Stimulation of Ras Guanine Nucleotide Exchange Activity of Ras-GRF1/CDC25\textsuperscript{Mm} upon Tyrosine Phosphorylation by the Cdc42-regulated Kinase ACK1*

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Ras-GRF1 is a brain-specific guanine nucleotide exchange factor (GEF) for Ras, whose activity is regulated in response to Ca\textsuperscript{2+} influx and G protein–coupled receptor signals. In addition, Ras-GRF1 acts as a GEF for Rac when tyrosine-phosphorylated following G protein–coupled receptor stimulation. However, the mechanisms underlying the regulation of Ras-GRF1 functions remain incompletely understood. We show here that activated ACK1, a nonreceptor tyrosine kinase that belongs to the focal adhesion kinase family, causes tyrosine phosphorylation of Ras-GRF1. On the other hand, kinase-deficient ACK1 exerted no effect. GEF activity of Ras-GRF1 toward Ha-Ras, as defined by in vitro GDP binding and release assays, was augmented after tyrosine phosphorylation by ACK1. In contrast, GEF activity toward Rac1 remained latent, implying that ACK1 does not represent a tyrosine kinase that acts downstream of G protein–coupled receptors. Consistent with enhanced Ras-GEF activity, accumulation of the GTP-bound form of Ras within the cell was shown through the use of Ras-binding domain pull-down assays. Furthermore, Ras-dependent activation of ERK2 by Ras-GRF1 was enhanced following co-expression of activated ACK1. These results implicate ACK1 as an upstream modulator of Ras-GRF1 and suggest a signaling cascade consisting of Cdc42, ACK1, Ras-GRF1, and Ras in neuronal cells.

The Ras family of small GTP-binding proteins has been implicated as a molecular switch that directs diverse cellular responses, such as cell cycle progression, transformation, and cell death (1, 2). In most cases, the activity of Ras is regulated through the action of guanine nucleotide exchange factors (GEFs),\textsuperscript{1} which increase the active GTP-bound form of Ras in living cells depending upon upstream signals. In mammalian cells, several proteins that exhibit GEF activity toward Ras, such as mSos, Ras-GRF, and Ras-GRP, have been identified (3, 4). All of these proteins contain conserved catalytic sequences, termed CDC25 homology domains (3). In particular, the role of mSos in Ras regulation has been explored intensively, leading to the notion that mSos acts as a regulator downstream of a wide variety of receptors, including tyrosine kinase-type (5), cytokine (6, 7), and G protein–coupled (8) receptors.

The Ras-GRF family of GEFs consists of at least two members, Ras-GRF1 (9–12) and 2 (13). Ras-GRF1 is expressed exclusively in the brain, whereas Ras-GRF2 transcripts are detected in several tissues including brain and lung. The physiological functions of Ras-GRF1 in neuronal signal transduction remain largely unknown. However, Ras-GRF1 is thought to play an important role because mice lacking Ras-GRF1 show defects in brain functions such as memory consolidation, whereas learning and short term memory seem to be intact (14). In addition to the CDC25 homology domain, both Ras-GRF proteins possess a pleckstrin homology (PH) domain, an IQ motif, and tandem Dbl homology (DH)/PH domains.

The IQ motif is responsible for the interaction with the Ca\textsuperscript{2+}-binding protein calmodulin, which mediates signals triggered by an increase in the intracellular Ca\textsuperscript{2+} concentration. Thus, Ras-GRF1 is considered to be involved in Ca\textsuperscript{2+} influx-dependent activation of Ras, particularly in neuronal cells (15). On the other hand, Ca\textsuperscript{2+}-responsive, Ras-independent as well as constitutive, Ras-dependent pathways downstream of Ras-GRF1 were shown to direct Raf and extracellular signal-regulated kinase (ERK) activities (16).

