Radixin Stimulates Rac1 and Ca\(^{2+}\)/Calmodulin-dependent Kinase, CaMKII

CROSS-TALK WITH G\(\alpha_{13}\) SIGNALING*

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The ERM (ezrin, radixin, moesin) proteins function as cross-linkers between cell membrane and cytoskeleton by binding to membrane proteins via their N-terminal domain and to F-actin via their C-terminal domain. Previous studies from our laboratory have shown that the \(\alpha\)-subunit of heterotrimeric G\(\alpha_{13}\) protein induces conformational activation of radixin via interaction with its N-terminal domain (Vaiksnarke, R., Adarichev, V., Furthmayr, H., Kozasa, T., Gudkov, A., and Voyno-Yasenetskaya, T. A. (2000) J. Biol. Chem. 275, 26206–26212). In the present study, we tested whether radixin can regulate G\(\alpha_{13}\)-mediated signaling pathways. We determined that the N-terminal domain (amino acids 1–318) and C-terminal domain (amino acids 319–583) of radixin on serum response element (SRE)-dependent gene transcription initiated by a constitutively activated G\(\alpha_{13}\), potentiated SRE activation induced by G\(\alpha_{13}\)Q226L. The N-terminal domain potentiated SRE activation induced by G\(\alpha_{13}\)Q226L. A pull-down assay demonstrated that G\(\alpha_{13}\)Q226L activated Rac1 but not RhoA or Cdc42 proteins. By contrast, G\(\alpha_{13}\)Q226L activated RhoA but not Rac1 or Cdc42. We have also shown that both the C-terminal domain of radixin and G\(\alpha_{13}\)Q226L can stimulate Ca\(^{2+}\)/calmodulin-dependent kinase, CaMKII. Activated mutant that mimics the phosphorylated state of radixin (T564E) stimulated Rac1, induced the phosphorylation of CaMKII, and stimulated SRE-dependent gene transcription. Down-regulation of endogenous radixin using small interference RNA inhibited SRE-dependent gene transcription and phosphorylation of CaMKII induced by G\(\alpha_{13}\)Q226L. Overall, our results indicated that radixin via its C-terminal domain mediates SRE-dependent gene transcription through activation of Rac1 and CaMKII. In addition, the radixin–CaMKII signaling pathway is involved in G\(\alpha_{13}\)-mediated SRE-dependent gene transcription, suggesting that radixin could be involved in novel signaling pathway regulated by G\(\alpha_{13}\) protein.

Disruption of the gene encoding the G\(\alpha_{13}\) subunit in mice impairs the ability of endothelial cells to develop into an organized vascular system and results in embryo lethality, which underlines the physiological importance of this G\(\alpha\) subunit (3). In cells, G\(\alpha_{13}\) plays a role in multiple cellular functions, such as stress fiber formation, cellular transformation, regulation of Na\(^{+}\)/H\(^{+}\) exchanger (NHE1), induction of mitogenesis and apoptosis, and regulation of the extracellular signal-regulated kinase and c-Jun N-terminal kinase pathways (4–9). Identifying the Rho guanine nucleotide exchange factor, p115RhoGEF, as an effector for G\(\alpha_{13}\) has contributed to the understanding of the same cellular events mediated by G\(\alpha_{13}\) (10, 11). For instance, activation of RhoA by G\(\alpha_{13}\) is responsible for actin stress fiber formation, SRE-dependent gene transcription, and NHE1 activity (4, 12, 13).

However, a number of signaling events regulated by G\(\alpha_{13}\) are Rho-independent. Thus, G\(\alpha_{13}\)-mediated activation of big mitogen-activated protein kinase (also known as extracellular signal-regulated kinase 5) is Ras- and Rho-independent (14). Similarly, G\(\alpha_{13}\)-mediated chloride conductance (15) and G\(\alpha_{13}\)-mediated activation of protein kinase A (16) are both RhoA-independent. In order to identify novel putative G\(\alpha_{13}\) effectors, our laboratory had used yeast two-hybrid screening and demonstrated that G\(\alpha_{13}\) subunit interacts with cytoskeleton-associated protein radixin.

