Kinase, and MAPK Families—To determine the effects of TCR engagement on the CD3 ε-associated kinase activity, thymocytes were stimulated with α-CD3 ε for varying time points, followed by CD3 ε precipitations and kinase reactions. The level of CD3 ε-associated kinase activity increased by 16–21% after 10 and 30 min of stimulation, respectively (Fig. 2A, lanes 1–3). The constitutively associated kinase activity was reduced when cells were cultured for 4 h at 37 °C (Fig. 2A, lane 4). When cultured cells were stimulated, the CD3 ε-associated kinase activity increased by 62% (Fig. 2A, lane 6). Such findings are in accordance with previously published reports and suggest that the kinase association was constitutive in thymocytes as a consequence of ongoing TCR interactions with self-peptide/major histocompatibility complex (21).

To elucidate the type(s) of kinase(s) associating with CD3 ε, the phosphoamino acid content of several of the prominent 32P-labeled bands and/or the exogenous substrate MBP was determined (Fig. 2B). A 1:1 ratio of 32P-serine and 32P-threonine was detected in all the proteins analyzed from CD3 ε immunoprecipitations (Fig. 2C, bands a and c). 32P-Tyrosine was only observed in ZAP-70 immunoprecipitates (Fig. 2C, band b).

Kinase reactions were also performed in the presence of selective kinase inhibitors. Piceatannol, a resveratrol analog that blocks the activity of certain Ser/Thr kinases as well as Syk family members, was the only inhibitor that consistently decreased the level of the CD3 ε-associated kinase activity (Fig. 2D, lanes 5 and 6) (25, 26). Kinase reactions in the presence of wortmannin inhibited the CD3 ε-associated kinase activity to a
small degree. However, this outcome was not consistent. Taken together, these results demonstrate that CD3ε associated with a Ser/Thr kinase that was distinct from the PKC, phosphatidylinositol 3-kinase, and MAPK families.

The Membrane-proximal BRS of CD3ε Mediates Interactions between a Kinase Other than HPK1—Next we wanted to identify the region(s) within the cytoplasmic tail of CD3ε responsible for the Ser/Thr kinase association. Several sequence motifs within the cytoplasmic tail of CD3ε are known to mediate protein interactions. These include the previously mentioned PRS and ITAM, as well as a region that we termed the basic-rich stretch (BRS) because of its high lysine and arginine content (Fig. 1A; BRS, PRS, and ITAM) (11, 14, 16). GST fusion proteins consisting of the BRS, PRS, or ITAM were used in pulldown and kinase assays. GST-BRS was the only fusion protein that associated with the kinase activity (Fig. 3A).

To identify the Ser/Thr kinase interacting with the BRS, Western blot analyses of CD3ε immunoprecipitates were performed with mAbs against several Ser/Thr kinases known to be involved in TCR-mediated signaling events, including HPK1, PAK, PKG, PKCθ, NIK, MEKK1, ERK 1/2, and p38. Of these, only HPK1 was detected in the CD3ε precipitate (Fig. 3B and data not shown). However, this interaction required α-CD3ε stimulations (Fig. 3B, lane 7). Furthermore, HPK1 was not found in GST-BRS pulldowns from either unstimulated or stimulated T cells (Fig. 3C and data not shown). This indicated that another Ser/Thr kinase was constitutively associated with CD3ε.

Identification of GRK2 as a BRS-associated Kinase—Because we were unable to identify a BRS-associated, Ser/Thr kinase by Western blotting, a protein purification approach was used. Murine thymocyte homogenates were applied to a Q-Sepharose™ anion-exchange column. Proteins retained on the column were eluted with a linear salt gradient, and a total of 80 fractions was collected (Fig. 4A). An aliquot from each fraction, including the flow-through, was incubated with GST-BRS, followed by kinase reactions in the presence of MBP. PO4-MBP was then measured to determine which fractions contained the highest concentration of the associating kinase. Fractions 27–32 (eluting between 0.1 and 0.2 M NaCl) contained two closely spaced peaks of kinase activity (Fig. 4A, dashed line). These fractions were pooled and applied to a GST-BRS affinity column. Proteins retained on the affinity column were eluted with a linear salt gradient, and kinase reactions were performed in the presence of MBP. Phosphoproteins were visualized by autoradiography (for piceatannol inhibition, n = 4 assays).