Tandem DH/PH domains are conserved among Dbl family proteins, GEFs that target Rho family GTP-binding proteins (17). Recently, it has been revealed that Ras-GRFs exhibit GEF activity toward Rac as well, for which the DH/PH domains are required. While Rac-GEF activity of Ras-GRF1 becomes evident following stimulation by G protein βγ subunit (Gβγ) signals (18), Ras-GRF2 shows constitutive and Ca\textsuperscript{2+}-enhanced Rac-GEF activities (19). Additionally, the tyrosine kinase Src phosphorylates Ras-GRF1, thereby inducing its Rac-GEF activity like Gβγ (20). In response to G protein–coupled receptor signals, not only Rac-, but also Ras-GEF activity is promoted (21). Phosphorylation of a serine residue is important for triggering Ras-GEF activation (22), whereas tyrosine phosphorylation is indispensable for Rac-GEF activation (18, 20). Recently, oligomerization of Ras-GRFs through their DH domains was reported (16). Oligomerization seems to be required for biological functions, because a mutation within the DH domain that abolishes oligomerization rendered Ras-GRFs incapable of inducing transformation of NIH 3T3 cells.

ACK1 was isolated as a target of the Rho family GTP-binding protein Cdc42 (23). Like other target molecules, ACK1 binds to the GTP-bound form of Cdc42 through the Cdc42/Rac interactive binding (CRIB) region (24). On the basis of the sequence similarity of the catalytic domains, ACK1 is thought to belong

\textsuperscript{1} The abbreviations used are: GEF, guanine nucleotide exchange factor; CRIB, Cdc42/Rac interactive binding; DH, Dbl homology; EGF, epidermal growth factor; ERK, extracellular signal-regulated kinase; Gβγ, G protein βγ subunits; GST, glutathione S-transferase; PH, pleckstrin homology; WT, wild type; IB, immunoblot; Ki-Ras, Kirsten Ras.

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to the focal adhesion kinase family of nonreceptor tyrosine kinases (23). In addition to the kinase catalytic domain and the CRIB motif, ACK1 possesses an Src homology 3 domain and several proline-rich motifs. ACK1 interacts with the Grb2 adaptor protein through its proline-rich motifs and thus is thought to act downstream of receptor tyrosine kinases such as the epidermal growth factor (EGF) receptor as well (25). Indeed, the tyrosine phosphorylation level of ACK1 was transiently increased upon EGF treatment (25). In contrast, the binding partner of the Src homology 3 domain of ACK1 remains to be identified. Tyrosine phosphorylation of ACK1 is also induced in response to temperature shift down or an increase in osmolarity (25). We recently found that ACK1 leads to phosphorylation of DbI, a GEF toward Rho family proteins, thereby promoting its activity (26). Thus, ACK1 may link Rho family GTP-binding proteins through the action of specific GEFs. However, the physiological function as well as specific target molecules of ACK1 are still obscure.

ACK2, a close relative of ACK1, is activated following treatment with fetal bovine serum, EGF, and bradykinin but neither platelet-derived growth factor, tumor necrosis factor α, nor interleukin-1 (27). Recently, it has been shown that ACK2 mediates cell adhesion signals initiated by integrin β1 in a Cdc42-dependent manner (28). Hence, ACK2 is likely to participate in signaling pathways directed by a variety of extracellular stimuli. Another ACK-related kinase, Tnk1, was isolated from umbilical cord blood hematopoietic stem/progenitor cells (29). Aside from the lack of the CRIB motif, overall structural features of Tnk1 are similar to ACK1. Tnk1 may play a pivotal role in fetal development because its transcripts are widely detected in fetal tissues examined.

Through investigation of the regulatory mechanism of Ras-GRF1 upon tyrosine phosphorylation, we identified ACK1 as an upstream kinase that induces tyrosine phosphorylation of Ras-GRF1. Subsequent to ACK1-dependent tyrosine phosphorylation, GEF activity toward Ras was enhanced. In contrast, Rac-GEF activity remained latent after tyrosine phosphorylation by ACK1, suggesting that ACK1 is not implicated in Gβγ-dependent signaling pathways.

