Activation of JNK by EPAC is independent of its activity as a Rap Guanine Nucleotide Exchanger

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ABSTRACT

Guanine Nucleotide Exchange Factors (GEFs) and their associated GTP binding proteins (G-proteins) are key regulatory elements in the signal transduction machinery that relays information from the extracellular environment into specific intracellular responses. Among them, the MAPK cascades represent ubiquitous downstream effector pathways. We have previously described that, analogous to the Ras-dependent activation of the Erk-1/2 pathway, members of the Rho family of small G-proteins activate the JNK cascade when GTP is loaded by their corresponding GEFs. Searching for novel regulators of JNK activity we have identified EPAC as a strong activator of JNK-1. EPAC (Exchange Protein Activated by cAMP) is a member of a growing family of GEFs that specifically display exchange activity on the Rap subfamily, of Ras small G-proteins. We report here that while EPAC activates the JNK several fold, a constitutively active (G12V) mutant of Rap1b does not, suggesting that Rap-GTP is not sufficient to transduce EPAC-dependent JNK activation. Moreover, EPAC signaling to the JNKs was not blocked by inactivation of endogenous Rap suggesting that Rap activation is not necessary for this response. Consistent with these observations, domain deletion mutant analysis shows that the catalytic GEF domain is dispensable for EPAC-mediated activation of JNK. These studies identified a region overlapping the REM domain as critical for JNK activation. Consistent with this, an isolated REM domain from EPAC is sufficient to activate JNK. We conclude that EPAC signals to the JNK cascade through a new mechanism that does not involve its canonical catalytic action, i.e. Rap-specific GDP/GTP exchange. This represents not only a novel way to activate the JNKs but also a yet undescribed mechanism of downstream signaling by EPAC.
INTRODUCTION

Epac (exchange protein activated by cAMP) is a newly discovered guanine nucleotide exchange factor (GEF) \(^1\) that selectively activates members of the Rap family of G-proteins (1-3). Rap proteins, like all G-proteins, are biochemical transducers (4,5) which function as allosteric regulatory elements, switching between an inactive GDP-bound and an active GTP-bound conformation (4). The switch mechanism consists of activation by exchange of bound GDP for GTP, and inactivation by hydrolysis of GTP into GDP, catalyzed by GEFs and GTPase-activating proteins (GAPs), respectively. Similar to all GEFs, Epac acts catalytically on the rate-limiting step for G protein activation, i.e. dissociation of bound GDP (4).

The involvement of Rap1 in signaling mechanisms is demonstrated by the variety of second messengers mediating its activation. Recently, a number of exchange factors were identified as mediators of these specific activities (6). Among them, Epac was characterized as a molecule responsible for cAMP-dependent Rap1 activation (1,2). Epac is a multidomain protein comprised of a C-terminal catalytic and an N-terminal regulatory module. The C-terminal module encompasses the Rap-specific GEF catalytic core and the Ras exchange motif (REM) (1). According to the structure solved for SOS GEF, the REM domain does not provide any catalytic residues but rather participates in GEF stability (7). The N-terminal regulatory module contains a DEP domain whose function seems to be related to membrane/cytoskeleton association, and a cAMP-binding domain (CBD) homologous to the CBD originally identified in the regulatory subunit of PKA (1). Although cAMP binding and GEF activity are energetically uncoupled processes (8), deletion of the N-terminal regulatory module renders a constitutively active (cAMP-independent) GEF, which is suggestive of an auto-inhibitory mechanism that is relieved upon cAMP binding (1).

The mitogen-activated protein kinases (MAPKs) represent an example of fundamental biochemical processes regulated by G-proteins (9). Three major MAP kinase cascades have been described so far (10): The Ras-dependent MAPKs as ERK-1/2 are typically associated with cell proliferation (11,12). The stress activated proteins kinases (SAPKs) as the c-Jun kinase or JNK and the p38 kinases are triggered in response to environmental stresses (13,14) although they have also been linked to
proliferative conditions depending on the cellular setting (9,12). The discovery of additional MAPKs as ERK-5 and others add diversity to the MAPK scenario (15).

The signaling cascades that end in MAPKs and regulate their activity include several sequential events of phosphorylation in the cytoplasm that define phosphorylation cascades. MAPKs are activated by dual phosphorylation at two residues, a tyrosine and a threonine by kinases known as MAPKKs (16). Particularly JNK is activated by the prototypical JNKKs, M KK4 (SEK1) and M KK7 (SEK2), which are in turn activated by upstream phosphorylation events. A large group of JNKKKs has been reported (13). However, the mechanisms involved in the activation of these JNKKKs, and the link between a specific stress signal and its mediators are still ill defined. The module SOS/Ras/Raf/MEK/Erk1-2 constitutes a typical example of a MAPK signaling pathway (10). In recent years we have contributed to the development of the dominant concept that probably all of the MAPKs are activated in an analogous fashion when we discovered that Dbl, a GEF specific for the Rho family of small G-proteins can activate the JNK pathway (17) through the Rho family members Rac and Cdc42 and a series of kinases that include MLK3, MEKK and SEK (17-20).

