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T Cell Receptor Engagement Leads to the Recruitment of IBP, a Novel Guanine Nucleotide Exchange Factor, to the Immunological Synapse*

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Reorganization of the actin cytoskeleton is crucial to the formation and function of the immunological synapse. Rho GTPases are critical mediators of cytoskeletal reorganization, and their activity at the synapse is likely to be stringently regulated. Guanine nucleotide exchange factors (GEFs) belonging to the Dbl family of proteins represent one major class of proteins that regulate the activity of Rho GTPases. Here we demonstrate that IBP, a homologue of SWAP-70, is a novel GEF for Rac1 and Cdc42 in T lymphocytes, which is recruited to the immunological synapse upon engagement of the antigen receptor. Mutational analysis supports a model whereby IBP is inactive in unstimulated cells. Upon engagement of the T cell receptor, its GEF activity is enhanced by tyrosine phosphorylation, as well as by binding newly generated phosphatidylinositol 3,4,5-trisphosphate. Although it is known that T cell receptor engagement leads to the recruitment of Vav to the immunological synapse, these findings indicate that other GEFs, such as IBP, also relocalize to this intercellular region. The recruitment and activation of distinct classes of GEFs may allow for precise control of Rho GTPase function at the crucial interface between T cells and antigen presenting cells.

Engagement of the T cell receptor (TCR)[1] initiates a complex cascade of biochemical events that culminates in the expansion and differentiation of T cells (1, 2). Activation of protein tyrosine kinases (PTKs) of the Src family, Lck and Fyn, constitutes one of the most proximal and crucial signaling events that couples receptor engagement to downstream biochemical pathways (3). These Src family kinases phosphorylate specific tyrosine residues within the immunoreceptor tyrosine-based activation motifs of the CD3 and TCR ζ chain leading to the recruitment and activation of additional kinases, adaptor proteins, and enzymes. Pharmacological and genetic studies have indicated that stimulation of the activity of one enzyme, phosphoinositide 3-kinase (PI3K), is particularly important in mediating the propagation and amplification of the TCR-mediated signaling cascade (4, 5). The products of PI3K, PI(3,4,5)P_3, and PI(3,4)P_2 bind to pleckstrin homology (PH) domains contained in a variety of crucial signaling intermediates inducing the relocalization of these proteins to specific areas of the plasma membrane.

T cell recognition of antigen presenting cells (APCs) results in the formation of a specialized interface, termed the immunological synapse (IS) (6–8). The immunological synapse may function to integrate and/or stabilize TCR-generated signaling pathways and to promote the restricted delivery of secretory products, such as cytokines, to the target cell. Assembly of the immunological synapse is accompanied by the large scale redistribution of receptors and signaling proteins, processes that are critically dependent on TCR-mediated actin cytoskeletal remodeling and polarization. Actin cytoskeletal reorganization in the immunological synapse requires the activity of the Rho family of GTPases (9, 10).

Rho GTPases function as molecular switches that cycle between an inactive GDP-bound form and an active GTP-bound form (11–13). Guanine nucleotide exchange factors (GEFs) belonging to the Dbl family of proteins represent major regulators of the activation state of Rho GTPases (14). One member of this family, Vav, is critically important in orchestrating TCR-mediated actin cytoskeletal reorganization (15–17). Like other members of the Dbl family, Vav contains a sequence of ~200 amino acids termed the Dbl homology (DH) domain, responsible for catalyzing the GDP/GTP exchange reactions, followed by a C-terminal PH domain necessary for proper intracellular localization and function (18). Binding of PI(3,4,5)P_3 to the PH domain serves to activate the GEF activity of Vav, which is further enhanced by multiple tyrosine kinases. Interestingly, recent studies in *Drosophila melanogaster* indicate that cells employ multiple GEFs to elicit specific subsets of Rho GTPase-mediated responses (19). Whether GEFs other than Vav are activated and recruited to the immunological synapse is, however, not known.

Although the great majority of GEFs for Rho GTPases contain the canonical DH-PH module, recent studies (13) have revealed the existence of novel classes of these regulators. In

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[1] The abbreviations used are: TCR, T cell receptor; PTK, protein tyrosine kinase; PI3K, phosphoinositide 3-kinase; PI(3,4,5)P_3, phosphatidylinositol 3,4,5-trisphosphate; PI(3,4)P_2, phosphatidylinositol 3,4-biphosphate; PH, pleckstrin homology; APC, antigen presenting cells; IS, immunological synapse; GEF, guanine nucleotide exchange factor; DH, Dbl homology; mAb, monoclonal antibody; HA, hemagglutinin; GST, glutathione *S*-transferase; PKC, protein kinase C; FITC, fluorescein isothiocyanate; TRITC, tetramethylrhodamine isothiocyanate; Ab, antibody; PI(4,5)P_2, phosphatidylinositol 4,5-biphosphate; SEE, *Staphylococcus* enterotoxin E; PAK1, p21-activated kinase 1.

particular, SWAP-70 is a novel type of Rac-GEF, in which the catalytic domain is flanked at its N terminus, rather than at its C terminus, by a PH domain (20). The DH domain of SWAP-70 exhibits only a very low degree of homology to that of other Rho-GEFs such as Vav, further suggesting that this class of GEFs may activate a unique subset of Rho GTPase-mediated functions. We recently identified a novel protein termed IBP (IRF-4-binding protein) that exhibits significant similarity to SWAP-70 (21). IBP is highly expressed in lymphoid tissues, and, interestingly, the T cell compartment preferentially expresses IBP rather than SWAP-70 (21, 22). This finding prompted us to investigate the regulation and function of IBP in T cells. Here we report that IBP is rapidly tyrosine-phosphorylated by Lck in response to T cell activation. Tyrosine phosphorylation of IBP enables it to bind PI(3,4,5)P_3. IBP is recruited to the T cell:APC contact region in a manner that is dependent on the activities of both Lck and PI3K, and these signals control the ability of IBP to function as a GEF toward Rac1 and Cdc42. The recruitment of IBP to the immunological synapse coupled with the finding that its GEF activity is controlled by TCR-mediated signals suggest that IBP is a novel type of GEF that participates in lymphocyte activation.

MATERIALS AND METHODS

Cell Cultures and Transfections—The various Jurkat (human T cell leukemia) cell lines, including JE6–1 (wild-type), J.CaM1.6 (Lck-deficient), and J-TAg (SV40 large T-antigen-transfected) cell lines, were obtained from American Type Culture Collection (ATCC, Manassas, VA). All the cell lines were grown in RPMI 1640 medium supplemented with 10% heat-inactivated fetal calf serum (Atlanta Biologicals, Inc.), 2 mM L-glutamine, 10 mM HEPES, and antibiotics. 293T (a human embryonic kidney cell line) cells were a kind gift of Dr. Chris Schindler, Columbia University, New York, NY and were grown in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum. COS-7 cells (a generous gift of Dr. Steven Greenberg, Columbia University, New York, NY) were grown in RPMI 1640 medium supplemented with 10% fetal calf serum. Raji cells were obtained from Dr. Raphael Clynes (Columbia University) and were grown in RPMI 1640 medium supplemented with 10% fetal bovine serum. Jurkat T cells were washed with serum-free RPMI 1640 medium, serum-starved for 5 h, and then stimulated at 37 °C for the indicated time periods with a mouse anti-human CD3 mAb (clone OKT3) followed by cross-linking with a secondary goat anti-mouse Ig antibody, as described previously (23). For expression of recombinant proteins, 293T cells and COS-7 cells were transfected with expression plasmids by the calcium phosphate/DNA precipitation method or the SuperFect transfection reagent (Qiagen, Inc.), respectively. After 24 h of incubation, the transfected cells were harvested for cell extract preparation.