Radixin belongs to the conserved ERM (ezrin, radixin, moesin) protein family. ERM proteins play a role in multiple cell functions, such as cell shape maintenance, formation of microvilli, cell-cell adhesion, cell migration, membrane trafficking, and cell polarity (see reviews in Refs. 17–20). ERM proteins consist of the high homology N-terminal FERM (band 4.1, ERM) domain and the C-terminal domain (21). Interaction between the N-terminal FERM domain and the C-terminal domain maintains a dormant inactive state of the ERM proteins. In an active open state, the N-terminal domain binds to the membrane proteins such as CD43, CD44, ICAM1–3, Na\(^{+}\)/H\(^{+}\) exchanger regulator (NHE3), the cystic fibrosis transmembrane conductance regulator, and the 2-adrenergic receptor, whereas C-terminal domain binds to F-actin. Therefore, ERM proteins were originally regarded as cross-linkers between cytoskeletal actin and the plasma membrane.

Recently, it was suggested that ERM proteins function as signaling molecules. Interaction of ezrin with the regulatory p85 subunit of phosphatidylinositol 3-kinase is required for phosphatidylinositol 3-kinase-dependent cell survival (19). ERM proteins have been shown to interact with and to be involved in Syk-mediated tyrosine kinase signaling (22). Importantly, ERM proteins are involved in the regulation of the Rho pathway. Binding of the guanine nucleotide dissociation inhibitor (RhoGDI) by the N-terminal domain of radixin promotes Rho activation in vitro (2). Interestingly, phosphorylation of ERM at the C terminus by Rho kinase maintains radixin in a relaxed active state (23), suggesting that the ERM proteins can act both upstream and downstream of the Rho signaling pathway. The fact that G\(\alpha_{13}\) interacts with radixin

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2 The abbreviations used are: SRE, serum response element; GDI, guanine nucleotide dissociation inhibitor; siRNA, small interference RNA; CaMK, Ca\(^{2+}\)/calmodulin-dependent kinase; GST, glutathione S-transferase; BAPTA/AM, 1,2-bis(2-aminophenoxy)ethane-N,N\(^{\prime}\),N\(^{\prime}\)-tetraacetic acid acetoxyethyl ester; GEF, guanine nucleotide exchange factor; PBD, p21 binding domain; RBD, Rho binding domain.
and induces the open active state of radixin, resulting in the increased binding for F-actin (1), leads us to propose that radixin may function as a signaling molecule for \(G_{13}\). To test this hypothesis, we examined the role of radixin on \(G_{13}\)-mediated SRE-dependent gene transcription.

Extracellular signals such as serum and lysophosphatidic acid cause Rho activation and subsequently actin dynamics change; thereafter, the decrease of free G-actin pool initiates the activation of SRE-dependent gene transcription (24, 25). Other members of the small Rho GTPase family, Rac and Cdc42, also stimulate the SRE gene transcription (24). In addition, the increase in intracellular \(Ca^{2+}\) leads to SRE-dependent gene transcription and is mediated by \(Ca^{2+}\)/calmodulin-dependent kinases, CaMKII and CaMKIV (26). Here, we report that radixin via its C-terminal domain mediated SRE-dependent gene transcription through activation of Rac1 and CaMKII. We have also shown that radixin-CaMKII signaling is involved in \(G_{13}\)-protein-mediated SRE-dependent gene transcription.

**EXPERIMENTAL PROCEDURES**

**Materials**—Full-length, N-terminal, and C-terminal domains of radixin have been described before (1). pGEX-2T containing rhotekin-Rho binding domain and pGEX4T3 containing p21-binding domain of PAK1 were provided by Dr. A. Schwartz and G. Bokoch (The Scripps Research Institute), respectively. The internal EE-tagged G13_Q226L, the constitutively activated RhoA and Rac, and the dominant negative RhoA, Rac1, and Cdc42 were purchased from the Guthrie Research Institute (Sayre, PA). The dominant negative CaMKII (K42M) and dominant negative CaMKIV (dCTK?SE) were gifts from Dr. M. Rosner (University of Chicago) and Dr. J. Xie (University of Manitoba), respectively. Genistein, piceatannol, FL-613, AP-2, AP-3, and spinocorine A were purchased from Calbiochem.

Monoclonal antibodies against RhoA, Rac1, Cdc42, polyclonal antibodies against total and phosphorylated CaMKII, and polyclonal antibody against radixin were from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Monoclonal antibody against EE epitope was from Covance (Berkeley, CA).

**Cell Culture and Transfection**—NIH 3T3 fibroblasts were grown in Dulbecco's modified Eagle's medium supplemented with 10% heat-inactivated calf serum, 100 units/ml streptomycin, and 100 units/ml penicillin. Lipofectamine 2000 reagent (Invitrogen) was used for transfection. Activated calf serum, 100 units/ml streptomycin, and 100 units/ml penicillin (Berkeley, CA). Monoclonal antibody against EE epitope was from Covance (Berkeley, CA).