**EXPERIMENTAL PROCEDURES**

**Materials**—Antibodies including anti-Ras-GRF antibody (sc-226), anti-Ha-Ras antibody (sc-520), anti-Ki-Ras antibody (sc-30), anti-N-Ras antibody (sc-31), and anti-phosphotyrosine antibody (PY20, sc-7020) were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Antibodies including anti-Myc antibody (9E10, Babco), anti-HA antibody (12CA5, Eastman Kodak Co.), anti-FLAG antibody (M2, Sigma), anti-mouse Ig antibody (55480, Cappel), and horseradish peroxidase-conjugated anti-mouse Ig antibody (NA931; Amersham Pharmacia Biotech) were purchased from the indicated commercial suppliers. EGF was purchased from Takara Biomedicals (Tokyo, Japan). The tyrosine kinase inhibitor herbinycin A was purchased from Calbiochem.

**Cell Culture and Transfection**—Human embryonic kidney 293 cells were maintained in Dulbecco’s modified Eagle’s medium supplemented with 10% (v/v) fetal bovine serum. Transfection of 293 cells with various combinations of plasmids was serum-starved for 24 h. Cells were rinsed with ice-cold phosphate-buffered saline (8.1 mM Na2HPO4, 1.45 mM KH2PO4, 137 mM NaCl, 2.7 mM KCl), dissolved in RIPA buffer (50 mM Tris-HCl (pH 8.0), 150 mM NaCl, 1% (v/v) Nonidet P-40, 0.1% (w/v) SDS, 0.5% (w/v) sodium deoxycholate, 0.1 mM phenylmethylsulfonyl fluoride, 2 μg/ml leupeptin, 2 μg/ml aprotinin, 1 mM Na3VO4, 50 mM NaF, 50 mM β-glycerophosphate, 20 mM Na2PO4, and centrifuged at 15,000 × g for 10 min. Subsequently, the anti-Myc antibody 9E10 (2 μg) and a rabbit anti-mouse Ig antibody conjugated to protein A-Sepharose (Amersham Pharmacia Biotech) were added to cell lysates, followed by incubation for 1 h at 4 °C with gentle mixing. Immunoprecipitates were washed twice with RIPA buffer and twice with exchange buffer (10 mM Hepes–NaOH (pH 7.4), 5 mM MgCl2, 5 mM KCl, 1 mM EGTA) and subjected to GTP/GTP exchange assays.

For [3H]GDP binding assays, immunoprecipitated proteins were incubated with Ha-Ras (40 ng) on ice or Rac1 (200 ng) at 30 °C, respectively, in exchange buffer supplemented with 2 mM dithiothreitol, 0.2 mg/ml bovine serum albumin, 1 mM ATP, and 1 μM [3H]GDP (1,265 TBq/mol). After incubation for specified periods, ice-cold wash buffer (10 mM Tris–HCl (pH 7.5), 10 mM MgCl2) was added, and samples were filtered through a nitrocellulose membrane, which was subjected to extensive washing with wash buffer. Radioactivity remaining on the filter was quantitated by the liquid scintillation counter.

For [3H]GDP release assays, Ha-Ras-[3H]GDP complex was prepared by incubation of Ha-Ras in exchange buffer supplemented with 2 mM dithiothreitol, 5 mM EDTA, 0.2 mg/ml bovine serum albumin, 1 mM ATP, and [3H]GDP (1,265 TBq/mol) for 10 min at 30 °C. 5 mM MgCl2 was added to terminate the GDP/GTP exchange reaction. Immunoprecipitated proteins were incubated with the Ha-Ras-[3H]GDP complex on ice in exchange buffer supplemented with 2 mM dithiothreitol, 0.2 μg/ml bovine serum albumin, 1 mM ATP, and 10 mM GDP. After incubation for specified periods, radioactivity remaining in the complex was quantitated by filter binding assays as described above.