DNA Constructs—The full-length wild-type human IBP expression plasmids (pCEP4-HA-IBP and pIRES2-EGFP-HA-IBP) were constructed by cloning the entire coding region of the human IBP cDNA, fused in-frame with a hemagglutinin (HA) epitope coding sequence at its 5′ terminus, into the pCEP4 expression vector (Invitrogen) or pIRES2-EGFP bicistronic expression vector (Clontech), respectively. The point mutations Y210F or R236C in the full-length IBP were introduced by the site-directed mutagenesis method (Stratagene) and confirmed by DNA sequencing. Various deletion mutants of human IBP were generated by PCR using appropriate primers and confirmed by DNA sequencing. For preparation of various glutathione *S*-transferase (GST)-IBP fusion proteins, the corresponding GST-IBP expression plasmids were generated by cloning either the entire coding sequence of the human IBP cDNA or its appropriate segments, in-frame, into the pGEX-KG *Escherichia coli* expression vector (Amersham Biosciences). The in-frame junction in the GST-IBP fusion constructs was confirmed by DNA sequencing. The constitutively active Lck(Y505F) expression construct in pcDNA3 mammalian expression vector (24) was a kind gift of Dr. Jerry Siu.

Protein Purification, Antibodies, Cell Extracts, and Protein Assays—GST-IBP fusion proteins were expressed in *E. coli* DH5α and affinity-purified on glutathione-agarose beads (Sigma), as described previously (25). HA epitope-tagged IBP (full-length wild-type) was expressed in 293T cells and affinity-purified on immobilized anti-HA monoclonal antibody (clone 3F10; anti-HA affinity matrix; Roche Applied Science) according to the procedures recommended by the manufacturer. The polyclonal anti-IBP antibody was generated by immunizing rabbits with purified GST-IBP (amino acids 410–631) fusion protein (Covance, Inc., Princeton, NJ). This GST fusion protein contains a portion of the human IBP protein, which is least homologous to SWAP-70 and thus minimizes cross-reactivity of the antibody with SWAP-70 (21). The anti-IBP antibody was utilized at 1:1000 in Western blotting and at 1:200 in the immunofluorescence experiments. An anti-phosphotyrosine monoclonal antibody (clone 4G10) was obtained from Upstate Biotechnology. The rat monoclonal antibody against HA epitope (clone 3F10) was purchased from Roche Applied Science. The PKC-θ and β-actin antibodies were purchased from Santa Cruz Biotechnology, Inc.

Whole cell extracts were prepared as described previously (26). Cell lysates were immunoprecipitated with an anti-IBP antibody or anti-HA mAb (clone 3F10) as described previously (26). The immunoprecipitates were resolved by 7% SDS-PAGE. The gel was transferred to a nitrocellulose membrane and then immunoblotted with an anti-phosphotyrosine antibody (4G10) or the anti-IBP antiserum. The bands were visualized by ECL (Amersham Biosciences). Pull-down assays with phosphoinositide analogue beads (Echelon Research Laboratories Inc.) were performed according to the manufacturer's instructions.

Lck-mediated tyrosine phosphorylation of IBP was assessed by *in vitro* Lck kinase assay using purified Lck kinase (Upstate Biotechnology) and either purified HA epitope-tagged IBP (wild-type) or immunoprecipitated recombinant IBP proteins (wild-type or Y210F mutant) according to previously described protocols (27). Briefly, purified HA-IBP (~150 ng) or immunoprecipitates of recombinant IBP proteins were incubated with 5 units of purified Lck in 30 μl of 1× kinase buffer (20 mM HEPES, pH 7.4, 100 mM NaCl, 5 mM $MnCl_2$, 10 mM $MgCl_2$, 50 μM Na_3VO_4, 1 mM dithiothreitol) containing 1 μM cold ATP and 10 μCi of [γ-^{32}P]ATP for 30 min at 30 °C. The reactions were terminated by adding SDS-PAGE sample buffer and boiling. The reaction samples were resolved on a 7% SDS-polyacrylamide gel. The gel was fixed, soaked in 1 N KOH at 55 °C for 2 h, refixed, dried, and then autoradiographed to visualize tyrosine-phosphorylated products.

In Vivo Rac1/Cdc42 Activation Assay and in Vitro GDP Release Assay—For the *in vivo* activation assays for Rac1 and Cdc42, COS-7 cells were transiently transfected with an appropriate expression vector for either wild-type HA-IBP or various IBP deletion mutants. The cells were washed with ice-cold phosphate-buffered saline containing 5 mM $MgCl_2$ and then lysed in 1× lysis buffer (25 mM HEPES, pH 7.5, 150 mM NaCl, 1% Nonidet P-40, 10 mM $MgCl_2$, 1 mM EDTA, 10% glycerol, 2 mM dithiothreitol, 1 mM Na_3VO_4, protease inhibitors). Activated GTP-bound Rac1 (or Cdc42) was affinity-precipitated from the cell lysates by using GST-PAK1 PBD (p21 Rac/Cdc42-binding domain) fusion protein immobilized onto glutathione-agarose beads (Upstate Biotechnology) according to the manufacturer's instructions. The precipitated Rac1-GTP or Cdc42-GTP was resolved by 12.5% SDS-PAGE and then visualized by Western blot analysis with either an anti-Rac1 antibody (Upstate Biotechnology) or an anti-Cdc42 antibody (Transduction Laboratories), respectively.

The *in vitro* GDP release assays were carried out by filter-binding method as described previously (28–31). To prepare [^{3}H]GDP-loaded GTPases, 40 pmol of bacterially expressed and purified GST-Rac1, GST-Cdc42, or His-RhoA (Calbiochem-Novabiochem) was incubated with 1 μM [^{3}H]GDP in 100 μl of a binding buffer (10 mM HEPES, pH 7.5, 100 mM NaCl, 5 mM $MgCl_2$, 1 mM EGTA, 1 mM dithiothreitol, 50 μg/ml bovine serum albumin) for 60 min at 30 °C. The nucleotide exchange reaction was initiated by adding 100 μM nonradioactive GTP and 4 pmol of purified GST alone or GST-IBP fusion proteins (or when appropriate, 4 pmol of purified wild-type HA-IBP either non-phosphorylated or *in vitro* phosphorylated by purified Lck and cold ATP) to the [^{3}H]GDP-loaded GTPase and then equally splitting and incubating the reaction mixture for the indicated time periods at room temperature. When indicated, control exchange reactions were also performed with purified Lck alone using the same amount of Lck as was used to phosphorylate purified HA-IBP. In some experiments, a water-soluble analogue of PI(3,4,5)P_3 (Echelon Research Laboratories Inc.) was added to the exchange reaction mixtures to a final concentration of 1 μM. The exchange reactions were stopped by adding 1 ml of ice-cold dilution buffer (20 mM Tris-HCl, pH 7.5, 100 mM NaCl, 20 mM $MgCl_2$) to the reaction mixtures. The amount of [^{3}H]GDP remaining bound to the GTPases was determined by filtering the quenched reaction samples over nitrocellulose membranes followed by extensive washing of the filters and then quantification of the membrane-bound radioactivity by scintillation counting.