**Radixin Down-regulation**—Expression of the endogenous radixin was down-regulated by siRNA using the BD™ Knock-out RNAi System (BD Biosciences). The target sequence is the 19 nucleotides corresponding to the coding region between nucleotides 188 and 207 of the mouse radixin. The siRNA sequence contains the target sense sequence, hairpin loop, and target antisense sequence; the same sequence without the target antisense sequence was designed as control siRNA. The primers were chemically synthesized by Sigma-Genosys (The Woodlands, TX). After annealing, they were subcloned into the vector pSIREN-RetroQ (BD Biosciences). The sequences were confirmed by DNA sequencing.

**Reporter Gene Assay**—SRE-mediated gene expression was determined by the SRE.L reporter system (Stratagene) as described previously (27). Briefly, NIH 3T3 cells at 90% confluence grown on 24-well plates were transfected with the following plasmids (per well): 50 ng of pSRE.L reporter, 50 ng of pCMV-galactosidase (LacZ) (control plasmid for transfection efficiency), and 50–200 ng of other plasmids indicated in each experiments as described under “Results.” The cells were serum-starved overnight before assays. Washed with phosphate-buffered saline buffer, the cells were lysed and assayed following the manufacturer's instruction for luciferase activity and \(\beta\)-galactosidase activity with the Promega assay kit (Promega, Madison, WI). Luciferase activity was normalized to the activity of \(\beta\)-galactosidase to correct the difference caused by different transfection efficiency.

**In Vivo Rho GTPase Activation Assay**—Activation of Rho proteins in vivo was determined by using pull-down assays as described by us earlier (28). These assays involve the use of glutathione S-transferase (GST) fusion proteins containing the GTP-dependent binding domains from effectors that bind the various Rho GTPases. Rhotekin interacts with GTP-bound RhoA but not with Rac1 or Cdc42. Conversely, the PAK serine/threonine kinase interacts with activated Rac1 and Cdc42 but not with RhoA. pGEX expression vectors encoding GST fusion proteins that contain the isolated GTP-dependent binding domains of the Rac1 and Cdc42 effector PAK1 (amino acids 70–132 of PAK1; PAK PBD) or the RhoA effector rhotekin (amino acids 7–89 of rhotekin; rhotekin RBD) were used for the bacterial expression of GST fusion proteins. Confluent NIH 3T3 cells (100-mm dishes) were transfected with the indicated cDNAs for 48 h. Cells were serum-starved 24 h prior the experiment. Cells were then quickly washed with ice-cold Tris-buffered saline and lysed in lysis buffer (50 mM Tris, pH 7.4, 1% Triton X-100, 0.5% sodium deoxycholate, 0.1% SDS, 500 mM NaCl, 10 mM MgCl2, 10 \(\mu\)g/ml each of aprotinin and leupeptin, and 1 mM phenylmethylsulfonyl fluoride). Cell lysates were clarified by centrifugation at 14,000 \(\times\) g at 4°C for 2 min, and equal volumes of cell lysates were incubated with GST-RBD rhotekin beads or GST–RBD PAK beads (15 \(\mu\)g) at 4°C for 1 h. The beads were washed three times with wash buffer (50 mM Tris, pH 7.4, 1% Triton X-100, 150 mM NaCl, 10 mM MgCl2, 10 \(\mu\)g/ml each of aprotinin and leupeptin, and 0.1 mM phenylmethylsulfonyl fluoride), and bound Rho, Rac1, or Cdc42 were eluted by boiling in Laemmli sample buffer. Samples eluted from the beads and the total cell lysates were then separated on 12.5% SDS–polyacrylamide gel electrophoresis, transferred to nitrocellulose, and analyzed by Western blotting using appropriate antibodies.

**Western Blotting for Detection of Phosphorylation of CaMKII**—Cells were washed with ice-cold phosphate-buffered saline, lysed in ice-cold radioimmune precipitation buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS, 10 \(\mu\)l/ml protease inhibitor mixture), and sonicated on ice. The insoluble material was removed from the lysates by centrifugation at 14,000 \(\times\) g for 10 min. Protein concentration of the total cell lysates was determined using a Bio-Rad DC protein assay kit (Bio-Rad). 50 \(\mu\)g of proteins were subjected to SDS-PAGE, transferred to a polyvinylidene difluoride membrane, and analyzed by immunoblotting with the appropriate antibodies.