**Affinity Precipitation of Ras Using GST-c-Raf-1-RBD**—Affinity precipitation of Ras using GST-c-Raf-1-RBD was performed essentially as described elsewhere (32). E. coli BL21 cells transformed with pGEX-c-Raf-1-RBD were grown at 30 °C to early logarithmic phase. Expression of GST-c-Raf-1-RBD was induced by 1 mM isopropyl-1-thio-β-D-galacto- side for 4 h at 30 °C. Harvested cells were suspended in lysis buffer (50 mM Tris–HCl (pH 7.5), 20 mM MgCl2, 150 mM NaCl, 0.5% (v/v) Nonidet P-40, 20 μg/ml aprotinin, 1 mM Na3VO4) supplemented with 1 mM dithiothreitol, and disrupted by sonication. Disrupted cells were centrifuged at 18,000 × g for 20 min at 4 °C, and the supernatant was stored at −80 °C. Glutathione–Sepharose beads were mixed with the lysate containing 20 μg of GST-c-Raf-1-RBD for 30 min at 4 °C and washed twice with lysis buffer. beads were lysed in lysis buffer and centrifuged at 15,000 × g for 10 min at 4 °C. Supernatant was added for 1 h at 4 °C with GST-c-Raf-1-RBD conjugated to glutathione-Sepharose beads. Slurries were washed twice with lysis buffer and twice with washing buffer (50 mM Tris–HCl (pH 7.5), 20 mM MgCl2, and 150 mM NaCl). Precipitated Ras was detected by immunoblotting using specific antibodies.

**ERK2 Kinase Assay**—Ectopically expressed HA-ERK2 was immunopositive in the membrane.
precipitated from 293 cells with 0.2 μg of an anti-HA antibody conjugated to protein A-Sepharose. ERK2 assays were performed as described previously (18).

RESULTS

Tyrosine phosphorylation of Ras-GRF1 was examined using a transient cDNA expression system (Fig. 1). Both full-length Ras-GRF1 and its N-terminally deleted mutant (Ras-GRF1ΔN582) were tyrosine-phosphorylated when co-expressed with wild-type ACK1 and GTPase-deficient Cdc42(G12V) (Fig. 1A). Cdc42(G12V/T35A), an effector domain mutant that is unable to bind and activate ACK1 (26), did not show any stimulatory effect. The expression level of Cdc42(G12V/T35A) as determined by immunoblotting was virtually the same as that of Cdc42(G12V) (data not shown). The constitutively active mutant ACK1(L543F) (26) induced tyrosine phosphorylation of Ras-GRF1 in the absence of Cdc42(G12V), whereas kinase-deficient ACK1(K214R) exhibited no effect (Fig. 1B). The tyrosine phosphorylation level of Ras-GRF1 in ACK1(L543F)-expressing cells was not further enhanced by co-expression of Cdc42(G12V) (data not shown). We are currently unsuccessful in detecting in vitro phosphorylation of Ras-GRF1 by the ACK1 kinase catalytic domain produced in E. coli as a GST fusion protein (23). However, an autocrine mechanism for Ras-GRF1 tyrosine phosphorylation seems quite unlikely, because conditioned medium of 293 cells expressing ACK1(WT) and Cdc42(G12V) exhibited markedly enhanced GEF activity. Likewise, co-expression with ACK1(L543F) stimulated Ras-GEF activity of Ras-GRF1 (Fig. 4), whereas ACK1(K214R) exerted no effect (data not shown). Cdc42(G12V/T35A) also did not enhance Ras-GEF activity (Fig. 4). Overall, tyrosine phosphorylation of Ras-GRF1 was paralleled with its activation as determined by immunoblotting (data not shown).

EGF transiently induces tyrosine phosphorylation and subsequent activation of ACK1 (25) and thus is thought to regulate the ACK1 pathway. Therefore, we next examined the effect of EGF on Ras-GRF1 tyrosine phosphorylation. As shown in Fig. 2, EGF rapidly triggered tyrosine phosphorylation of Ras-GRF1(ΔN690) in an ectopically expressed ACK1-dependent manner with maximal phosphorylation detected at 1 min, diminishing thereafter. EGF-induced tyrosine phosphorylation of full-length Ras-GRF1 was also observed with a similar time course (data not shown). We are currently unable to block EGF-induced Ras-GRF1 tyrosine phosphorylation by dominant-negative Cdc42(T17N), because Cdc42(T17N) by itself activates ACK1 by unknown mechanisms.