Conjugate Formation and CD3 Capping—Raji cells were used as the APCs for conjugation with Jurkat T cells. Raji cells were labeled with 10 μM 7-amino-4-chloromethylcoumarin cell tracker blue dye (Molecular

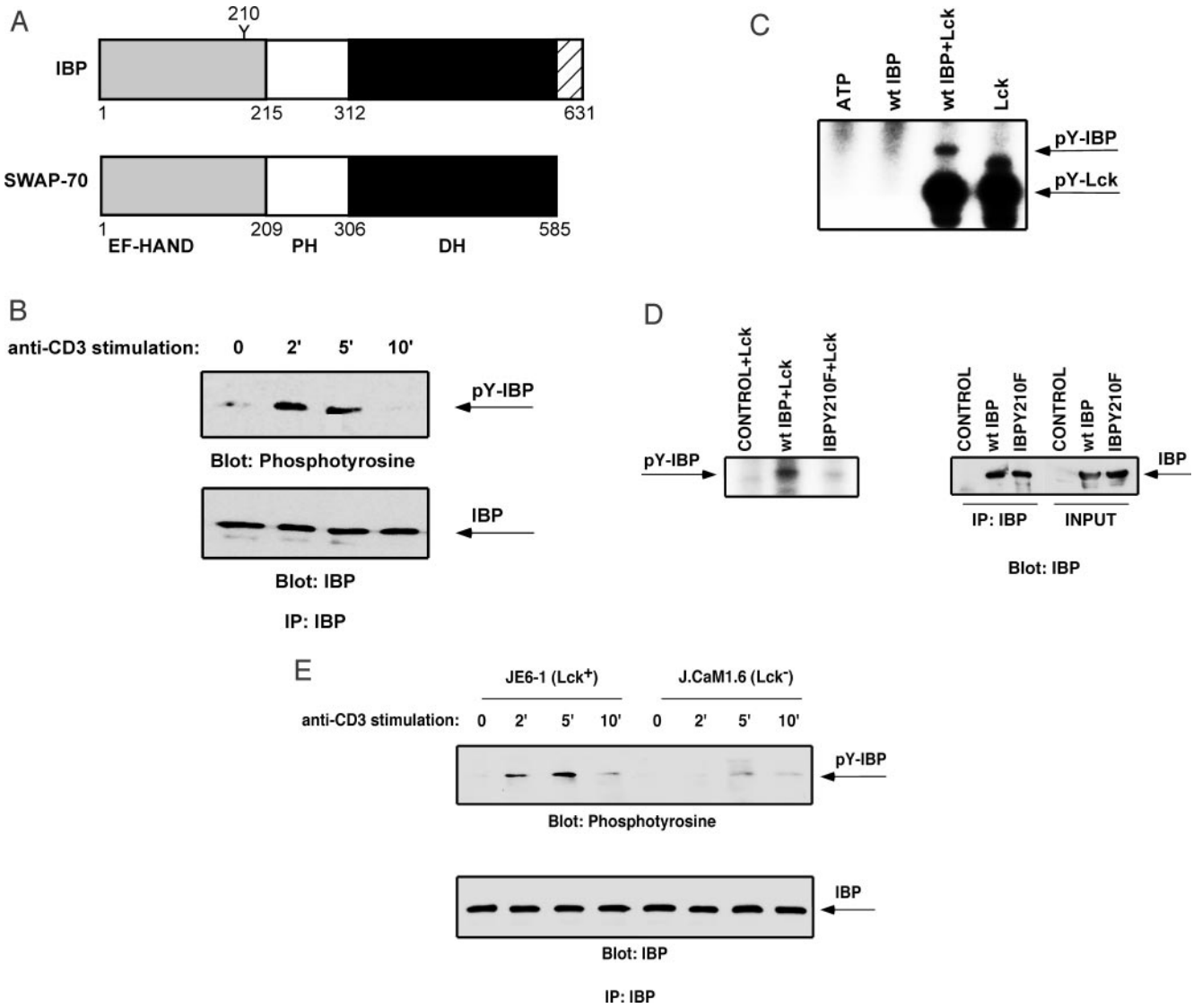

FIG. 1. **IBP is tyrosine phosphorylated in response to TCR engagement in an Lck-dependent manner.** *A*, schematic of domains of human IBP and SWAP-70 proteins. The location of the potential Lck-mediated tyrosine phosphorylation site in IBP is indicated. *B*, Jurkat T cells (J-Tag) were either left unstimulated or stimulated with cross-linked anti-CD3 mAb (OKT3) for the indicated time periods. Whole cell lysates were then prepared and immunoprecipitated (*IP*) with an anti-IBP antiserum. The immunoprecipitates were resolved by 7% SDS-PAGE and then analyzed by Western blotting using an anti-phosphotyrosine antibody (4G10) (*upper panel*). The blot was later stripped and reprobed with the anti-IBP antiserum to ensure equal loading of immunoprecipitates (*lower panel*). *C*, purified wild-type (*wt*) HA-tagged IBP (150 ng) was subjected to *in vitro* kinase reactions either alone (IBP) or together with purified Lck kinase (5 units) in the presence of [γ-^{32}P]ATP. Control kinase reactions included [γ-^{32}P]ATP alone (*ATP*) or purified Lck alone (*Lck*). The reactions were resolved by 7% SDS-PAGE. The phosphorylated products were subsequently detected by autoradiography. *D*, 293T cells were transiently transfected with an empty vector (*CONTROL*) or with an expression vector for either wild-type (*wt*) IBP or the IBPY210F mutant. Whole cell lysates were prepared and immunoprecipitated with an anti-IBP antiserum. The immunoprecipitates were extensively washed and then subjected to *in vitro* kinase reactions with purified Lck kinase (5 units) in the presence of [γ-^{32}P]ATP. One-half of each reaction was resolved by 7% SDS-PAGE, and the phosphorylated products were subsequently detected by autoradiography (*left panel*). The remaining half of the reaction was analyzed by Western blotting with the anti-IBP antibody to ensure for equivalent expression levels of wild-type and mutant IBP proteins in the different transfectants (*right panel*). *E*, cells from wild-type Jurkat (*JE6–1 (Lck$^+$)*) and a Jurkat variant (*J.CaM1.6 (Lck$^-$)*) were stimulated with cross-linked anti-CD3 mAb (OKT3) for the indicated periods of time. Whole cell lysates were obtained, immunoprecipitated with an anti-IBP Ab, and then analyzed by Western blotting as described for *A*.

Probes, Eugene, OR) followed by pulsing with or without 5 μg/ml SEE (Toxin Technology, Sarasota, FL) for 30 min at 37 °C. To induce conjugate formation, 1×10^5 B cells were combined with 1×10^5 Jurkat E6–1 or 1×10^5 Lck-deficient Jurkat (J.CaM1.6) T cells at 37 °C for 5 min. Conjugates were pipetted onto poly-L-lysine-coated coverslips and then fixed in 3.7% formaldehyde, washed, and permeabilized with 0.5% Triton X-100/phosphate-buffered saline. Conjugates were then stained with antibodies to IBP and PKC-θ followed by a secondary staining with Alexa-Fluor 568-conjugated donkey anti-rabbit (Molecular Probes) and FITC-conjugated donkey anti-mouse (Jackson Immunoresearch Laboratories, IgAb), respectively. For treatments with wortmannin (Calbiochem), T cells were resuspended in serum-free medium containing 100 nM wortmannin and incubated for 30 min at 37 °C followed by incubation with APCs. Conjugates were examined by a Zeiss LSM 510 laser scanning confocal microscope (Thornwood, NY) with a ×100/1.3 Plan-Neofluor objective lens. FITC, TRITC, and 7-methyl-4-chloromethylcoumarin (in two-photon mode) were excited at 488, 543, and 800 nm, respectively, and emission was collected at 500–550, above 585, and 435–485 nm, respectively. Optical section thickness was ~1 μm. Image enhancement and analysis were performed using the public domain program NIH Image 1.6 and Adobe Photoshop 6.0. Approximately 100 conjugates were scored visually for polarized IBP or PKC-θ at the synapse from two independent scores of three different experiments.