**Statistical Analysis**—For statistical analysis, Student’s \(t\) test was used to compare data between two groups. Values are expressed as mean ± S.D. of three independent experiments. \(p < 0.05\) was considered statistically significant.

**RESULTS**

**Activation of SRE-dependent Gene Transcription by the C-terminal Domain of Radixin**—Rho proteins, including Rho, Rac, and Cdc42, are regulated by the guanine nucleotide dissociation inhibitors (GDI)s, which inhibit the dissociation of the nucleotide bound to these proteins. The dissociation of GDI from the protein is a prerequisite for membrane association and activation of these Rho proteins by guanine nucleotide exchange factors (29). Based on the evidence that \(G_{13}\) interacts with and activates radixin (1) and that RhoGDI interacts with N-terminal domain of radixin, thereby displacing RhoGDI from Rho proteins in \textit{in vitro} studies (2), it was reasonable to expect that domain(s) of radixin
Radixin Stimulates Serum Response Factor

FIGURE 1. Stimulation of SRE-dependent gene transcription by the C-terminal domain of radixin. A, effect of Gα13Q226L on SRE activity induced by N-terminal and C-terminal domains of radixin. NIH 3T3 cells were co-transfected with 50 ng of SRE-luciferase reporter, 50 ng of β-galactosidase, 50 ng of Gα13Q226L, 100 ng of N-terminal domain, and the C-terminal domain of radixin as indicated. Empty vector pcDNA3.1 was used to make the final DNA amount equal in all transfections. The cells were incubated in serum-free medium for 18 h and then lysed for luciferase and β-galactosidase assays. Luciferase activity was normalized to β-galactosidase activity and was expressed as fold stimulation from the basal level. Data represent mean ± S.D. from three experiments performed in triplicate. *, p < 0.05, significant difference. B, expression of Gα13Q226L is transfected cells. NIH 3T3 cells were transfected with Gα13Q226L with internal EE tag, Gα13Q226L-EE. The cell lysates were subjected to 10% SDS-PAGE. Expression of Gα13Q226L-EE was detected with monoclonal antibody against EE tag. The total levels of Gα13 were detected with polyclonal antibody against Gα13. C, dose-dependent stimulation of SRE transcription by C-terminal domain of radixin. NIH 3T3 cells were co-transfected with 50 ng of SRE-luciferase reporter, 50 ng of pcMV-β-gal, and the indicated amounts of the C-terminal domain of radixin in the absence or presence of 50 ng of Gα13Q226L. The luciferase activity was determined as described above. Data represent mean ± S.D. from one of at least three experiments performed in triplicate.

...may regulate Gα13-mediated activation of RhoA and subsequent cellular signaling.

We tested how expression of the N-terminal (amino acids 1–318), or C-terminal (amino acids 319–583) domains of radixin affected the Gα13-mediated SRE-dependent gene transcription. As shown in Fig. 1A, RhoGDI inhibited SRE activation induced by Gα13Q226L expression, supporting the notion that Rho proteins mediate SRE activation induced by Gα13Q226L. The N-terminal domain of radixin did not exhibit any basal activity compared with the empty vector. However, the N-terminal domain of radixin potentiated SRE activation induced by Gα13Q226L. RhoGDI inhibited SRE activation induced by the combination of Gα13Q226L and the N-terminal domain of radixin (Fig. 1A).

Interestingly, the C-terminal domain of radixin itself induced a 2-fold increase of the SRE-dependent gene transcription that was inhibited by RhoGDI (Fig. 1A). Surprisingly, when co-transfected with Gα13Q226L, the C-terminal domain radixin synergistically stimulated the SRE activation (Fig. 1A). This activation was also inhibited by RhoGDI, suggesting that Rho proteins are involved in this signaling pathway.

To rule out the possibility that observed synergistic stimulation of SRE was due to change in expression of transfected proteins, we analyzed whether the expression of Gα13Q226L with internal EE epitope was affected by the C-terminal domain of radixin. Detection of Gα13Q226L-EE with EE antibody showed that the C-terminal domain of radixin did not change the expression of Gα13Q226L-EE (Fig. 1B), suggesting that observed stimulation of SRE activity was not due to increased expression of Gα13Q226L-EE showed the similar to Gα13Q226L stimulation of SRE-dependent gene transcription (data not shown). Reprobing of the same blots with Gα13 antibody did not reveal a detectable difference of Gα13 expression in cells transfected with Gα13Q226L-EE or vector alone, suggesting that overexpression of the transfected construct was minimal.