As a first step to assess the physiological significance of ACK1-dependent tyrosine phosphorylation, GEF activity toward Ha-Ras was measured. Time courses of [3H]GDP binding and release in the presence of anti-Myc immunoprecipitates from mock- or Myc-tagged Ras-GRF1-transfected 293 cells lysates are illustrated in Fig. 3. Ectopically expressed Myc-Ras-GRF1 stimulated the exchange rate, which represents basal GEF activity of Ras-GRF1. Notably, tyrosine-phosphorylated Myc-Ras-GRF1 recovered from cells co-expressing ACK1(WT) and Cdc42(G12V) exhibited markedly enhanced GEF activity. Likewise, co-expression with ACK1(L543F) stimulated Ras-GEF activity of Ras-GRF1 (Fig. 4), whereas ACK1(K214R) exerted no effect (data not shown). Cdc42(G12V/T35A) also did not enhance Ras-GEF activity (Fig. 4). Overall, tyrosine phosphorylation of Ras-GRF1 was paralleled with its activation as a Ras-GEF. The deletion mutant Ras-GRF1(ΔN582) showed GEF activity toward Ha-Ras, which was also enhanced upon activated ACK1-dependent tyrosine phosphorylation (Fig. 4). Effects of the tyrosine kinase inhibitor herbimycin A are shown in Fig. 5. Tyrosine phosphorylation of Ras-GRF1 following co-expression with ACK1(WT) and Cdc42(G12V) was potently inhibited by herbimycin A (Fig. 5A). The expression level of Ras-GRF1 remained unchanged after treatment with herbimycin A (data not shown). Correspondingly, herbimycin A treatment abrogated GEF activity toward Ha-Ras to the level of unphosphorylated Ras-GRF1 (Fig. 5B). Thus, activated ACK1-dependent tyrosine phosphorylation seems to be required for enhancement of GEF activity of Ras-GRF1 toward Ha-Ras.

2 J. Kato, Y. Kaziro, and T. Satch, unpublished results.
were subjected to \([3H]\)GDP binding (ing serum starvation. Anti-Myc antibody 9E10 immunoprecipitates (filled squares

cells

), or pCMV5-Myc-Ras-GRF1 with pCMV5-ACK1(WT) and pCMV5-

FLAG-Cdc42(G12V) (filled squares

293 cells were transfected with

phosphorylated by activated ACK1.

N582) with indicated plasmids were subjected to the \([3H]\)GDP binding assay for Ha-Ras. Amounts of \([ 3H]\)GDP bound to Ha-Ras after a 1-min incubation are shown as mean ± S.E. \((n = 3)\).

**Fig. 3. GEF activity toward Ha-Ras of Ras-GRF1 tyrosine-phosphorylated by activated ACK1.** 293 cells were transfected with a control vector (open circles), pCMV5-Myc-Ras-GRF1 alone (filled circles), or pCMV5-Myc-Ras-GRF1 with pCMV5-ACK1(WT) and pCMV5-FLAG-Cdc42(G12V) (filled squares). Cell lysates were prepared following serum starvation. Anti-Myc antibody 9E10 immunoprecipitates were subjected to \([3H]\)GDP binding (A) or release (B) assays for Ha-Ras. Data are shown as mean ± S.E. \((n = 3)\).

**Fig. 4. GEF activity toward Ha-Ras of full-length and N-terminally deleted Ras-GRF1.** Anti-Myc 9E10 immunoprecipitates from 293 cells transfected with pCMV5-Myc-Ras-GRF1 or pCMV5-Myc-Ras-GRF1(N582) with indicated plasmids were subjected to the \([3H]\)GDP binding assay for Ha-Ras. Amounts of \([3H]\)GDP bound to Ha-Ras after a 1-min incubation are shown as mean ± S.E. \((n = 3)\).

Formation of the GDP-bound form of endogenous Ras proteins was assessed by a pull-down assay using a GST-fused Ras-binding domain construct (Fig. 6). When Ras-GRF1 was expressed with ACK1 activated by co-expression with Cdc42(G12V) or the L543F mutation, the GDP-bound form of all three subtypes of Ras (Ha-, Ki-, and N-Ras) was significantly accumulated. In contrast, GTP-bound Ras yielded in response to unphosphorylated Ras-GRF1 was virtually undetectable under these conditions.