It is well now established that the Rho-like small G-proteins Rac and Cdc42 represent a link between environmental stimuli and JNK activation (21). These G-proteins as well as their corresponding GEFs are also regulators of the actin cytoskeleton dynamics (22). Expression of the small G-protein Rap1b in Swiss3T3 fibroblasts induces an anchorage-dependent transformed phenotype (23), accompanied by changes in focal contacts and actin cytoskeleton (unpubl. observ.). These results prompted us to address whether Rap G-proteins, like Rac and Rho, might participate in signaling to the JNKs. Unexpectedly, we have found that Epac effectively signals to the JNK pathway while its cognate G-protein Rap1b does not. Our studies identified a new function for the Rap-selective guanine nucleotide exchange factor Epac: JNK activation, which is independent of Rap1 activation and unrelated to its GEF activity. Moreover, this signal is specific for JNK since other SAPKs like p38 MAPK are not activated. We can circumscribe downstream signaling to JNK to a region in EPAC devoid of exchange activity on Rap, the REM domain, which is sufficient to activate JNK. This is to our knowledge not only the first report of a transduction mechanism
that connects this GEF to the MAPKs acting independently of its G-protein counterpart but also describes a novel, Rap-independent, EPAC function.
EXPERIMENTAL PROCEDURES

Cell Lines and Transfections: HEK 293T cells, were maintained in Dulbecco’s modified Eagle’s medium (DMEM, Life Technologies) supplemented with 10% fetal bovine serum. Transfections were performed by the calcium phosphate precipitation technique, adjusting the total amount of plasmid DNA to 3-6 µg/plate with empty vector. Additional transfections were performed using FuGene trasfection reagent (Roche-Boehringer), adjusting the total amount of DNA plasmid to 0.5-1 µg/plate as directed by the manufacturer.

Kinase Assays: 48hs after transfection, cells were starved with serum free media for 2 hours, stimulated if necessary, washed with cold phosphate-buffered saline, and lysed at 4°C in a buffer containing 25mM HEPES, pH 7.5; 0.3M NaCl; 1.5 mM MgCl₂; 0.2mM EDTA; 0.5mM DTT; 1% Triton X-100; 0.1% SDS; 20mM β-glycerophosphate; 1mM sodium vanadate and 1mM phenylmethylsulfonylfluoride (PMSF). AU5-JNK was immunoprecipitated from the cleared lysates by incubation with the specific antibody against AU5 (MMS-135R, Covance) for 1.5 hours at 4°C. Immunocomplexes were recovered with the aid of Gamma-Bind sepharose beads (Santa Cruz) and washed one time with PBS containing 1% NP-40 and 2mM sodium vanadate, once with 100mM TRIS, pH 7.5; 0.5M LiCl, and three times in kinase reaction buffer (12.5mM MOPS, pH 7.5; 12.5mM β-glycerophosphate; 7.5mM MgCl₂; 0.5mM EGTA; 0.5mM sodium fluoride and 0.5mM sodium vanadate). The JNK activity present in the immunoprecipitates was determined by resuspension in 30 µl of kinase-reaction buffer containing 10 µCi [γ³²P] ATP per reaction and 20 µM of unlabeled ATP, using 1 µg of purified bacterially expressed, GST-ATF2 protein as substrate. After 30 min at 30°C, reactions were terminated by addition of 10 µl 5X Laemmli buffer. Samples were heated at 95°C for 5 min and analyzed by SDS-gel electrophoresis on 12% acrylamide gels. Autoradiography was performed with the aid of an intensifying screen. Parallel immunoprecipitates were processed for western-blot analysis using the same antiserum as described (17). To assay p38 activity, cells were transfected with a plasmid that expresses HA-p38 which was immunoprecipitated from the cell lysates with a specific
antibody against the HA epitope (MMS-101R, Covance) and processed as described above for JNK (ref. Teramoto et al.)

**DNA constructs:** pCEFLAU5-JNK was constructed transferring the JNK cDNA insert from pCDNA3HA-JNK (ref.) as a BglII-NotI fragment to the corresponding expression vector. Plasmids pCDNAonc-Dbl, pCDNA3Rac1QL and pCEFL HA-p38 have already been described (ref.). pCMVmyc-EPAC and pCMVmyc-[EPAC were generated transferring SalI-NotI fragments from pMT2SMHA-EPAC (ref.); pCMVmyc-N-EPAC was cloned using a SalI-BglII fragment, which contains the DEP, CBD and REM domains (1-1270 nucleotides). pCGNHA-Rap1bN17, pCGNHA-Rap1bG12V and pMT2SMHA-Rap1GAP have already been described (24) (25). The EPAC REM and DEP domains were isolated by PCR amplification using oligonucleotides 5’acGGATCCacagtgatgtctggcacc3’ and 5’tgGAATTCtcactgctcgctgccacccgc3’ for REM and 5’acGGATCCatgacccgagaccggaagtacc3’ and 5’gaGAATTCgtagaattgggcatctcg3’ for DEP respectively. The PCR products were digested with BamHI and EcoRI enzymes and ligated in the corresponding sites in a pCEFL-GST vector. pCEFL-GSTREM expresses aminoacids 345 to 410 from EPAC as a GST fusion protein. The same is true for aminoacids 74 to 140 from EPAC in pCEFL-GSTDEP.