CD3ε Association with GRK2

FIGURE 2. CD3ε constitutively associates with a serine/threonine kinase. A, thymocytes were either untreated or stimulated immediately ex vivo for 10 or 30 min with anti-CD3ε mAbs (lanes 1–3). Alternatively, the cells were cultured at 37 °C for 4 h followed by CD3ε stimulations (lanes 4–6). The cells were washed, lysed, and subjected to in vitro kinase reactions in the presence of MBP. Associated kinase activity was assessed by quantifying the amount of PO4-labeled substrate (MBP). B, indicated molecules were isolated and assayed by in vitro kinase (i.v.k) reactions. C, indicated 32P-labeled bands (bands a–c) were subjected to phosphoamino acid analysis (n = 5 assays). D, CD3ε was immunoprecipitated (IP) from murine thymocyte lysates. The samples were then incubated with the indicated kinase inhibitors or media alone (lanes 13 and 14) for 10 min at room temperature, and kinase reactions were performed in the presence or absence of MBP. Phosphoproteins were visualized by autoradiography (for piceatannol inhibition, n = 4 assays).
affinity-purified fractions containing the enzymatic activity were precipitated with acetone, resolved by SDS-PAGE, and silver-stained to visualize their protein content. Several protein bands were detected (Fig. 5A). Six bands (Fig. 5A, labeled a–f) were extracted, trypsin-digested, and analyzed by mass spectrometry. One peptide sequence (LLDSLQELER, derived from band b at 80 kDa) corresponded to a protein known as GRK2 (Fig. 5A and Table 2). GRK2 is a Ser/Thr kinase involved in the negative regulation of GPCRs (reviewed in Ref. 27). Western blot analysis of the affinity column active fractions submitted for mass spectrometry confirmed that GRK2 was present in these samples (Fig. 5B, lane 4). Many of the other proteins identified by mass spectrometry were DNA- or RNA-binding proteins (Table 2). Several of these proteins, such as DEAD (Asp-Glu-Ala-Asp)-box polypeptide 5, have been shown to be involved in RNA processing and translation, while others, such as heterogeneous nuclear ribonuclear protein L, are involved in CD45 isoform splicing (28, 29). The relevance of these protein interactions has yet to be investigated.

**CD3 ε Association with GRK2**

To characterize the association between GRK2 and CD3 ε, transient transfection/co-precipitation experiments were undertaken. GRK2 was cloned from murine thymocytes and inserted into a pcDNA3.1/myc-HIS expression vector. HEK 293T cells were then co-transfected with Myc-tagged GRK2 and a Tac fusion protein consisting of the Tac extracellular and transmembrane domains fused to the cytoplasmic domain of CD3 ε (Tac-ε) (30). Forty eight hours post-transfection, the cells were lysed, and Tac-ε was immunoprecipitated (IP) and analyzed by immunoblotting (IB) with α-HPK1 mAb. C, various GST fusion proteins were used in pulldown assays with lysates prepared from thymocytes (lanes 2–5) or lymphocytes (lanes 6–9). WCLs (lane 1), and the indicated pulldowns were immunoblotted with α-HPK1 Abs. B and C are representative of 2–3 independent assays.
Direct CD3 ε immunoprecipitations were also carried out in both primary human and murine thymocytes. When these immunoprecipitations were Western-blotted using an α-GRK2/3 antibody, a band corresponding to GRK2 could be detected (Fig. 5D, lane 4). This indicated that endogenous GRK2 could co-precipitate with CD3 ε.

**The Effect of GRK2 siRNA Treatment on the BRS/CD3 ε-associated Kinase Activity**—Next, siRNA treatment of Jurkat T cells was used to determine whether decreasing GRK2 expression affected the CD3 ε-or BRS-associated kinase activities. Electroporation of Jurkat T cells with siRNAs directed against GRK2 consistently decreased GRK2 protein levels by 40–75% compared with control transfected cells (Fig. 6A, lane 2, and data not shown). Lysates from the siRNA-treated cells were then subjected to GST-BRS pulldowns or CD3 ε immunoprecipitations, followed by kinase reactions. As seen, the BRS-associated kinase activity was reduced by 16.8% when GRK2 siRNA-treated cells were compared with control transfected cells (average of 14.6 ± 7.3%; n = 4 independent assays) (Fig. 6B). Although modest, the average decrease of 14.6 ± 7.3% was statistically significant (p value < 0.05) (Fig. 6B). CD3 ε immunoprecipitations from cells in which GRK2 protein expression had been reduced by 75% exhibited at most a 4% decrease in the level of associated kinase activity and was not found to be statistically significant (data not shown). As detailed under the “Discussion,” there are several possibilities for why a more dramatic decrease in the associating kinase activity was not seen following GRK2 siRNA treatment.