For capping experiments, naïve CD4$^+$ T cells were isolated from splenocytes of D0.11.10 TCR transgenic mice by negative selection using naïve CD4$^+$-specific T cell enrichment columns (R & D Systems). The purity of naïve CD4$^+$ cells was assessed by flow cytometry and was found to be >90%. T cells were stimulated with 5 μg/ml anti-CD3ϵ Ab (Pharmingen) for 1 h on ice, followed by cross-linking with FITC-labeled

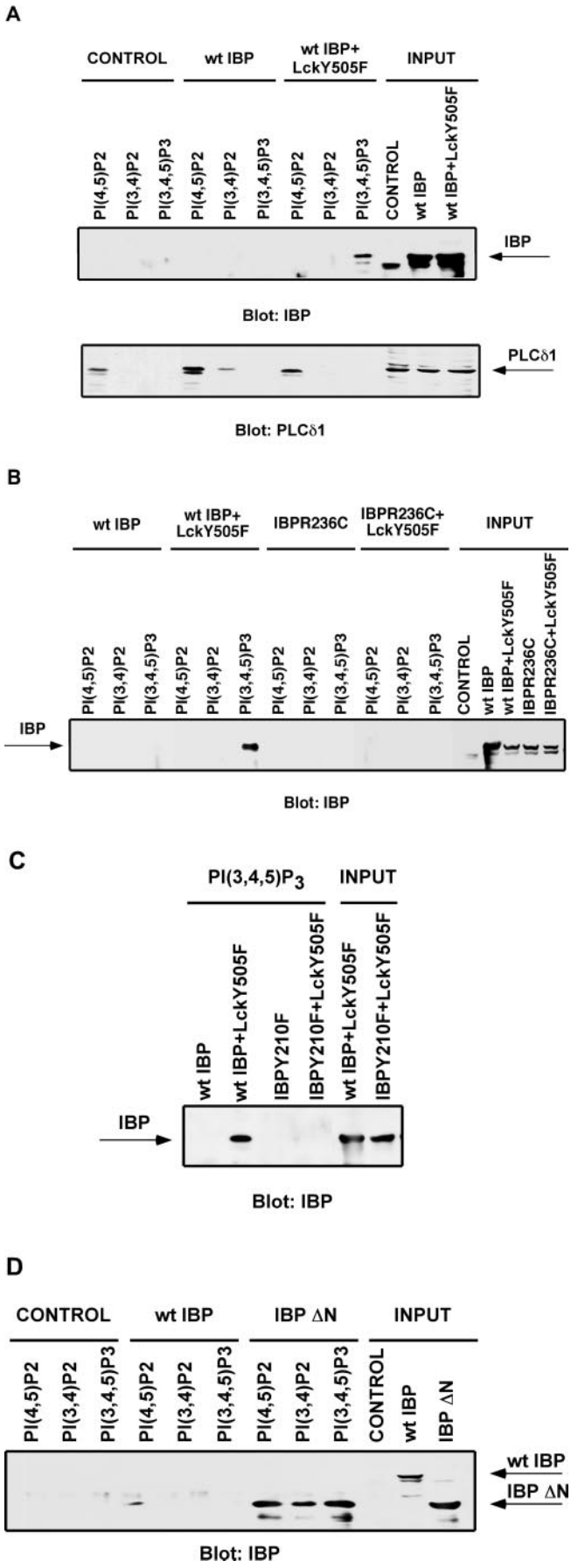

FIG. 2. **Interaction of IBP with phosphoinositides upon Lck stimulation.** *A*, 293T cells were transiently cotransfected with appropriate vector controls (*CONTROL*) or with a wild-type IBP expression vector, together with either an empty vector (*wt IBP*) or a constitutively active Lck expression vector (*wt IBP+LckY505F*). Whole cell lysates were prepared and incubated with $PI(4,5)P_2$, $PI(3,4)P_2$, or $PI(3,4,5)P_3$ analogue beads. The bead-bound proteins were resolved by 7% SDS-PAGE and then analyzed by Western blotting using an anti-IBP Ab (*upper panel*). The blot was later stripped and reprobed with an anti-PLCδ1 Ab as a control (*lower panel*). *B*, 293T cells were transiently cotransfected with an expression vector for either wild-type IBP or the IBP R236C mutant, together with either an empty vector or a constitutively active Lck expression vector (LckY505F). Whole cell lysates were prepared and incubated with $PI(4,5)P_2$, $PI(3,4)P_2$, or $PI(3,4,5)P_3$ analogue beads and then the bound proteins were analyzed by Western blotting using an anti-IBP Ab. *C*, 293T cells were transiently cotransfected with an expression vector for either wild-type IBP or the IBPY210F mutant, together with either an empty vector or a constitutively active Lck expression vector (LckY505F). Whole cell lysates were prepared and incubated with $PI(3,4,5)P_3$ analogue beads and then the bound proteins were analyzed by Western blotting using an anti-IBP Ab. *D*, 293T cells were transiently transfected with an empty vector (*CONTROL*), a wild-type IBP expression vector (*wt IBP*), or an expression vector encoding an IBP deletion mutant lacking the N-terminal region (*IBP ΔN*). Whole cell lysates were obtained and incubated with $PI(4,5)P_2$, $PI(3,4)P_2$, or $PI(3,4,5)P_3$ analogue beads and then the bound proteins were analyzed by Western blotting as described above.

mouse anti-hamster Ig Ab (Molecular Probes, Eugene, OR) for 5 min at 4 or 37 °C. Cells were pipetted onto poly-L-lysine-coated coverslips and then fixed with 3.7% formaldehyde. Cells were then washed, permeabilized, and stained with antibodies against IBP followed by a secondary antibody stain of anti-rabbit Alexa-Fluor 568 (Molecular Probes). Capped T cells were examined by a Zeiss LSM 510 laser scanning confocal microscope (Thornwood, NY) with a ×100/1.3 Plan-Neofluor objective lens. FITC and TRITC were excited at 488 and 543 nm, respectively, and emission was collected at 500–550 and above 585 nm, respectively. Optical section thickness was ~1 μm. Image enhancement and analysis were performed using the public domain program NIH Image 1.6 and Adobe Photoshop 6.0. The percentage of cells displaying caps, in which the Alexa-Fluor condenses to less than 25% of the cell surface was determined by counting 10 fields per treatment composed of ~150–250 cells. D0.11.10 TCR transgenic mice were obtained from Jackson Immunoresearch Laboratories and were maintained under specific pathogen-free conditions.

RESULTS

IBP Is Tyrosine-phosphorylated upon TCR Stimulation in an Lck-dependent Manner—The rapid activation of PTKs of the Src family is one of the earliest signaling events triggered by engagement of the TCR (1, 2). Because a survey of the IBP sequence utilizing the Scansite algorithm (32) revealed the presence of a potential tyrosine phosphorylation site, which fits the consensus motif for Lck-mediated phosphorylation (Fig. 1*A*) (33), we first determined whether IBP underwent enhanced tyrosine phosphorylation in response to TCR stimulation. Jurkat T cells, which express endogenous IBP, were stimulated with anti-CD3 mAb for varying periods of time, and whole cell lysates were obtained and then immunoprecipitated with an Ab directed against IBP (Fig. 1*B*). Western blot analysis of the immunoprecipitates with an anti-phosphotyrosine Ab revealed that TCR engagement led to the rapid and transient tyrosine phosphorylation of IBP. To address more directly the possibility that IBP might be a substrate for the Src family of tyrosine kinases, we determined whether purified Lck could phosphorylate purified recombinant IBP in an *in vitro* kinase assay (Fig. 1*C*). Coincubation of IBP and Lck resulted in the phosphorylation of IBP in addition to the known autophosphorylation of Lck. Furthermore, transient cotransfection of an IBP expression construct in 293T cells, together with a constitutively active form of Lck (LckY505F) (24), led to the tyrosine phosphorylation of IBP (data not shown). Given that the N terminus of IBP contains a tyrosine (Tyr-210) that represents a potential consensus motif for Lck-mediated phosphorylation, we then proceeded to determine whether inactivating this residue would affect the ability of Lck to phosphorylate IBP. As