**Western Blots:** Lysates taken from transfected cells, containing similar amounts of protein were analyzed for protein expression levels by PAGE-SDS followed by Western blotting, with the corresponding antibodies. Immunocomplexes were visualized by enhanced chemiluminescence detection using Horse Radish Peroxidase conjugated secondary antibodies (Santa Cruz) and Luminol as a substrate (Sigma) as described (17). Mouse monoclonal antibodies anti-HA and anti-AU5 epitopes were purchased from Covance.

**Rap activation assay using RalGDS-RBD:** Cells transfected with a plasmid expressing HA-Rap1b were lysed with a buffer containing 200mM NaCl, 50mM Tris-HCl pH 7.5, 1% NP-40, 10% glycerol, 2mM MgCl₂, 1mM phenylmethylsulfonyl fluoride (PMSF), 2μM leupeptin and 2μM aprotinin. Lysis was performed at 4°C for
10-30 min. Lysates were clarified by centrifugation at maximal speed in an Eppendorf centrifuge for 10 min at 4°C. 10µg of bacterially expressedRalGDS-RBD (26) coupled to Glutathione-sepharose beads (Amersham Pharmacia Biotech) were added to the supernatants and incubated at 4°C for 60 min with slight agitation. Beads were washed four times in the same lysis buffer. After the final wash, Laemmli sample buffer was added to the samples. Proteins were fractionated in a 15% SDS-PAGE and transferred to a nitrocellulose membrane. HA-Rap1-GTP was visualized by western blotting with an anti-HA antibody as previously described (17).
RESULTS

**EPAC is a JNK activator:**

Expression of the Rap1-specific GEF Epac into HEK293T cells triggered a strong JNK response, about a six-fold increase in its kinase activity. This Epac-mediated effect was similar in magnitude to the one produced by canonical JNK activators as Onc-Db1 and the protein synthesis inhibitor anisomycin (fig.1, upper panel and bars). The lower panel of fig. 1 shows that co-expression of the GEFs did not alter the JNK protein levels which is indicative of changes in specific JNK activity rather than changes in the amount of enzyme present. As reported previously for MAPK activation (27), parallel experiments showed that a GFP-JNK fusion protein translocates to the nucleus upon cotransfection with plasmids that express Epac or Onc-Db1 (data not shown).

**Opposite to Epac, a constitutively active mutant of Rap1b fails to stimulate JNK activity**

We have previously found that expression of constitutively active forms of small G-proteins of the Rho subfamily, were highly effective in triggering JNK activation to levels comparable as its corresponding GEFs, Ost and Db1 (17). Epac is a Rap-specific GEF that turns out to be a significant JNK activator (fig 1). If its effects on JNK activity are conveyed through Rap activation, we reasoned that expression of a constitutively active Rap form should fully mimic Epac’s action. We found that while Rac1QL and Epac display a five-fold induction in JNK activation, (fig 2A), expression of a GTPase deficient form of Rap1b (RapG12V) did not produce any changes in JNK activity, despite high levels of expression as measured in western blots (fig 2A and 2B). The HA-RapG12V construct is expressed on its active form when transfected into HEK293T cells as shown by its ability to bind to the Rap binding domain of RalGDS, which binds Rap1 on its active GTP-bound conformation (not shown). These results demonstrate that in contrast to Epac, Rap1b-G12V does not activate JNK, suggesting that the enzymatic activity described for Epac (GTP loading unto Rap) is not sufficient to transduce Epac’s effects on JNK activity.
Rap activity is not necessary for EPAC-mediated JNK activation.

Results from the two preceding sections suggest two potential alternatives for Epac signaling. One of them would be mediated by Rap activation, as has been previously demonstrated, while the other one would be Rap-independent. The first scenario should be mimicked by expression of RapG12V; meanwhile the second one should not be altered by the presence of Rap inhibitory molecules. We observed no JNK activation by RapG12V; therefore in order to assess the second possibility, endogenous Rap1 was inactivated and the effects of Epac on JNK were tested. Rap1 inactivation was attained by expression of two inhibitory molecules: dominant negative RapN17 and RapGAP which inactivate Rap by different mechanisms.

As shown in Fig.3A, we found that RapGAP that inactivates active Rap-GTP by driving the equilibrium towards its inactive Rap-GDP form (25), did not modify Epac-mediated JNK activity despite these protein being expressed at high levels (fig.3B) and able to inhibit Epac-mediated Rap1 activation (see below). Like the so-called dominant negative RasN17 mutant (28), RapN17 inhibits activation of endogenous Rap by binding and sequestering GEFs, upstream activators of Rap1. As expected, expression of RapN17 partially inhibited Epac activation of JNK activity (fig.3A). This seemingly contradictory result could be explained assuming that RapN17 is actually an inhibitor of the upstream GEF regulator Epac, and not of the small G-protein itself, acting presumably through titration of “free” Epac molecules, thereby preventing Epac actions including JNK activation.

Since RapG12V does not mimic Epac’s action on JNK, and expression of a downstream negative regulator of Rap1 (RapGAP), has no effect on Epac-stimulated JNK activity, we conclude that Rap1 activity should not be necessary for Epac-mediated JNK activation.

Stimulation of JNK by EPAC is independent of its cAMP-dependent GEF activity.