To obtain further evidence for the enhancement of Ras-GEF activity in response to ACK1-dependent tyrosine phosphorylation, we measured the activity of ERK2, a serine/threonine kinase downstream of Ras (Fig. 7). Whereas Ras-GRF1 alone enhanced ERK2 activity, which may be ascribed to the basal Ras-GEF activity, ACK1 did not exert any stimulatory effect in response to unphosphorylated Ras-GRF1. Anti-Myc 9E10 immunoprecipitates from 293 cells transfected with indicated plasmids were subjected to the \([3H]\)GDP binding assay for Ha-Ras. Amounts of \([3H]\)GDP bound to Ha-Ras after a 1-min incubation are shown as mean ± S.E. \((n = 3)\).

**Fig. 5. Effect of herbimycin A on tyrosine phosphorylation and activation of Ras-GRF1.** A, tyrosine phosphorylation of Ras-GRF1. Proteins within anti-Myc antibody 9E10 immunoprecipitates from 293 cells transfected with pCMV5-Myc-Ras-GRF1 alone or pCMV5-Myc-Ras-GRF1 with pCMV5-ACK1(WT) and pCMV5-FLAG-Cdc42(G12V) were stained with the anti-phosphotyrosine antibody PY99. Where indicated, cells were treated with herbimycin A (1 μg/ml) for 24 h. Representative results of three independent experiments are shown. B, GEF activity toward Ha-Ras of Ras-GRF1. Anti-Myc 9E10 immunoprecipitates from 293 cells transfected with indicated plasmids were subjected to the \([3H]\)GDP binding assay for Ha-Ras. Amounts of \([3H]\)GDP bound to Ha-Ras after a 1-min incubation are shown as mean ± S.E. \((n = 3)\).

**Fig. 6. Formation of the GDP-bound form of Ras proteins.** Endogenous Ras proteins complexed with GTP were affinity-precipitated by the use of GST-c-Raf-1-RBD as a probe from 293 cells expressing indicated proteins. Precipitated proteins were stained with subtype-specific anti-Ras antibodies as indicated. Representative results of three independent experiments are shown.

**DISCUSSION**

The GDP/GTP cycle of Ras is regulated by GEFs in response to extracellular stimuli. Virtually all types of receptors that trigger the accumulation of Ras-GTP employ mSos, a ubiquitously expressed GEF for Ras, as a regulator of the GDP/GTP state (5–8). Translocation of mSos from the cytosol to the plasma membrane through the interaction with the adaptor Grb2 is critical, while GEF activity itself remains unaffected (5). In support of this, forced association of mSos with the plasma membrane resulted in constitutive activation of the Ras pathway (34, 35). In contrast to mSos, the signaling mechanism through Ras-GRF1 seems to be more complicated. Although an artificial modification leading to membrane localization rendered Ras-GRF1 constitutively active, it is unclear whether such translocation occurs within the cell in response to upstream signals (35). On the other hand, Ca\(^{2+}\)/calmodulin-binding-dependent or serine phosphorylation-dependent activation was reported (15, 21, 22).

Herein, we show tyrosine phosphorylation-dependent activation of Ras-GEF activity of Ras-GRF1. Transient co-expression
of Ras-GRF1 with ACK1 activated by the interaction with GTPase-deficient Cdc42 or a single amino acid mutation resulted in tyrosine phosphorylation and remarkable enhancement of GEF activity toward Ras. However, we are currently unable to demonstrate that ACK1 directly phosphorylates Ras-GRF1 as a substrate in a cell-free system using purified recombinant proteins. For this, we utilized the GST-fused catalytic domain of ACK1, which was indeed active in phosphorylating myelin basic protein or glutamate/tyrosine polymers. We speculate that domains other than the catalytic domain of ACK1 may be required for recognition of physiological substrates. Alternatively, the involvement of other tyrosine kinases downstream of ACK1 is also possible. In either case, ACK1 plays a crucial role in modulating Ras activity through Ras-GRF1.

As shown in Fig. 6, activated Ras-GRF1 acts on all three subtypes of Ras within 293 cells. Furthermore, unphosphorylated Ras-GRF1 also stimulated the formation of the GTP-bound form of Ha-, Ki-, and N-Ras, when expressed alone at higher levels than in Fig. 6 (data not shown). In contrast, Jones and Jackson (36) reported that Ras-GRF1 activated Ha-Ras, but not Ki- and N-Ras, in NIH3T3 cells. Although we did not compare activation levels of three Ras subtypes quantitatively, our observation suggests that Ras-GRF1 may not absolutely discriminate Ras subtypes at least in our systems.