To further analyze the SRE-dependent gene transcription regulated by the C-terminal domain of radixin, we evaluated how different amounts of C-terminal domain of radixin cDNA affected Gα13Q226L-dependent SRE activation (Fig. 1C). Data showed that the C-terminal domain of radixin stimulated SRE-dependent gene transcription in a dose-dependent manner both alone and in the presence of Gα13Q226L (Fig. 1C).

Involvement of Rac1 in SRE Activation Induced by the C-terminal Domain of Radixin—To evaluate the effect of radixin on activation of individual Rho GTPases, we used the Rho-binding domain of the RhoA effector, rhoetin, to affinity-precipitate active RhoA, as a direct readout for RhoA activation. We have also used the Rac1- and Cdc42-binding domains of Rac1 and Cdc42 effector PAK to affinity-precipitate active Rac1 and Cdc42 as a direct readout for Rac1 and Cdc42 activation. NIH 3T3 cells were transfected with either the C-terminal domain of radixin or Gα13Q226L. Data showed that Gα13Q226L induced a 5–6-fold increase in RhoA activity (Fig. 2A). Importantly, Gα13Q226L did not activate Rac1 or Cdc42 (Fig. 2A). The C-terminal domain of radixin expressed alone or in the presence of Gα13Q226L did not affect RhoA activity (Fig. 2A; data not shown). In addition, wild-type and N-terminal domain of radixin did not affect the activity of Rho proteins (data not shown).

By contrast, the C-terminal domain of radixin activated Rac1 but not RhoA and Cdc42 (Fig. 2A). The C-terminal domain of radixin expressed in the presence of Gα13Q226L did not further enhanced Rac1 activity (data not shown). Equal protein expression of the C-terminal domain of radixin or Gα13Q226L was controlled by Western blotting (data not shown), which confirmed that the different effects of these proteins on activation of Rho GTPases was not due to difference in the amount of expressed protein. These data provided the direct evidence that in mammalian cells, Gα13Q226L stimulated RhoA, whereas the C-terminal domain of radixin stimulated Rac1 proteins.

To further support the observation that the C-terminal domain of radixin stimulated Rac1 protein, we have used dominant negative mutants of these GTPases. Data showed that dominant negative mutants of Rac1 (T17N) but not RhoA (T19N) or Cdc42 (T17N) inhibited SRE activation induced by the C-terminal domain of radixin (Fig. 2B). Thus, this result further corroborated the finding that C-terminal
The presented data are the mean ± S.D. from three independent experiments. *, p < 0.05, significant difference.

domain of radixin regulated activity of Rac1 but not RhoA or Cdc42 proteins.

In addition, the data showed that both C3 toxin and dominant negative Rac1(T17N) inhibited Gα13-induced SRE activation (Fig. 2C). The different sensitivities of reporter assay and pull-down assay may have contributed to the differences in the data.

Involvement of CaMKII in SRE Activation Induced by C-terminal Domain of Radixin and Gα13Q226L—Partial inhibition by dominant negative Rac1 of the SRE activation induced by the C-terminal domain of radixin suggested that an additional pathway involved in SRE activation might exist. Since some tyrosine kinases and two isoforms of Ca2+/calmodulin-dependent kinase, CaMKII and CaMKIV, have been shown to stimulate SRE gene transcription (26, 30–35), we determined how inhibitors of tyrosine kinases and inhibitors of CaMK affect the SRE activation induced by the C-terminal domain of radixin. At concentrations chosen for the activity against the respective target enzymes, general tyrosine kinase inhibitor genistein (50 μM) and selective Syk tyrosine kinase inhibitor piceataneriod (10 μM) did not affect the transcriptional activity (data not shown), suggesting that tyrosine kinases are probably not involved in the SRE activation induced by the C-terminal domain of radixin.

To test the involvement of the Ca2+/calmodulin-dependent kinase pathway, we examined how intracellular calcium chelator (BAPTA/AM), calmodulin kinase inhibitor (KN-93), and calcineurin inhibitor (cyclosporine) affect SRE activation induced by the C-terminal domain of radixin. Our results showed that BAPTA/AM and KN-93 inhibited SRE activation (Fig. 3A), suggesting that the Ca2+/calmodulin-dependent kinase pathway may be involved in the regulation of SRE gene transcription induced by the C-terminal domain of radixin.