The general organization of Epac is schematized in Fig.4A. The C-terminal domain contains the catalytic exchange core (GEF), responsible for the GDP/GTP exchange on Rap. The N-terminal regulatory domain negatively modulates this catalytic
activity. Binding of cAMP to the CBD site in this N-terminal domain relieves the negative constrain, leading to GEF-dependent Rap activation (1). Accordingly, deletion of the N-terminal domain, including the CBD, renders a constitutively active GEF mutant (Δ-Epac), which maximally activates Rap1 even in the absence of agonist stimulation (cAMP) (1). We reasoned that if Rap exchange activity is involved in JNK signaling, maximum JNK activation should be observed when Δ-EPAC dose-responses are compared to full length EPAC’s, working in the absence of cAMP stimulation. In other words, when measuring JNK activity the dose response curve to Epac should present a higher EC50 relative to that of ΔEpac. In addition, cAMP-signaling agents should potentiate Epac’ but not ΔEpac’ dose response curves.

As shown in fig.4B, Epac and ΔEpac stimulation of JNK activity showed identical profiles in the absence of cAMP stimulation. A 15 min. treatment with forskolin, a cAMP-elevating agent did not increase JNK activity induced by either Epac versions (not shown). We verified that ΔEpac is functional as a Rap1GEF, as shown by GST-RalGDS-RBD pull-down assays (fig.4C). Moreover, co-expression of RapGAP reduced the amount of pulled HA-Rap to values below basal, thus confirming that the observed ΔEpac-induced increase in HA-Rap represents active GTP-loaded Rap. In addition, RapGAP co-expression did not affect JNK activation by ΔEpac (data not shown). We conclude that Epac stimulation of JNK is independent of cAMP-mediated Epac exchange activity on Rap1b.

*Epac-mediated JNK activation is dissociated from Epac’s GEF activity*

Although the results described above strongly suggest that Epac’s nucleotide exchange on Rap1 is not involved in Epac-mediated JNK activation, we directly tested this premise experimentally. We asked whether the presence of the GEF domain was necessary for EPAC-mediated JNK activation. A construct containing the N-terminal regulatory domain (N-EPAC) but devoid of the catalytic GEF domain was sufficient to activate JNK at levels comparable to both EPAC and Δ-EPAC (fig.5A, compare to fig. 3A and 4B). As expected, ΔEpac stimulated the loading of GTP onto Rap1, while N-Epac did not (fig.5b). Most importantly, while the JNK stimulating activity of full length Epac was partially sensitive to RapN17 (fig.3A), the JNK response to N-Epac was completely resistant to both Rap inhibitory molecules RapN17 and RapGAP.
These results demonstrate unequivocally that Epac activation of JNK is independent of its guanine nucleotide exchange function.

**Finding a new effector domain in EPAC: A role for the REM domain in JNK activation.**

What is the minimal domain of Epac responsible for downstream signaling into JNK? The three EPAC versions used in this study (Epac, N-Epac and ΔEpac) activate JNK comparably to known JNK activators as Dbl or anisomycin. That N-Epac fully mimics the effect of full length Epac on JNK suggests that the JNK-activation domain of Epac resides outside the GEF domain in a region common to the three mutants. That ΔEpac is as a potent JNK activator as N-Epac, suggests that the minimal Epac domain responsible for downstream signaling into JNK is a region encompassing the REM domain (amino acids 345 to 410), which is common to these three constructs (fig.4A). In order to assess this possibility we decided to isolate the REM domain and challenge its ability to activate JNK, another Epac region that contains the DEP domain was also isolated as a control and both were expressed as GST fusion proteins. Fig 6 shows that while being expressed to the same levels (fig. 6A), GST-DEP did not induce changes in JNK activity but GST-REM exerted an effect comparable to N-Epac (fig. 6B). We understand that Epac’s REM domain is responsible for this JNK activation, as GST alone did not alter either JNK activity or expression levels (fig. 6B bars, lower and middle panels). Thus, we have identified REM as the minimal Epac domain endowed with capability to fully stimulate JNK activity.

**Signaling specificity: EPAC mediated JNK activation is not reproduced by other SAPKs as p38 MAPK.**

To assay for JNK signaling specificity and to discard any effect on cell stress due to the over expression of Epac mutants we decided to test p38 kinase activity, a SAPK clearly distinct but closely related to JNK. Figure 7 shows that cotransfection with increasing amounts of a plasmid expressing N-Epac fails to augment p38 kinase activity (bars and upper panel) while it strongly activates the phosphorylating activity of
JNK (insert to figure 7 and figure 5). N-Epac was readily expressed and cotransfection with different amounts of N-Epac expressing DNAs had no effect on p38 expression (figure 7, middle and lower panels). We conclude that Epac signals to the JNK through its REM domain and does it in a specific manner that does not affect other SAPKs.
DISCUSSION

Signal transduction mediated by proteins that bind and hydrolyze GTP and its associated GEFs has been receiving growing interest. The discovery of the MAPKs has contributed to outline how their signaling pathways convey messages to the cell nucleus. Linkages between a variety of GEFs and the MAPK cascades have been established. We describe here novel properties for both JNK and Epac. First, Epac activates JNK independently of Rap1. Second, Epac stimulation of JNK is unrelated to its cAMP-dependent GEF activity. Third, the REM region of Epac is endowed with positive signaling capabilities.