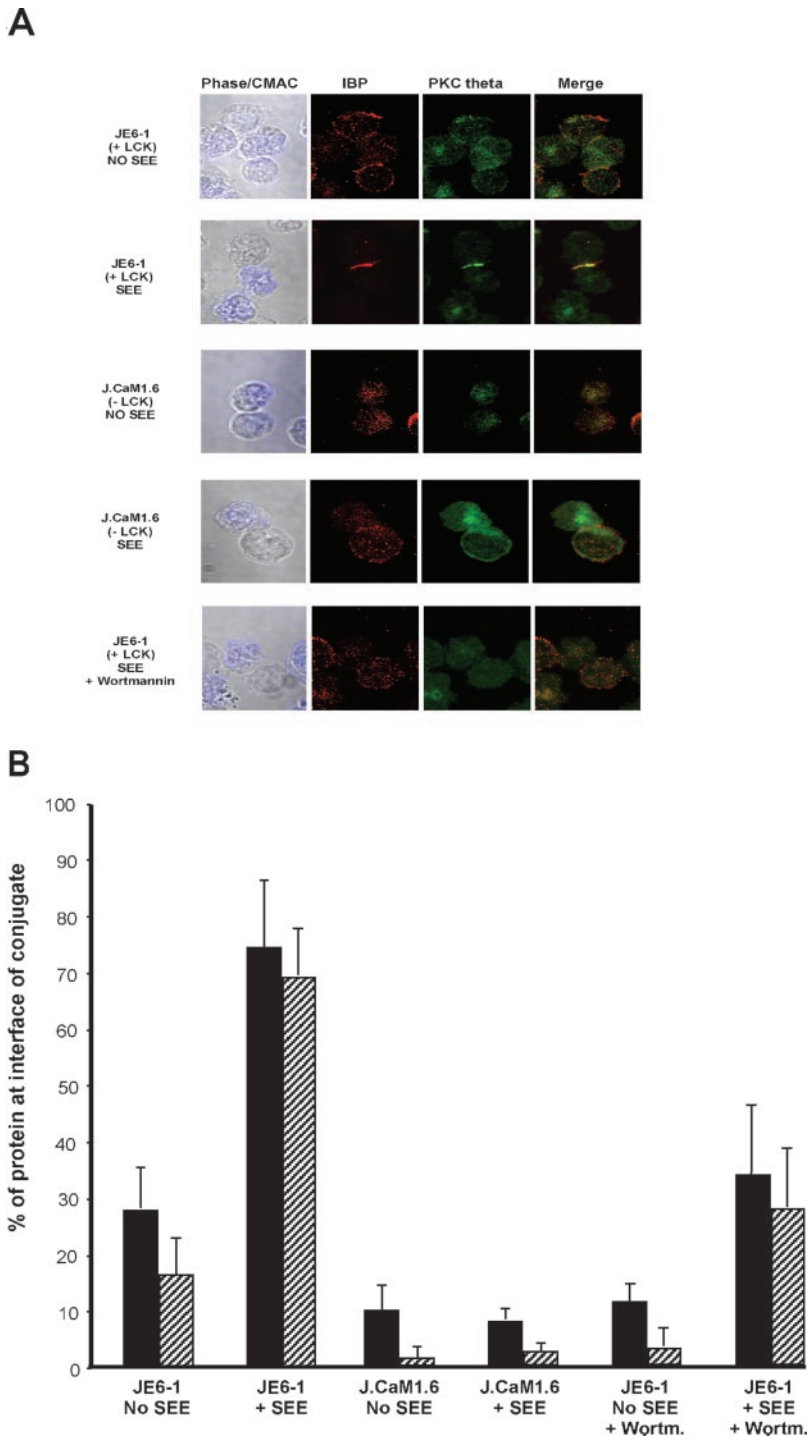

FIG. 3. **Recruitment of IBP to the immunological synapse.** *A*, 1×10^5 Jurkat E6–1 (Lck^+) or 1×10^5 J.CaM1.6 (Lck^-) cells were mixed in a 1:1 ratio with the B cell lymphoma cell line, Raji cells, that had been prepulsed with medium alone or with 5 μg/ml of SEE superantigen. Raji cells were labeled with the 7-methyl-4-chloromethylcoumarin cell tracker dye (Molecular Probes). Cell conjugates were pelleted, plated on poly-L-lysine-coated slides, fixed, permeabilized, and stained with antibodies to IBP and PKC-θ, followed by a secondary staining with Alexa-Fluor 568-conjugated anti-rabbit Ig (*red*) and FITC-conjugated anti-mouse Ig (*green*), respectively. Conjugates were then examined by confocal microscopy. Fluorescence and phase images are shown. In the wortmannin-treated samples, T cells had been pretreated with 100 nM wortmannin prior to incubation with APCs. *B*, quantitative analysis of data presented in *A*. Cells were scored visually for localization of IBP (*solid bars*) or PKC-θ (*hatched bar*) at the synapse. The *graphs* represent the mean percentages of imaged cells showing IBP or PKC-θ at the synapse ± S.D. of two independent scores from three different experiments. Approximately 100 conjugates per stain were examined, and those showing a distinct band of labeling at the contact site were scored.

shown in Fig. 1*D*, *in vitro* kinase assays indeed demonstrated that Lck can phosphorylate wild-type IBP but not the Y210F mutant. To assess whether Lck was required for TCR-induced phosphorylation of IBP in lymphocytes, we compared the ability of IBP to undergo tyrosine phosphorylation in cells from the Lck^+ Jurkat cell line JE6.1 and in cells from J.CaM1.6, an Lck-deficient subline of JE6.1 (34). The anti-CD3-induced tyrosine phosphorylation of IBP was markedly diminished in the Lck-deficient as compared with the Lck^+ Jurkat T cells (Fig. 1*E*). Taken together these data indicate that IBP is rapidly tyrosine-phosphorylated upon TCR stimulation and that IBP can serve as a substrate for Src kinases.

IBP Binds PI(3,4,5)P_3 upon Phosphorylation by Lck—Because IBP contains a PH domain we determined whether IBP binds specific phosphoinositides. We prepared whole cell lysates from 293T cells cotransfected with an HA-tagged IBP expression construct and either an empty vector or a vector expressing a constitutively active Lck (LckY505F). Lysates were then subjected to pull-down assays with a panel of different phosphoinositides conjugated to agarose beads (Fig. 2*A*). IBP bound efficiently to the PI3K product, PI(3,4,5)P_3, only when coexpressed with constitutively active Lck. We did not detect association of IBP with PI(4,5)P_2. To confirm the specificity of the interaction of PI(3,4,5)P_3 with the PH domain of IBP, we generated a point mutation within the PH domain of IBP (R236C). This residue has been shown previously (35) to be critical for the interaction of PH domains with PI(3,4,5)P_3, and a similar mutation was previously demonstrated to prevent the