Because both CaMKII and CaMKIV stimulate SRE gene transcription and KN-93 inhibits the activity of both kinases, next we examined whether the dominant negative mutants of CaMKII and CaMKIV can affect the SRE activation induced by the C-terminal domain of radixin. As shown in Fig. 3B, the dominant negative mutant of CaMKII but not of CaMKIV inhibited the SRE activation induced by the C-terminal domain of radixin.

Because dominant negative Rac1 induced partial inhibition of SRE activity (Fig. 2B), we tested whether simultaneous inhibition of Rac1 and CaMKII inputs will further decrease the SRE activity induced by the C-terminal domain of radixin. Data showed that both dominant negative Rac1 and kinase-dead CaMKII partially inhibited SRE activity induced by C-terminal domain of radixin (Fig. 3C). Simultaneous inhibition of Rac1 and CaMKII by dominant negative constructs further inhibited SRE activity (Fig. 3C), suggesting that both inputs contributed to the SRE activity induced by the C-terminal domain of radixin.

We tested the involvement of the Ca2+/calmodulin-dependent kinase pathway in Gα13-mediated signaling. Our results showed that BAPTA/AM and KN-93 inhibited SRE activation (Fig. 4A), suggesting that the Ca2+/calmodulin dependent kinase pathway may be involved in the regulation of SRE gene transcription induced by the Gα13 protein. Interestingly, dominant negative mutants of CaMKII and CaMKIV inhibited the SRE activation induced by Gα13Q226L (Fig. 4A), suggesting that both CaMKII and CaMKIV are involved in Gα13-mediated SRE-dependent gene transcription.

The phosphorylation state of CaMKII was analyzed using specific antibody targeted to the autophosphorylation site of CaMKII at residue of Thr286. We observed significant increase of the endogenous CaMKII phosphorylation (Fig. 4, B and C) in cells transfected with the C-terminal domain of radixin, whereas total CaMKII expression remained constant. Wild-type radixin or its N-terminal domain did not induce CaMKII phosphorylation (data not shown). Importantly, Gα13Q226L also induced CaMKII phosphorylation (Fig. 4, B and C). Co-transfection of C-terminal domain of radixin together with Gα13Q226L resulted in a further increase of CaMKII phosphorylation. These data provided evidence that both the C-terminal domain of radixin and Gα13Q226L stimulated CaMKII.

Radixin Mutant Mimicking Phosphorylated State Activates Rac1 and Induces CaMKII Phosphorylation and SRE-dependent Gene Transcription—Activation of radixin requires release of the intramolecular association, and this is believed to involve phosphorylation of threonine 564 (17). To determine whether the C-terminal radixin accurately reflects an activated state of radixin, we made the mutant T564E radixin, which mimics the phosphorylated state of radixin (17). Data showed that radixin (T564E) induced activation of Rac1 (Fig. 5A), phosphorylation of CaMKII (Fig. 5B), and SRE activation (Fig. 5C). Together, these
results suggested that the C-terminal domain of radixin can reflect the activated state of radixin.

Involvement of Radixin in Gα13-mediated SRE Gene Transcription—To test the involvement of radixin in Gα13-mediated signaling, we used siRNA technology to reduce the expression of endogenous radixin in NIH 3T3 cells. Cells were transfected with constructs containing either radixin or control siRNA, and 24 h later cell lysates were analyzed using Western blotting with antibodies against radixin, ezrin, or moesin. Densitometry analysis showed that expression of the endogenous radixin was down-regulated by ~50% upon transfection of the NIH 3T3 cells with vector containing radixin siRNA (Fig. 6A). The transfection of the control siRNA

FIGURE 3. C-terminal domain of radixin stimulates SRE via CaMKII. A, effects of pharmacological inhibitors on SRE activation induced by C-terminal domain of radixin. NIH 3T3 cells were co-transfected with 50 ng of SRE.L luciferase reporter, 50 ng of pCMV-βgal, and 100 ng of the C-terminal domain of radixin. The cells were incubated with either vehicle or BAPTA/AM (50 μM), KN-93 (1 μM), and cyclosporin (400 nM) in serum-free medium for 18 h. The luciferase activity was determined as described in the legend to Fig. 1. *, p < 0.05, significant difference. B, dominant negative CaMKII inhibited radixin-dependent SRE activation. NIH 3T3 cells seeded onto 24-well plates were transfected with 50 ng of pSRE.L, 50 ng of pCMV-βgal, and 100 ng of the C-terminal domain of radixin, and 200 ng of dominant negative CaMKII as indicated. The luciferase activity was determined as described in the legend to Fig. 1. The presented data are the mean ± S.D. from three independent experiments. *, p < 0.05, significant difference.