Epac is not the only Ras/Rap GEF able to stimulate JNK. It was previously shown that C3G, a non-selective GEF for Rap proteins, stimulated JNK activity through activation of R-Ras (29). However, unlike Epac, C3G stimulation of JNK requires its GEF activity; a C3G construct, C3G-ACD, which lacks the catalytic GEF domain does not activate JNK (29). Similarly, other GEFs such as Dbl, Ost, Vav, mSos1, Ras-GRP/CalDag-GEFII, and, Ras-GRF also stimulate JNK (17,29-31) Yet, in all cases, unlike Epac, these GEFs activate JNK in ways that depend on nucleotide exchange on their cognate G-proteins.

Unlike other GEFs, Epac’s exchange activity can be regulated by interaction with a soluble ligand. Binding of cAMP to the regulatory domain induces a conformational change that exposes the previously occluded exchanger domain (1). It is generally accepted that Epac exhibits a strict dependence on cAMP in regards to both its activation and its nucleotide exchange on Rap (1). To our knowledge no effect of Epac unrelated to cAMP and/or Rap G-proteins has been described to date other than JNK stimulation, as described here. This is a provocative conclusion with respect to Epac, although a precedent example of a cAMP-unrelated action pertinent to a protein that is typically activated by binding of cAMP exists— LPS induced PKA-mediated phosphorylation of p65 NFkB is independent of cAMP (32).

As is the case with Epac, binding of cAMP to PKAr relieves inhibitory constrains imposed by the CBD of PKAr, resulting in allosteric activation of PKAc (33). In this context, it is noteworthy to mention some similarities noted between the
cAMP-independent actions of Epac and PKA. As with Epac, the cAMP-independent action of PKA was first observed by transient transfection experiments in which over expression of desired proteins are achieved. This approach assumes that, in the cell, regulated proteins are in equilibrium between an inactive and an active state, the rate of which is controlled by the binding of activating molecules, such as cAMP for Epac and PKA. What over-expression of Epac (or PKA) actually achieves is an increase in the amount of the active conformation that would otherwise be attained by the activating molecules. As with PKA phosphorylation of p65-NFkB, Epac stimulation of JNK is not seen with cAMP-signaling agents, (data not shown) suggesting that a novel, cAMP-independent way of activating Epac might exist. Mirroring the cAMP-independent action of PKA on p65-NFkB, ubiquitination dependent phosphorylation that relieves a cAMP-independent auto-inhibitory component (IκB), we wonder if Epac activation of JNK might be regulated in an analogous fashion.

We have identified the REM domain of Epac as the minimal domain endowed with full signaling capabilities into the JNK pathway. The REM domain is the common feature between Epac, N-Epac and Δ-Epac, all of which fully stimulate JNK. Little is known about the role of REM in signal transduction processes. All Cdc25-like Ras and Rap GEFs contain REM domains (34) albeit at variant distances from their catalytic GEF core. However, to our knowledge, none of them are activators of JNK, suggesting that Epac’s REM domain displays unique features compared to the other REM domains. Based on the crystal structure of Ras-Sos it has been suggested that the REM domain does not play a direct role in the exchange catalytic step but instead, REM is thought to stabilize the GEF core domain (7). Our findings, however, revealed that Epac’s REM actively signals into JNK raising the question of what are the downstream target(s) linking Epac-REM to the JNK pathway.

Recently, an additional signaling function involving the REM domain of SOS has been described. Binding of Ras-GTP to a region that involves the REM domain of SOS provides allosteric regulation of the GDP/GTP exchange activity catalyzed by a distant domain on the same molecule (35). This novel discovery suggests a similar role for Epac’s REM domain in downstream signaling. However, Epac’s REM domain might facilitate exchange on a molecule in trans (perhaps a member of the Rho/Rac/Cdc42 family of guanine nucleotide exchangers) given the fact that: 1) GTP
loading on Rap does not activate JNK as we have shown and 2) an isolated REM domain from Epac fully activates the JNK. We, and others, have contributed to define the signaling module Rac/MLK3/SEK as a JNK activation pathway (17,19,20). Current efforts are aimed at understanding if Epac activation of JNK is related to this module.

A feasible scenario that might connect Epac to this JNK signaling cascade should involve Epac’s mediated GTP loading on small G proteins that are not its cognate. An example of this is the SOS catalized GTP loading on Rac (31,36). This interaction between a GEF and a non-canonical small GTP binding protein proceeds through the assembly of a multiprotein complex. Multidomain proteins like SOS (and also Epac) might provide a frame for the cross-talk of small G protein signaling pathways. The possibility that Epac mediated activation of JNK proceeds through an analogous complex is a hypothesis under intensive study and efforts to identify REM interacting proteins that could mediate Epac’s ability to activate JNK, are currently being developed.

A DEP domain located on the amino-terminal region of Epac has been described. Although previous reports indicate that some DEP domains can trigger JNK activation (37) we show that Epac’s DEP domain is not responsible for transducing signals to JNK. In our hands Δ-Epac, which lacks the N-terminus including the DEP and CBD domains, is able to induce JNK as strongly as full length Epac. Moreover, a GST-DEP fusion protein fails to promote JNK activation while full activation was observed with GST-REM. Our studies suggest that the REM domain, and not the DEP domain from Epac, is fully responsible for JNK stimulation.