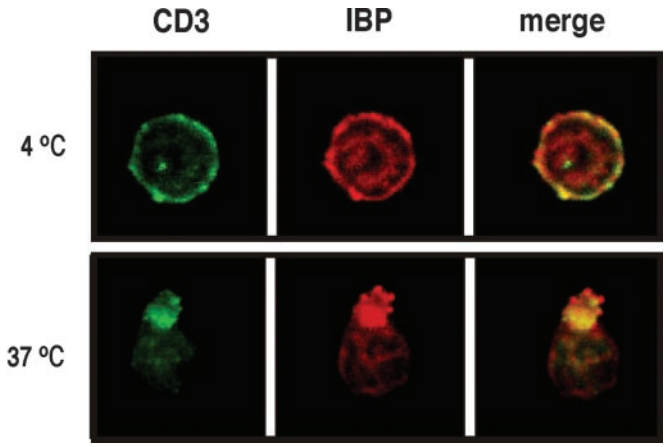

FIG. 4. **IBP colocalizes with the TCR/CD3 cap in primary murine TCR transgenic T cells.** Purified T lymphocytes from the D0.11.10 TCR transgenic mouse were incubated with 5 μg/ml anti-CD3ϵ (145–2C11; Pharmingen) and then an FITC-labeled cross-linking secondary Ab at 4 or 37 °C. Cells were then fixed and stained with an antibody against IBP followed by an Alexa-Fluor 568-conjugated secondary antibody staining. Cells were visualized by confocal microscopy. A representative single cell enlarged image before (4 °C) and after (37 °C) activation for 5 min is shown.

association of SWAP-70 with phosphoinositides (20). As shown in Fig. 2*B*, the R236C mutation completely abolished the ability of IBP to interact with PI(3,4,5)P_3. To further investigate whether phosphorylation of Tyr-210 by Lck was critical for the ability of IBP to efficiently associate with PI(3,4,5)P_3 we then assayed the ability of the IBPY210F mutant to bind PI(3,4,5)P_3 (Fig. 2*C*). Consistent with the notion that Tyr-210 constitutes the major Lck phosphorylation site within the IBP molecule, cotransfection of Lck with IBPY210F did not lead to binding of this mutant to PI(3,4,5)P_3. Interestingly, a deletion mutant of IBP lacking the entire N terminus (IBP ΔN) bound to PI(3,4,5)P_3 even in the absence of constitutively active Lck (Fig. 2*D*). Surprisingly, this mutant also acquired the ability to bind PI(4,5)P_2 and PI(3,4)P_2. Further studies are in progress to determine whether the binding of IBP ΔN to PI(4,5)P_2 and PI(3,4)P_2 is mediated directly by the PH domain of IBP or by association of this mutant with additional PH domain-containing proteins of different specificities. Collectively, these data are thus consistent with a model whereby phosphorylation of Tyr-210 by Lck leads to a conformational change in IBP that allows its PH domain to bind to the appropriate phosphoinositide molecule.

Recruitment of IBP to the IS Requires the Activities of Lck and PI3K—To determine whether IBP is recruited to the immunological synapse, we examined the distribution pattern of endogenous IBP in conjugates formed by Jurkat T cells incubated with APCs (Raji) in the presence or absence of *Staphylococcus* enterotoxin E (SEE) superantigen (Fig. 3*A*). The localization of PKC-θ, a key molecule known to be recruited to the IS upon T cell activation, was examined, as well (36). In most conjugates formed in the absence of SEE, both IBP and PKC-θ displayed a uniform speckled pattern. However, conjugates formed using SEE-pulsed APCs displayed a striking redistribution of IBP to the synapse. Redistribution of IBP paralleled that of PKC-θ, and the two proteins were found to colocalize. Relocalization of IBP and PKC-θ to the IS was observed in ~75% of activated T cells (Fig. 3*B*). Interestingly, in ~20–30% of conjugates formed in the absence of SEE, we observed some redistribution of both IBP and PKC-θ to the cell:cell contact zone, suggesting that TCR-independent pathways may also contribute to the redistribution of IBP. Consistent with the biochemical studies described above, no recruitment of IBP to the T cell-APC interface could be detected in Lck-deficient mutant Jurkat T cells (J.CaM1.6) upon incubation with SEE-pulsed B cells. Furthermore, addition of a PI3K inhibitor, wortmannin, to Lck^+ Jurkat T cells (JE6–1) inhibited recruitment of IBP and of PKC-θ to the IS. Taken together, these data indicate that IBP relocalizes to the T cell-APC contact area upon activation and that the activities of both Lck and PI3K are required for the recruitment of IBP to the immunological synapse.

We also examined whether in primary T cells IBP colocalizes with TCR/CD3 caps, asymmetric structures formed by the clustering of T cell receptors in response to T cell activation. Formation of TCR/CD3 caps closely mirrors the molecular mechanisms, which are necessary for synapse formation (37). TCR/CD3 capping was induced in purified $CD4^+$ T cells from DO.11.10 TCR Tg mice. In unstimulated cells, both CD3 and IBP molecules were distributed uniformly (Fig. 4, *upper panel*). Once T cell stimulation was induced, a clear polarization of both TCR/CD3 and IBP to one side of the cell could be observed (Fig. 4, *lower panel*). IBP colocalized with the TCR/CD3 cap in ~70% of the cells (data not shown). These results indicate that activation of primary T cells leads to the relocalization of IBP to areas of TCR clustering where critical downstream signaling events occur.

IBP Exhibits GEF Activity toward Rac1 and Cdc42 but Not Toward RhoA—Activation of Rho GTPases plays a critical role in the complex cytoskeletal dynamics that underlie the formation, as well as the function, of the IS (9, 10). Because IBP shares a high degree of similarity with SWAP-70, a protein known to possess GEF activity toward Rac1 (20), we determined whether IBP activates specific members of the Rho family of proteins. We generated a panel of IBP deletion mutants that removed either the potentially autoinhibitory N-terminal region (IBP ΔN) or the putative C-terminal DH domain (IBP Δ313–631) (Fig. 5*A*). We also generated a mutant (IBP 313–631) containing the putative DH domain alone. We utilized these constructs in *in vivo* assays of GEF activity (Fig. 5, *B* and *C*). Wild-type IBP, in the absence of any additional signals, was unable to mediate the activation of Rac1 or Cdc42. However, expression of the deletion mutants that either lacked the potential autoinhibitory N-terminal domain (IBP ΔN) or contained the potential DH domain alone (IBP 313–361) led to Rac1 activation (Fig. 5*B*) and, more modestly, Cdc42 activation (Fig. 5*C*). Activation of these GTPases was not detected in COS-7 cells expressing a deletion mutant of IBP lacking the potential DH domain (IBP Δ313–631). These results indicate that IBP displays GEF activity for Rac1 and Cdc42 *in vivo*. The GEF catalytic activity of IBP maps to its DH domain, and the N terminus of IBP may contain a regulatory domain that modulates its GEF activity.

To confirm that IBP can act as a GEF for Rho GTPases, we subsequently utilized purified recombinant wild-type or mutant IBP proteins to conduct *in vitro* GDP release assays on purified recombinant Rho proteins. Consistent with the *in vivo* activation assays, incubation of wild-type IBP with Rac1 (Fig. 6*A*) or Cdc42 (Fig. 6*B*) did not stimulate the dissociation of [^{3}H]GDP from these Rho GTPases. However, a mutant form of IBP containing only the putative DH domain (313–631) stimulated rapid GDP dissociation from both Rac1 (Fig. 6*A*) and Cdc42 (Fig. 6*B*) but not from RhoA (Fig. 6*C*). In contrast, an IBP deletion mutant lacking this C-terminal domain (IBP Δ313–631) did not exhibit any GDP/GTP exchange activity on Rac1 or Cdc42, further supporting the notion that the GEF activity of IBP is contained within its C terminus. Because our initial studies had demonstrated that both Lck and PI3K enzymes target IBP, we examined the potential impact of these signaling pathways on the GEF activity of IBP (Fig. 6*D*). Al-