Recently, we have shown that G protein-coupled receptor-mediated signals induced Rac-GEF activity of Ras-GRF1 through Gβγ (18). Tyrosine phosphorylation was required for the induction of Rac-GEF activity (18). Moreover, the nonreceptor tyrosine kinase Src phosphorylates and thereby stimulates Rac-GEF activity, leading to the notion that tyrosine phosphorylation of critical residues occurs in response to both Gβγ-dependent tyrosine kinase and Src (20). In contrast, ACK1 enhances Ras-GEF activity without affecting Rac-GEF activity. Therefore, phosphorylation of a set of tyrosines distinct from those phosphorylated by Src may be crucial for the enhancement of Rac-GEF activity. We are currently attempting to identify individual sets of phosphorylated tyrosine residues in Ras-GRF1. Our preliminary results by the use of an array of deletion mutants suggested that multiple but different tyrosines are indeed subjected to phosphorylation by Src and ACK1, respectively. As illustrated in Figs. 1, 4, and 7, the C-terminal half of Ras-GRF1 (Ras-GRF1ΔN582) consisting of the CDC25 domain and its flanking regions is also tyrosine-phosphorylated, thereby being activated. Hence, it is possible that enhancement of Ras-GEF activity may solely depend on a local conformational change in the CDC25 domain upon tyrosine phosphorylation. In addition, it is unclear whether one Ras-GRF1 molecule can stimulate Ras and Rac simultaneously when phosphorylated, for instance, by both ACK1 and Src. Presumably, subcellular localization and the interaction with unknown scaffold proteins like MP1 and JIP1 for mitogen-activated protein kinase cascade (37, 38) may determine the substrate specificity of an individual Ras-GRF1 molecule. Further studies will be required to reveal the precise regulatory mechanism of Ras- and Rac-GEF activities at the molecular level.

In contrast to diverse Rac/Cdc42 effectors that regulate cytoskeletal reorganization, the physiological role of ACK1 as a Cdc42-specific effector remains largely unknown. The involvement of ACK in integrin (28) and chondroitin sulfate proteoglycan (39) signaling was proposed, although downstream molecules are still obscure. Recently, we have shown that ACK1 triggers phosphorylation of Dbl, thereby promoting its GEF activity toward Rho family proteins (26). These results suggest that ACK1 may link Rho family proteins to compose a signaling cascade or a positive feedback loop. Moreover, ACK1 phosphorylates and activates Ras-GRF1 as described in this paper. Taken together, an important role for ACK1 may be the regulation of GTP-binding protein networks by targeting GEFs for...
The role of Ras and Rho family proteins in neuronal cells remains poorly understood. Possible regulators for Ras and Rac, such as Ras-GRF1 and synGAP, a synapse-specific GTP-ase-activating protein for Ras, are components of the postsynaptic density fraction, suggesting a role at the synaptic junction (40–42). In fact, Ras-GRF1 is implicated in signaling mediated by G protein-coupled receptors, such as receptors for a variety of neurotransmitters (18, 21, 22). Thus, Ras and Rac may regulate, for instance, the expression of specific genes and channel activity, thereby ultimately leading to the modulation of electrophysiological functions in postsynaptic cells. Furthermore, they may act downstream of specific channel-type receptors such as the N-methyl-D-aspartate receptor, which is localized in the postsynaptic density fraction. Recently, two DH/PH domain-containing proteins, termed collybistin I and II, were identified as binding partners of the peripheral membrane protein gephyrin (43). Gephyrin is associated with neurotransmitter receptors, such as glycine and γ-aminobutyric acid type A receptors and is required for receptor clustering. Hence, Rho family proteins, when activated by collybistins, may exert critical functions in the formation of postsynaptic receptor clusters. It will be important to investigate further the role of Ras and Rho family proteins and their regulatory mechanisms, particularly in the brain.

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