There is a previous report (29) that while analyzing JNK activation by other GEFs shows no JNK activation by Epac. We understand this difference might be due to a divergence in the pattern of proteins expressed by the cell lines being maintained in different labs. Our results provide strong evidence that EPAC signals to the JNK cascade. Moreover we provide evidences of the existence of a novel mechanism for EPAC signaling. Through a variety of complementary approaches, we prove that EPAC is acting, in this case, in a Rap-independent fashion. This is to our knowledge the first report of a GEF-independent signal transduction activity for EPAC and ends up adding a new ingredient in the increasingly complex scene of signaling by GEFs.
One important implication of this work is the intriguing possibility that Epac-REM/JNK stimulatory function is triggered upon stimulation with a yet unidentified stress signal. Although the upstream signaling agents feeding on Epac are not known from the present work, bona fide activators of JNK such as nutrients, growth factors, osmolarity, temperature, pH, radiation, etc.; may use Epac-dependent signal transduction processes independently of cAMP and Rap G-proteins. Our results strongly suggest that a cAMP-independent mechanism is involved in the transduction of environmental signals that are discriminated by Epac into activation of JNK. These predictions are currently being investigated.
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The abbreviations used are:

GEF: guanine nucleotide exchange factor
GDP: guanosine diphosphate
GTP: guanosine triphosphate
MAPK: mitogen-activated protein kinase
SAPKs: stress activated proteins kinases
JNK: c-Jun NH$_2$-terminal kinase
ERK: extracellular regulated kinase
cAMP: adenosine 3’-5’ cyclic monophosphate
DTT: dithiothreitol
SDS: sodium dodecyl sulphate
PAGE: polyacrylamide gel electrophoresis
PBS: phosphate-buffered saline
EGTA: ethylene glycol-bis(β-aminoethyl ether) N,N,N′,N′-tetraacetic acid
ATP: adenosine triphosphate
GST: glutathione S-transferase
DEP: dishevelled, Egl-10 and pleckstrin
REM: ras exchange motif
CBD: cAMP binding domain
GFP: green fluorescence protein
FIGURE LEGENDS

Figure # 1:
Overexpression of Wild – type Epac activates JNK.
HEK-293T cells were transfected with vectors that express the GEFs Onc-Dbl or Epac (empty vector was used in the controls) and stimulated or not with Anisomycin 10 mg/ml for 20 minutes as indicated. A plasmid carrying the cDNA for JNK (pCEFL-AU5-JNK, 250ng per plate) was introduced in all the transfection mixtures. Samples were immunoprecipitated using an anti-AU5 antibody and assayed for JNK activity using GST ATF-2 as a substrate. Quantitation was performed by scanning the autoradiograms and is expressed as fold induction relative to untreated cells (left bar). Data is representative of the mean (+/- S.D.) of three experiments. Samples run in parallel were assayed for JNK expression by western blot analysis using an antibody directed against the AU5 epitope (lower panel).

Figure # 2:
An activated mutant of Rap1b fails to augment JNK activity.
A) pCEFL-AU5-JNK was transfected into HEK-293T cells along with DNA constructs that express Rap1bG12V, Rac1QL or Epac in duplicate. Control samples were cotransfected with empty vector and stimulated with Anisomycin as in Fig # 1. Kinase assays and Western Blots were performed in the AU5 immunoprecipitates as in Fig. #1. Quantitation was performed by scanning the autoradiograms and is expressed as fold induction relative to untreated cells (left bar). Data is representative of the mean (+/- S.D.) of at least three experiments. B) Cells transfected with pCGN-HA-Rap1bG12V were assayed for Rap expression using an antibody directed against the HA epitope.

Figure # 3:
Effect of Rap-signaling interfering molecules on JNK activation triggered by Epac.
A) HEK-293T cells were transfected with expression plasmids for Epac, along with empty vector or constructs that express the inhibitory molecules RapN17 or RapGAP, as indicated in the figure. pCEFL-AU5-JNK was included in all mixtures and kinase assays were performed as in the preceding figures. The bars indicate JNK activity expressed as fold induction relative to cells transfected with empty vector alone. The panel in the middle and lower sections show levels of JNK and Epac expression as...
reported by anti-AU5 and anti-myc western-blots respectively. The data is representative of the mean (+/- S.D.) of at least three experiments. B) Expression of RapN17 and RapGAP as detected by immunoblotting against the HA epitope.

Figure # 4:
**GEF activity is not necessary for JNK activation triggered by Epac.**
A) The diagram represents the different functional domains identified in Epac. Names inside the open boxes indicate the approximate position of each domain. N-Epac and D-Epac are deletion mutants; the bars in the lower part show the extent of the remaining sections in each Epac mutant. B) Increasing amounts of wild type Epac or D-Epac were transfected in HEK-293T cells along with a fixed amount of pCEFL-AU5-JNK. Whenever needed, transfection mixtures were taken to 1μg of total plasmid DNA using empty vector. Samples were immunoprecipitated with anti-AU5 antibody and processed for JNK activity as in the preceding figures. Bars represent fold induction (+/- S.D.) referred to cells cotransfected with empty vector alone. Data is integrated from three different experiments. C) A plasmid expressing D-Epac was transfected along with another one expressing HA-Rap1b. Samples were collected and pull-down assays were performed on the lysates using a GST-RalGDS-RBD fusion protein. HA-Rap-GAP was cotransfected wherever indicated in the figure. The extent of GTP-loading on Rap is correlative to the strength of the signal in the anti-HA Western-Blots of the upper panel. The total amount of Rap present in the cell lysates is shown in the lower panel as HA-Rap1.