C, effect of dominant negative forms of Rac1 and CaMKII on radixin-dependent SRE activation. NIH 3T3 cells were co-transfected with 50 ng of SRE.L luciferase reporter, 50 ng of pCMV-βgal, 100 ng of the C-terminal domain of radixin, and 200 ng of dominant negative Rac1 or CaMKII as indicated. The luciferase activity was determined as described in the legend to Fig. 1. The presented data are the mean ± S.D. from three independent experiments. *, p < 0.05, significant difference compared with the cells transfected with the C-terminal domain of radixin and either dominant Rac1 or dominant CaMKII.
vector did not affect the expression of the endogenous radixin. However, expression of endogenous ezrin or moesin was not affected by either control or radixin siRNAs (Fig. 6A), suggesting that siRNA-induced down-regulation of radixin was specific.

Next, we tested whether down-regulation of the endogenous radixin can affect Gα13-mediated activation of SRE. Data showed that in the cells expressing vector containing radixin siRNA, SRE activity induced by Gα13-Q226L was reduced by ~50% (Fig. 6B). In the cells expressing control siRNA, Gα13-induced SRE activation was not affected. Importantly, SRE activity induced by a downstream effector of Gα13, p115RhoGEF, was not affected by down-regulation of radixin (Fig. 6B), indicating that the reduction of Gα13-Q226L-mediated SRE activation by radixin siRNA was specific. Furthermore, radixin siRNA but not control siRNA also reduced the phosphorylation of CaMKII by Gα13Q226L (Fig. 6C). Taken together, these data suggested that radixin-CaMKII signaling is involved in the Gα13-mediated SRE gene transcription.

DISCUSSION

In the present study, we have demonstrated for the first time that radixin can activate Rac1 and induce phosphorylation of CaMKII. Activation of these proteins resulted in the stimulation of SRE-dependent gene transcription. We have also demonstrated that Gα13 can induce CaMKII phosphorylation. In addition, reducing endogenous radixin with siRNA demonstrated that the radixin-CaMKII signaling pathway is involved in Gα13-mediated SRE-dependent gene transcription. The partial inhibition by dominant negative Rac1(T17N) suggests the possibility that the radixin-Rac1 signaling pathway is also involved in Gα13-mediated SRE-dependent gene transcription. Taken together, our studies suggest that radixin could be involved in a novel signaling pathway...
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FIGURE 7. \(\alpha\)-Radixin stimulates serum response factor (SRE) activation by Rac1. The C-terminal domain of radixin activates Rac1 and CaMKII. G\(\alpha_{13}\) may contribute to CaMKII activation in a radixin-independent manner.

Activation of Rac1 by C-terminal Domain of Radixin—In contrast to the G\(\alpha_{13}\) protein, which activates CaMKII in a radixin-independent manner (Fig. 7).

Activation of Rac1 by C-terminal Domain of Radixin—In contrast to the G\(\alpha_{13}\) protein, which activates CaMKII in a radixin-independent manner (Fig. 7).

Regulated by G\(\alpha_{13}\) protein. However, we cannot exclude the possibility that G\(\alpha_{13}\) also activates CaMKII in a radixin-independent manner (Fig. 7).

Activation of Rac1 by C-terminal Domain of Radixin—In contrast to the G\(\alpha_{13}\) protein, which activates CaMKII in a radixin-independent manner (Fig. 7).

The dominant negative mutant of CaMKII but not CaMKIV inhibited the SRE activity induced by the C-terminal domain of radixin (Fig. 3). Using the specific antibody against the autophosphorylation site at Thr286 of CaMKII, we determined that both the C-terminal domain of radixin and G\(\alpha_{13}\)Q226L increased the autophosphorylation of CaMKII (Fig. 4). These results provided strong evidence indicating the involvement of CaMKII in SRE activity induced by the C-terminal domain of radixin.