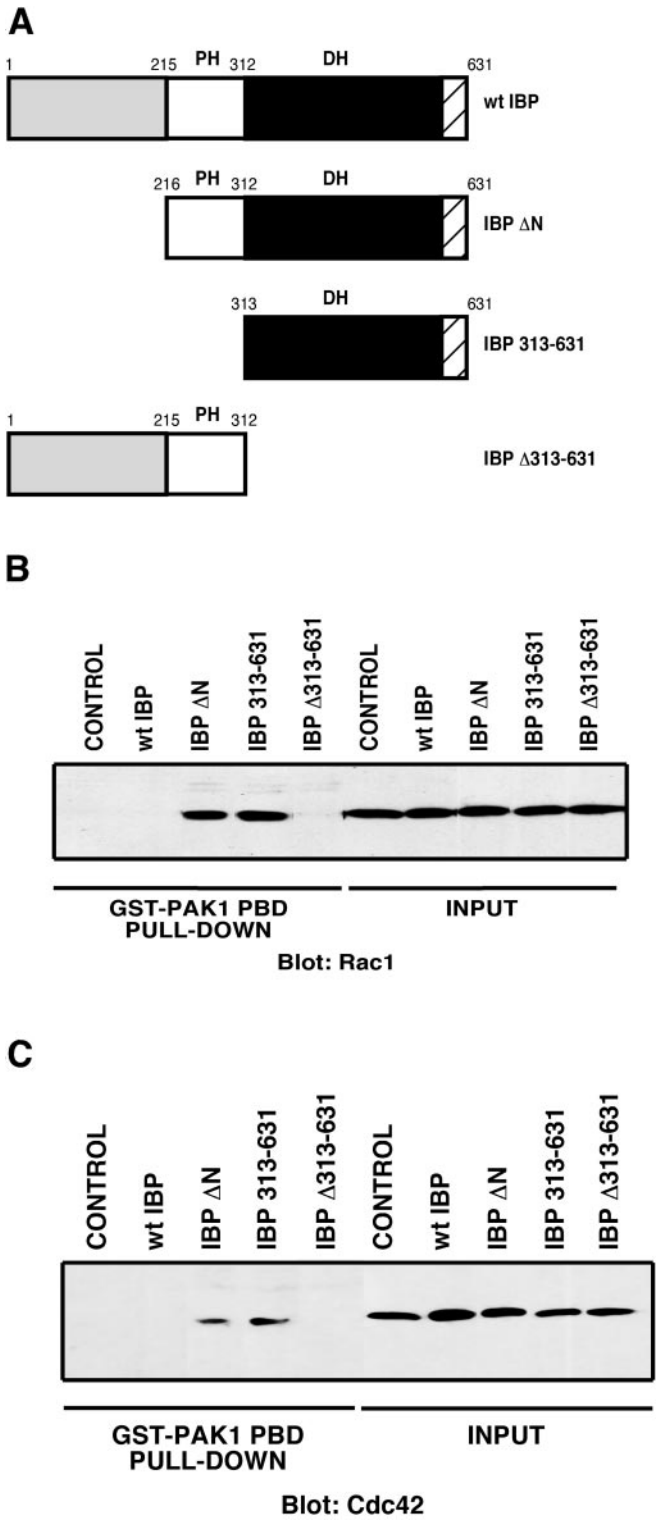

FIG. 5. ***In vivo* activation of Rac1 and Cdc42 by wild-type or mutant IBP proteins.** *A*, a schematic diagram of the IBP deletion mutants utilized in the Rac1/Cdc42 activation assays. *B*, levels of active Rac1 in cells expressing wild-type or mutant IBP proteins. COS-7 cells were transiently transfected with an empty vector (*CONTROL*) or with an expression construct for either wild-type IBP or the indicated IBP deletion mutants. Whole cell lysates were prepared and incubated with immobilized GST-PAK1 PBD fusion protein to affinity precipitate activated (*i.e.* GTP-bound) Rac1. The amount of Rac1-GTP that bound to the GST-PAK1 PBD was visualized by separation on an SDS-polyacrylamide gel followed by Western blotting with an anti-Rac1 antibody. The corresponding total cell lysates used for pull-down assays (*INPUT*) were simultaneously immunoblotted to ensure for equivalent expression levels of Rac1 in the different transfectants. *C*, levels of active Cdc42 in cells expressing wild-type or mutant IBP proteins. COS-7 cells were transiently transfected with an empty vector (*CONTROL*) or with an expression construct for either wild-type IBP or the indicated IBP deletion mutants. Whole cell lysates were prepared and incubated with immobilized GST-PAK1 PBD fusion protein to affinity precipitate activated (*i.e.* GTP-bound) Cdc42. The amount of Cdc42-GTP that bound to the GST-PAK1 PBD was visualized by separation on an SDS-polyacrylamide gel followed by Western blotting with an anti-Cdc42 antibody. Corresponding total cell lysates used for pull-down assays (*INPUT*) were simultaneously immunoblotted to ensure for equivalent expression levels of Cdc42 in the different transfectants.

though in the absence of any additional signal, the wild-type IBP molecule did not display any GEF activity, phosphorylation-mediated activation of IBP by Lck resulted in an enhanced ability of the wild-type IBP to promote GDP-GTP exchange on Rac1 (Fig. 6*D*). Addition of the PI3K product PI(3,4,5)P_3 in the exchange reactions stimulated the GEF activity of Lck-phosphorylated wild-type IBP on Rac1 even further (Fig. 6*D*). These results indicate that IBP can possess GEF activity toward Rac1 and Cdc42 but not toward RhoA. Furthermore, the signals that control the recruitment of IBP to the immunological synapse also regulate its GEF activity.

DISCUSSION

It has become clear that the productive interaction of a $CD4^+$ T cell with an antigen-presenting cell results in the profound reorganization of receptors and signaling molecules leading to the formation of what has been termed the immunological synapse (6–8). Remodeling of the actin cytoskeleton by the Rho family of GTPases is fundamental to the achievement of this three-dimensional structure. Because Rho GTPases are major regulators of the actin cytoskeleton, precise regulation of their activity is likely to be essential to the assembly/function of the immunological synapse. Here we report that IBP is a novel type of GEF that is activated in response to TCR engagement and is recruited to the immunological synapse.

The ability of IBP to activate Rac1 and Cdc42 is consistent with the fact that IBP displays a high degree of similarity to SWAP-70, a protein recently described to represent a novel type of GEF for Rac1 (20). SWAP-70 and IBP may be the only two members of this unique class of GEFs, because we have failed to identify any additional sequences sharing significant homology with these two proteins despite extensive searches. Interestingly, during the course of these studies Tanaka *et al.* (38) reported the identification of a protein homologous to SWAP-70, which they termed SLAT (SWAP-70-like adapter of T cells). A comparison of the sequences of these two proteins has revealed that murine IBP is identical to SLAT. Strikingly, expression studies have indicated that, unlike SWAP-70, IBP is expressed in T cells suggesting that IBP may be the predominant member of this unique class of GEFs to be found in T lymphocytes (21, 22, 38). Furthermore, although both SWAP-70 and IBP can be detected in B cells, these two molecules appear to be expressed at distinct stages of B cell differentiation as indicated by the fact that IBP is largely absent in germinal center B cells, which strongly express SWAP-70 (21, 22). The differential usage of these two GEFs by the immune system in specific compartments suggests that the functional outcome of the signaling events elicited by these two GEFs may be distinct. An alternative explanation is that the regulatory requirements for the activation of IBP and SWAP-70 are different. Indeed, whereas the GEF activity of IBP is controlled by both tyrosine phosphorylation and phosphoinositide binding (Fig. 6*D*), activation of SWAP-70 has been reported to depend primarily on the activity of PI3K (20). The generation of IBP-deficient mice should provide insight into whether these two GEFs play unique or redundant roles in the immune system.