Figure # 5:
**JNK activation is independent of EPAC’s GEF activity on Rap.**
A) HEK-293T cells were transfected with plasmid DNAs that express N-Epac, along with DNA constructs that express the inhibitory molecules RapN17 or RapGAP, as indicated in the figure. pCEFL-AU5-JNK was included in all mixtures and empty vectors served as controls whenever needed. The bars indicate JNK activity expressed as fold induction relative to cells transfected with empty vector alone. Data is representative of the mean (+/- S.D.) of at least three experiments. The panel in the middle and lower sections show levels of JNK and N-Epac expression respectively as in Fig # 3. B) Western Blots using anti-HA antibodies were performed upon pull-down
experiments on lysates containing HA-Rap originated in cells transfected with plasmid DNAs that express either N-Epac or [-]-Epac.

Figure # 6:  
**An isolated EPAC REM domain is able to induce JNK activation.**  
HEK-293T cells cotransfected with plasmids that express GST fusion proteins or N-Epac and HA-JNK. A) The Western-Blot shows GST fusion protein expression levels using an anti-GST antibody. B) Cells were lysed and JNK assays were performed in the HA immunoprecipitates. Quantitation was performed by scanning the autoradiograms and is expressed as fold induction relative to untreated cells (left bar). Data is representative of the mean (+/- S.D.) of at least three experiments. Western Blots using an HA antibody show JNK expression levels in the samples cotransfected with GST fusion proteins (lower panel).

Figure # 7:  
**JNK activation triggered by Epac is not mimicked by other kinases as p38.**  
HEK-293T cells were transfected with plasmid DNAs that express N-Epac and either of the epitope tagged SAPKs, AU5-JNK or HA-p38. Protein kinases were immunoprecipitated using the corresponding antibodies and kinase assays were performed in the immunoprecipitates. Western blots analysis was performed on a fraction of the total lysates for each sample in order to show expression levels of p38 MAPK or N-Epac (lower and middle panels). The bars in the graphic show p38 kinase activity expressed as fold induction relative to samples transfected with empty vectors. One representative gel (out of three independent experiments) is shown for p38 kinase activity. The insert in the upper part of the figure shows JNK activity in parallel samples cotransfected with the same amounts of N-Epac that were used in the p38 kinase assays.
REFERENCES