**SDF-1 Treatment of Primary Thymocytes Significantly Reduces the CD3 ε-associated Kinase Activity**—Kumar et al. (9) recently reported that when T cells are treated with stromal cell-derived factor-1α (SDF-1), an inducible association between the TCR and the GPCR, CXCR4, takes place. This interaction allows CXCR4 to utilize the CD3 invariant chains and their associating proteins for its own signal transduction. Because CXCR4 is known to both activate GRK2 and to be regulated by the catalytic activity of GRK2, we examined the effects of SDF-1 treatment on the CD3 ε-associated kinase activity. Murine thymocytes were incubated with recombinant SDF-1 for 30 min. CD3 ε, CXCR4, PKC θ, or CD28 were then immunoprecipitated and subjected to kinase reactions (Fig. 7A). Following SDF-1 treatment, the levels of CD3 ε- and CXCR4-associated kinase activities decreased by 33 and 36%, respectively (average of 24 ± 13.7 and 36 ± 8.5%, respectively; n = 6 independent assays; p value < 0.05) (Fig. 7A). Control immunoprecipitations using antibodies against PKC θ and CD28 revealed no significant variations in kinase activity.

Studies have shown that GPCR engagement induces the phosphorylation of GRK2 on tyrosine and/or Ser/Thr residues, thereby altering its intracellular trafficking, catalytic activity, and proteolytic degradation (31–36). To examine whether such
post-translational modifications of GRK2 were responsible for the decrease in the CD3 ε-associated kinase activity. Western blot analyses were performed. SDF-1 treatment did not result in the degradation of CD3 ε or GRK2 (Fig. 7B, upper and lower panels). Additionally, the amount of GRK2 associating with CD3 ε remained comparable in both treated and untreated cells (Fig. 7B, middle panel). In primary thymocytes, we observed no variations in the tyrosine phosphorylation of GRK2 after SDF-1 treatment (data not shown). Conversely, we did observe a modest increase in the phosphorylation of GRK2 on serine 670 (Ser-670) after 5 min of treatment (Fig. 7C, lane 2). Phosphorylation at this site has been demonstrated to inhibit the catalytic activity of GRK2 toward physiologically relevant substrates (36). However, the phosphorylation of Ser-670 was diminished below background levels after 30 min of treatment, suggesting that changes at this site may not account for the decreased kinase activity seen in the CD3 ε immunoprecipitates. Taken together, these experiments indicate that the regulation of GRK2 in association with CD3 ε may be a complex process, involving multiple post-translational modifications with varying kinetics.

**DISCUSSION**

The CD3 subunits of the TCR complex can interact with a variety of effector/adaptor proteins (2). Here we present evidence that the membrane-proximal BRS of CD3 ε constitutively associated with the Ser/Thr kinase GRK2. We also determined that a second Ser/Thr kinase, termed HPK1, could associate with CD3 ε after TCR stimulations.

A number of studies have emphasized a crucial role for the BRS of CD3 ε in T cell development and effector function (11, 30, 37, 38). However, most of these studies focused on the role of the PRS and the ITAM of CD3 ε. Mutations of these sequences have limited consequences for T cell development and/or TCR signaling (39, 40). Our current experiments suggest an important role for the BRS of CD3 ε. Notably, the first 13 amino acids within the BRS of CD3 ε are conserved in most species, including mouse and human. We propose that the BRS plays a paramount functional role for CD3 ε, in part through its interaction with GRK2. In terms of the immune system, GRK2 is highly expressed in leukocytes and is capable of interacting with a number of mol-

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**CD3 ε Association with GRK2**

**TABLE 2**

Proteins identified by mass spectrometry analysis of affinity-purified fractions

| Band | Protein name       | Mass (kDa) | Sequence identified          | Function                |
|------|--------------------|------------|------------------------------|-------------------------|
| a    | PTB-associated splicing factor | 83         | MGQGQTNMNDPYYSGQQK          | Pre-mRNA splicing factor |
| b    | DEAD box polypeptide 5 | 69         | LLDSDQELYR                  | Serine/threonine kinase |
| c    | Heterogeneous nuclear ribonucleoprotein L | 60         | SKPGAAVEMADGYAVDR          | RNA helicase            |
| d    | Regulator of differentiation 1 | 56         | NNFAQALLQYADPVNAYAK        | RNA-binding protein     |
| e    | Pigpen              | 52         | TQGEKNLTYDR                | DNA-stabilizing protein |
| f    | Farnesyl diphosphate synthetase | 40         | QILENYQSQKDEK              | Synthetase              |

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**FIGURE 6. Reduced expression of GRK2 decreases the BRS-associated kinase activity.**