The regulation of IBP in response to TCR signaling is remi-

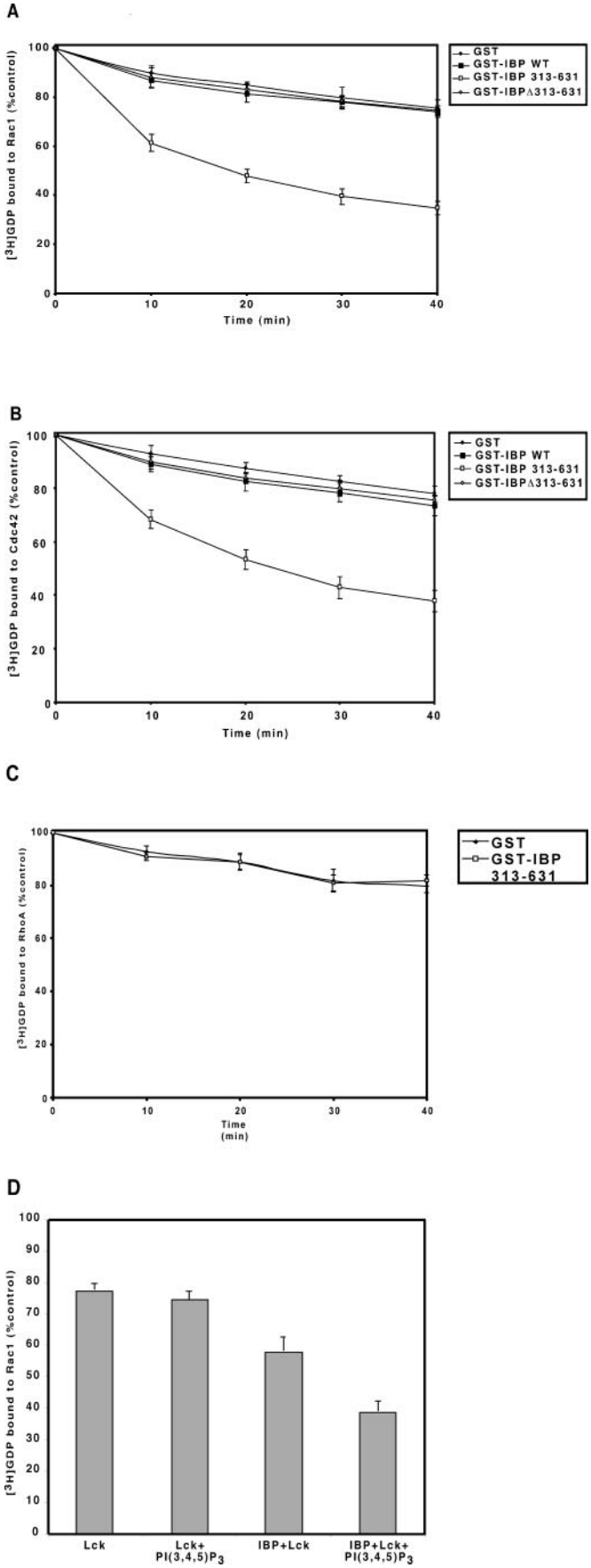

FIG. 6. **IBP exhibits GEF activity toward Rac1 and Cdc42 *in vitro*, which can be modulated by Lck and PI(3,4,5)P_3.** *A–C*, stimulation of GDP dissociation from Rho GTPases by wild-type or mutant IBP proteins. [^{3}H]GDP-loaded Rac1 (*A*), Cdc42 (*B*), or RhoA (*C*) was incubated with GST alone or with either wild-type or mutant GST-IBP fusion proteins, as indicated. The exchange reactions were stopped at the indicated *time points*, and the amount of [^{3}H]GDP remaining bound to the GTPases was then measured by filtering the quenched reaction samples over nitrocellulose membranes followed by scintillation counting of the membrane-bound radioactivity. The percentage of [^{3}H]GDP remaining bound at each *time point* was evaluated relative to the amount of radioactivity remaining at time 0 of the respective reaction, which was set to 100% in each experiment. The results shown are representative of at least three independent assays. *D*, regulation of IBP GEF activity by Lck and PI(3,4,5)P_3. [^{3}H]GDP-loaded Rac1 was incubated with Lck-phosphorylated wild-type HA-IBP (*IBP+Lck*) in the presence or absence of PI(3,4,5)P_3 (1 μM) for 0 or 40 min. Reactions without IBP (*Lck* and *Lck$^+$ PI(3,4,5)P_3*) were simultaneously carried out as control. The exchange reactions were then stopped, and the amount of [^{3}H]GDP remaining bound to Rac1 was measured by filter-binding method as described for *A–C*. The percentage of [^{3}H]GDP remaining bound after incubation for 40 min was evaluated relative to the amount of radioactivity remaining at time 0 of the respective reaction, which was set to 100% in each experiment. The results shown are representative of at least three independent assays.

niscent of the regulation of Vav, whose activity is controlled by both tyrosine phosphorylation and phosphoinositide binding (29, 30). As in the case of Vav, whose phosphorylation by either Src or Syk tyrosine kinases leads to the relief of an intramolecular inhibitory interaction (17), our results indicate that tyrosine phosphorylation of IBP by Lck is also likely to lead to a conformational change. This notion is supported by the finding that deletion of the N terminus leads to a constitutively active form of IBP whereas mutation of Tyr-210, the major Lck-phosphorylation site, prevented its ability to interact with PI(3,4,5)P_3. Although both Vav and IBP contain a PH domain and bind the PI3K product PI(3,4,5)P_3, our studies suggest that the exact mechanism by which phosphoinositides participate in the activation of these two GEFs may differ. Although binding of PI(3,4,5)P_3 is believed to enhance the tyrosine phosphorylation of Vav by PTKs (30), tyrosine phosphorylation appears to be a prerequisite for the ability of IBP to bind phosphoinositides. Therefore, we favor a model whereby the N-terminal region of IBP serves a regulatory function and maintains IBP in a closed conformation until its TCR-induced tyrosine phosphorylation relieves this autoinhibitory interaction. This would then expose the PH domain allowing it to bind PI(3,4,5)P_3. Binding of this phosphoinositide would lead to full activation of IBP by enabling its DH domain to interact with the appropriate Rho GTPase, as well as by directing IBP to the appropriate subcellular compartment. Given the unique structural features of IBP and SWAP-70, it remains to be determined whether the precise mechanism utilized by these two proteins to activate Rho GTPases differs from that utilized by conventional guanine nucleotide exchange factors.

The fact that IBP and Vav respond to TCR-mediated signals, and both activate Rac1 and Cdc42, raises the critical question of the exact role that IBP plays in T cells. Although genetic studies will be required to definitely address this issue, it has been suggested that GEFs may not only activate the GTPases but also regulate the downstream pathways that these GTPases control (13). This may be because of the fact that specific GEF/GTPase combinations, may elicit only a limited subset of the large number of downstream effector pathways activated by these GTPases. Given the complexities that underlie the assembly, as well as the functions of the immunological synapse, recruitment and activation of distinct GEFs would thus enable T cells to precisely control the molecular processes mediated by the synapse.

Although our initial studies have focused on two specific sets of signals, those mediated by Lck and PI3K, IBP function, like that of Vav (17), may be regulated by additional mechanisms and/or stimuli, as well. Vav can be tyrosine-phosphorylated not

only by Lck but also by another TCR-activated PTK, ZAP-70 (39). It is thus possible that additional kinases may also phosphorylate IBP. The presence of a putative EF-hand motif at the N terminus furthermore suggests that calcium may also play a role in the regulation of IBP function. Finally, we have noted in our conjugate experiments that even in the absence of SEE, redistribution of IBP to the contact zone was observed in 20–30% of cells. This is reminiscent of proteins such as ezrin whose localization is controlled not just by TCR signals but also by integrin engagement (40). Because cross-talk between TCR and additional coreceptors such as the integrin LFA-1 is crucial for synapse formation and T cell activation, determining the spectrum of receptors that activate IBP will be essential to understand the role of this novel GEF in T cell function.

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