1. Rooij, J., Zwartkruis, F. J. T., Verheijen, M. H. G., Cool, R. H., Nijman, S. M. B., Wittinghofer, A., and Bos, J. L. (1998) *Nature* **396**, 474-477
2. Kawasaki, H., Springett, G. M., Mochizuki, S. T., Nakaya, M., Matsuda, M., Housman, D. E., and Graybel, A. M. (1998) *Science* **282**, 2275-2279
3. Rooij, J., Rehmann, H., van Trest, M., Cool, R. H., Wittinghofer, A., and Bos, J. L. (2000) *J. Biol. Chem.* **275**, 20829-20836
4. Boguski, M. S., and McCormick, F. (1993) *Nature* **366**, 643-654
5. Kitashama, H., Sugimoto, Y., Matsuzaki, T., Ikawa, Y., and Noda, M. (1989) *Cell* **56**, 77-84
6. Bos, J. L., de Rooij, J., and Reedquist, K. A. (2001) *Nat. Rev.* **2**, 369-377
7. Boriack-Sjodin, A. P., Margarit, M. S., Bar-Sagi, D., and Kuriyan, J. (1998) *Nature* **394**, 337-343
8. Kraemer, A., Rehmann, H. R., Cool, R. H., Theiss, C., de Rooij, J., Bos, J. L., and Wittinghofer, A. (2001) *J. Mol. Biol.* **306**, 1167-1177
9. Gutkind, J. S. (2000) *Sci. STKE* **40**, 1-13
10. Pearson, G., Robinson, F., Beers Gibson, T., Xu, B., Karandikar, M., Berman, K., and Cobb, M. H. (2001) *Endocrine Rev.* **22**, 153-183
11. Sasaoaka, T., Langlois, W. J., Leitner, J. W., Draznin, B., and Olefsky, J. M. (1994) *J. Biol Chem.* **269**, 32621-32625
12. Chang, L., and Karin, M. (2001) *Nature* **410**, 37-40
13. Davis, R. D. (2000) *Cell* **103**, 239-252
14. Martin-Blanco, E. (2000) *BioEssays* **22**, 637-645
15. Kyriakis, J. M., and Avruch, J. (2001) *Phisiological Rev.* **81**, 807-869
16. Marshall, C. J. (1994) *Current Biology* **4**, 82-89
17. Coso, O. A., Chiariello, M., Yu, J. C., Termoto, H., Crespo, P., Xu, N., Miki, T., and Gutkind, J. S. (1995) *Cell* **81**, 1137-1146
18. Yan, M., Dai, T., Deak, J. C., Kyriakis, J. M., Zon, L. I., Woodgett, J. R., and Templeton, D. J. (1994) *Nature* **372**, 798-800
19. Sanchez, I., Hughes, R. T., Mayer, B. J., Yee, K., Woodgett, J. R., Avruch, J., Kyriakis, J. M., Zon, L. I. (1994) *Nature* **372**, 794-798
20. Teramoto, H., Coso, O. A., Miyata, H., Igishi, T., Miki, T., Gutkind, J. S. (1996) *J Biol Chem.* **271**, 27225-27228
21. Bar-Sagi, D., and Hall, A. (2000) *Cell* **103**, 227-238
22. Nobes, C. D., and Hall, A. (1995) *Cell* **81**, 53-62
23. Ribeiro-Neto, F., and Altschuler, D. L. (1998) *Proc. Natl. Acad. Sci* **95**, 7475-7479
24. Altschuler, D. L., and Lapetina, E. G. (1993) *J. Biol Chem.* **268**, 7527-7531
25. Reedquist, K. A., Ross, E., Koop, E. A., Wolthuis, R. M. F., Zwartkruis, F. J. T., van Kooyk, Y., Salmon, M., Buckley, C. D., and Bos, J. L. (2000) *J. Cell. Biol.* **148**, 1151-1158
26. Franke, B., Akkerman, J. W., and Bos, J. L. (1997) *EMBO J.* **16**, 252-259
27. Karim, M., and Hunter, T. (1995) *Current Biology* **5**, 747-757
28. Powers, S., O'Neill, K., and Wigler, M. (1989) *Mol. Cell. Biol.* **9**, 390-395
29. Mochizuki, N., Ohba, Y., Kobayashi, S., Otsuka, N., Graybiel, A. M., Tanaka, S., and Matsuda, M. (2000) *J. Biol Chem.* **275**, 12667-12671
30. Crespo, P., Bustello, X. R., Aaronson, D. S., Coso, O. A., Lopez-Barahona, M., Barbacid, M., and Gutkind, J. S. (1996) *Oncogene* **13**, 455-460
31. Nimnual, A. S., Yatsula, B. A., and Bar-Sagi, D. (1998) *Science* **279**, 560-563
32. Zhong, H., SuYang, H., Erdjument-Bromage, H., Tempst, P., and Ghosh, S. (1997) *Cell* **89**, 413-424
33. Francis, S. H., Poteet-Smith, C., Busch, J. L., Richie-Jannetta, R., and Corbin, J. D. (2002) *Front. Biosci.*
34. Bos, J. L. (2000) in *GTPases* (Hall, A., ed)
35. Margarit, S. M., Sondermann, H., Hall, B. E., Nagar, B., Hoelz, A., Pirruccello, M., Bar-Sagi, D., and Kuryan, J. (2003) *Cell* **112**, 685-695
36. Innocenti, M., Frittoli, E., Ponzanelli, I., Falck, J. R., Brachmann, S. M., Di Fiore, P., and Scita, G. (2003) *J cell Biology* **160**, 17-23
37. Boutros, M., Paricio, N., Strutt, D., and Mlodzik, M. (1998) *Cell* **94**, 109-118
Fig. 1

Kinase assay

GST ATF-2 (96) → Kinase assay

AU5 - JNK → W. Blot

Control  Aniso  Dbl  Epac
Fig. 2

(A) JNK activity (fold induction) for GST ATF-2 (96) and AU5 - JNK. The graph shows a comparison between control, Ariso, Rap G12V, Rac1 QL, and Epac conditions.

(B) Western blot showing HA - Rap G12V expression in control and Rap G12V conditions.
Fig. 3

A

GST ATF-2 (96)

Control  Vector  Rap - GAP  Rap N17

Epac

AU5 - JNK

myc - Epac
Fig. 4

A

EPAC

DEP CBD REM GEF

N-EPAC

□ - EPAC

B

JNK activity (fold induction)

Control Aniso.

0 1 2 3 4

plasmid DNA (ng)

0 100 250 500

Epac □ - Epac
Fig. 4

- + + myc Epac
- - + HA Rap - GAP

HA Rap1 - GTP

HA Rap1
Fig. 5

![Graph showing JNK activity fold induction with control, vector, Rap-GAP, and Rap-N17 conditions.](image)

- **GST ATF-2 (96)**
- **AU5 - JNK**
- **myc N - Epac**
Fig. 5

B

- - + myc N - Epac
- + - myc - Epac
Fig. 6

- GST
- GST-DEP
- GST-REM
- Control
- N-Epac

Gel Blot

JNK activity (fold induction)

A

B

GST

W. Blot □ □ GST

GST-DEP

GST-REM

HA-JNK

GST ATF-2 (96)
Fig. 7

The figure shows a bar graph representing p38 activity (fold induction) for Control and Aniso conditions with and without N-EPAC treatment. The graph indicates a significant increase in p38 activity under Aniso conditions compared to Control, especially with N-EPAC. The protein blots at the bottom confirm the expression levels of GST ATF-2 (96), myc N-EPAC, and HA-p38 under different conditions.
Activation of JNK by EPAC is independent of its activity as a Rap guanine nucleotide exchanger

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