A. Jurkat T cells were left untreated or were transfected with siRNAs specific for human GRK2 or luciferase (control). The cells were lysed, and GRK2 knockdown was assessed by Western blot analysis of WCLs. B, GST-BRS pull-downs were performed as described previously from the WCLs of Jurkat T cells transfected with the indicated siRNA. Kinase reactions were then performed in the presence of MBP. PO4-MBP was visualized by autoradiography (upper panel) and quantified using a PhosphorImager (RLU, relative light units). Immunoblotting (IB) against GST was performed to ensure equivalent levels of GST-BRS were used in each pull down (lower panel).
molecules known to be important for TCR signaling, such as protein kinase A, ERK 1/2, and c-Src (27). Furthermore, the activity and expression levels of GRK2 are regulated by T cell activation during both normal and inflammatory responses (41, 42).

To elucidate a functional link between CD3 ε signaling and GRK2, we undertook chemokine receptor stimulations and kinase reactions in murine thymocytes. SDF-1 treatment of thymocytes resulted in a statistically significant decrease in the level of CD3 ε-associated kinase activity. Although the kinetics of this decrease did not correlate with an increase in PO4-GRK2Ser670, a modification that has been shown previously to inhibit the catalytic activity of GRK2. It is still possible that alterations in GRK2 phosphorylation on other residues, including Ser-29 or Ser-685, may negatively effect GRK2 activity (35, 43). Notably, TCR stimulations of Jurkat T cells were reported previously to reduce the cell surface expression of CXCR4, thereby reducing the ability of T cells to migrate toward SDF-1 (44). Although this TCR-mediated down-regulation of CXCR4 was found to require PKC, to our knowledge, the role of GRK2 was never investigated. Therefore, our finding that GRK2 can interact with CD3 ε might prove to be a novel mechanism for the regulation of T cell migration following TCR engagement. Furthermore, because both SDF-1 and CXCR4 are known to play a critical role in T cell development, the identification of an interaction between GRK2 and CD3 ε in primary thymocytes may have important implications for thymocyte development (45).

In our studies, the interaction between GRK2 and CD3 ε was not always detected (present in 9 of 15 assays). There are several possible explanations for this variability. First, the association cates that interactions between GRK2 and CD3 ε within such compartments could be functionally important for regulating GPCR activity.

We also noted that a 14.6 ± 7.3% decrease in the BRS-associated kinase activity was observed when GRK2 protein expression was reduced by 40% or more in Jurkat T cells. This modest decrease in kinase activity may also be due to several factors. First, it is possible that only a minute amount of GRK2 is needed to generate the CD3 ε-associated kinase profile. This is supported by the fact that although a robust amount of GRK2 is present in the whole cell lysate of unstimulated thymocytes, only a small amount appears to interact with the TCR. In this case, even a 75% reduction in GRK2 protein levels may not be sufficient to eliminate the CD3 ε-associated kinase activity. It is also possible that interactions between CD3 ε and other kinases, such as HPK1, might mask the effects of decreasing GRK2 expression in Jurkat T cells. Therefore, siRNAs directed against multiple Ser/Thr kinases may be necessary to decrease the CD3 ε-associated kinase activity substantially.

In summary, the identification of two Ser/Thr kinases capable of interacting with CD3 ε adds to the complex array of proteins contributing to TCR-mediated signaling both before and after receptor engagement. With regard to CD3 ε interactions with GRK2, several reports have shown that signals generated through GPCRs and the TCR share a number of signaling components, including Gαq/11, ZAP-70, LAT, and SLP-76 (9, 44, 46). The finding that GRK2 associates with CD3 ε adds to the body of evidence that chemokine receptors and the TCR share molecules that enable them to cross-talk. To our knowledge, this is the first demonstration of a molecule known to

**CD3 ε Association with GRK2**

of GRK2 with CD3 ε may be indirect and require the presence of an intermediate protein, such as Gαq/11. Gαq/11, a well known binding partner of GRK2, has also been reported to interact with CD3 ε, facilitating TCR-mediated activation of phospholipase β (46, 47). Alternatively, CD3 ε-GRK2 interactions may be occurring within specialized compartments, such as endosomes. Because proteins in such intracellular organelles are difficult to extract, the solubilization conditions used in our procedures may not have been ideal. This conjecture is consistent with reports demonstrating that the co-localization of the TCR and CXCR4 takes place both at the cell surface and within intracellular organelles, including endosomes and the Golgi (48–51). This indications...
negatively regulate GPCRs in association with the TCR within primary cells.

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