Both CaMKII and CaMKIV have been shown to stimulate SRE activity (26). The mechanism for CaMKIV-mediated SRE-dependent gene transcription has been defined (35). Thus, CaMKIV phosphorylates transcriptional repressor histone deacetylase and dissociates it from serum response factor, removing this repression molecule from the transcription complex. Recently, CaMKII was shown to use the similar mechanism to stimulate the myocyte enhancer factor-2 transcription factor in neuron cells (41). It is conceivable that CaMK II might use a similar mechanism to activate SRE-dependent gene transcription.

The C terminus of radixin activates Rac1, but not RhoA or Cdc42 (Fig. 2). Because Rac1 can also stimulate SRE-dependent gene transcription, these data suggested that Rac1 might mediate the stimulation of SRE by the C-terminal domain of radixin. Inhibition of C-terminal domain-induced SRE activation by Rac1 (T17N) but not by RhoA (T19N) or Cdc42 (T17N) confirmed that the C-terminal domain of radixin stimulated SRE via Rac1 (Fig. 2B). Together, these data indicated that Rac1 is at least in part responsible for the stimulation of gene transcription by the C-terminal domain of radixin.

Activation of Rac1 by the C-terminal domain of radixin was consistent with the other studies. Overexpression of ezrin T567D, in which an aspartic acid mimics the constitutive threonine phosphorylation of the C terminus, resulted in increased membrane ruffling and lamellipodia (36). In addition, it was recently reported that ezrin T567D induced Rac1 activation in Madin-Darby canine kidney cells (37). Therefore, it seems that activated ERM proteins in an open relaxed state may activate Rac1. Our results indicated that the C-terminal domain of radixin mediates Rac1 activation.

Furthermore, ezrin was shown to be cleaved by calpain following the stimulation with phorbol 12-myristate 13-acetate (38). The physiological significance of this proteolytic cleavage is currently unclear, although it might be a mechanism of releasing N- and C-terminal domains.

How the C terminus of radixin activates Rac1 remains unknown. It is possible that the C-terminal domain of radixin may regulate Rac-specific guanine nucleotide exchange factors (GEFs). One candidate is Tiam, a specific GEF for Rac. Interestingly, CaMKII was shown to activate Tiam (39, 40). Since the C-terminal domain of radixin also induced the autophosphorylation of CaMKII (Fig. 6), it is possible that CaMKII and Tiam are involved in the activation of Rac1. This hypothesis is currently being tested in the laboratory.

Using the specific antibody against the autophosphorylation site at Thr286 of CaMKII, we determined that both the C-terminal domain of radixin and G\(\alpha_{13}\)Q226L increased the autophosphorylation of CaMKII (Fig. 4). These results provided strong evidence indicating the involvement of CaMKII in SRE activity induced by the C-terminal domain of radixin.

Both CaMKII and CaMKIV have been shown to stimulate SRE activity (26). The mechanism for CaMKIV-mediated SRE-dependent gene transcription is currently being tested in the laboratory. 

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Activation of CaMKII by C-terminal Domain of Radixin—Because dominant negative Rac1 induced partial inhibition of SRE activity (Fig. 2B), we tested whether other signaling pathways are involved in the regulation of SRE activity induced by the C-terminal domain of radixin. We determined that intracellular calcium chelator BAPTA/AM and CaMK inhibitor KN-93 inhibited SRE activation induced by the C-terminal domain of radixin (Fig. 3). Furthermore, we demonstrated that
activation by G13Q226L could be transient, and this would be more difficult to detect by the pull-down assay. By contrast, interference with the function of endogenous Rac1 by the dominant negative mutant could be relatively stable after the mutation is expressed. (vi) While this paper was in revision, a study was published describing that G13-mediated reactive oxygen species production by Rac activation (45). Direct activation of Rac1 by G13 was not shown in this paper. However, the authors determined that an inhibitor of G12/13 activates reactive oxygen species production by Rac activation (45). Direct activation of Rac1 by G13, as mediated by the Rac activator domain, may activate Rac1 when transiently transfected in NIH 3T3 cells. If this were the case, G13 would not use radixin in the signaling. Instead, G12/13 activates the downstream SRE gene transcription. Our data do not exclude this possibility. Taken together, it should be pointed out that direct signaling from G13 to radixin is likely, but further evidence is needed (Fig. 7), and further studies are also needed to distinguish among the above possibilities.

In summary, our studies indicate that radixin stimulates SRE-dependent gene transcription. This activity requires activation of Rac1 and CaMKII by radixin via its C-terminal domain. Our results also suggest that the radixin-CaMKII signaling is involved in G13-mediated SRE-dependent gene transcription.

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