CDC25^{Mm}/Ras-GRF1 regulates both Ras and Rac signaling pathways

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Abstract The Ras-GRF1 exchange factor molecule contains in addition to the catalytic domain two pleckstrin homology (PH1 and PH2), one IQ and one Dbl homology (DH) domains. In this study we investigated the role of such additional domains. We found that a Ras-GRF1 mutant lacking PH1 and IQ domains is sufficient to activate c-fos promoter in response to lysophosphatidic acid (LPA). The same mutant did not increase external stimuli-regulated kinase (ERK) activity, suggesting an additional mechanism for the induction of gene transcription. Isolated DH-PH2 module activates c-Jun NH_2-terminal kinase and the c-fos promoter in response to LPA, providing the basis for an ERK-independent mechanism. These results provide evidence that Ras-GRF1 acts as a bifunctional molecule on both ERK-dependent and independent pathways.

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Key words: External stimuli-regulated kinase; c-Jun NH_2-terminal kinase; Lysophosphatidic acid; Ras; Ras-GRF1; Rac

1. Introduction

Ras proteins function as molecular switches, cycling between active GTP-bound and inactive GDP-bound states. They are activated by guanine nucleotide exchange factors (GEFs) which in turn are regulated by extracellular signals. In mammalians three classes of Ras-specific GEFs have been characterized. Sos proteins are ubiquitously expressed and their involvement in coupling growth factor receptors to Ras-dependent signaling pathways is well understood [1–3]. Ras-GRF1, or Cdc25^{Mm} [4–6], is the prototype of the second class, that also includes Ras-GRF2 [7]. Ras-GRF1 is expressed postnatally in brain neurons [8,9] and appears to play a role in long term memory formation [10]. It induces Ras signaling in response to calcium influx [11] and to activated G protein-coupled receptors [12–14]. Recently a third type of Ras exchange factor called Ras-GRP has been described containing calcium and diacylglycerol-binding motifs [15].

Ras-GRFs and Sos have similar Ras-GEF catalytic domains and both contain a Dbl homology (DH)-pleckstrin homology (PH) module [16]. Ras-GRFs contain in addition an N-terminal PH domain followed by a putative coiled coil and a calcium calmodulin-binding motif (IQ) [11].

Recently the DH domains of Sos and of Ras-GRF2 have been reported to be associated with Rac-specific exchange activity [17,18], while no activity toward Rho family of small GTPases has been reported for DH domain of Ras-GRF1 [19]. However Rac1-specific GEF activity has been recently reported for Ras-GRF1 when expressed together with G protein βγ subunits [20].

We have previously shown that in fibroblasts, ectopically expressing Ras-GRF1, lysophosphatidic acid (LPA) stimulates the activation of the c-fos promoter and of external stimuli-regulated kinase 1 (ERK1) much more efficiently than in parental NIH-3T3 cells. This effect is abrogated by the expression of the dominant negative RasN17 mutant and of the N-terminal region of Ras-GRF1 (aa 1–600, containing PH1, IQ, DH-PH2 module) [13].

In the present paper we tested the ability of deletion mutants of Ras-GRF1, to induce ERK signaling and activation of the c-fos promoter in response to LPA. The results obtained indicate that the N-terminal region of Ras-GRF1 spanning from the PH1 to the IQ (PCCQ) is necessary for the LPA-induced activation of ERK1, but is dispensable for the upregulation of the c-fos promoter. In addition parts of the molecule containing the DH domain are capable to induce gene transcription without activating ERK signaling. This effect may be due to the ability of the DH domain to activate c-Jun NH_2-terminal kinase (JNK) in a Rac-dependent manner. This indicates that Ras-GRF1 belongs to a class of bifunctional GEFs that can regulate both the Ras-Raf-ERK and the Rho GTPases stress-activated protein kinase (SAPK) transduction pathways [21].

2. Materials and methods

2.1. Plasmids and constructs

pHC21-VSV was obtained from pHC21 [13] by insertion of the blunted 2.1 kb *Bam*H1/*Eco*R1 fragment into a *Sal*1 digested and filled pMT2-VSV-G tag vector. The mutant resulted C-terminally tagged. pDP-VSV was derived from pHC21-VSV by *Pst*1 digestion. The fragment amplified by polymerase chain reaction (PCR) with oligonucleotides 5′-catccggcttaacgatggccac-3′ and 5′-ggaattcttccactttcgccggcac-3′, was digested with *Eco*R1/*Xho*1 and inserted in a *Eco*R1/*Xho*1 digested pHC21-VSV. The resulting construct was called pPCCQ. DH domain amplified by PCR with oligonucleotides 5′-cgggatcccgaaagtggaagaacatcatccaggactacactcgg-3′ and 5′-cggaattctcactggctggtgtccaggaggatctcacagcc-3′ was digested with *Bam*H1/*Eco*R1 and subcloned in a double myc-tagged *Bam*H1/*Eco*R1 digested pBS vector. The resulting N-terminally myc-tagged DH domain was inserted by *Sal*1 digestion into pMT2 expression vector. pMT2-GRF-HA is described in [22]. pMT2-Mm1-HA was derived by subcloning the *Eco*R1 fragment of pMT2-GRF-HA in the pMT2 vector. DPC was derived by insertion of pHC21 *Pst*1/*Eco*R1 fragment in a pGEM-3Z containing the C-terminal *Eco*R1 fragment of Ras-GRF. By partial *Pst*1 digestion and blunted *Xho*1 digestion, the DPC was subcloned in a *Pst*1/*Sma*1 digested pMT2 vector. The resulting plasmid was called pMT2-DPC. pSRα-HA-JNK1 and pSRα-RacV12 were kindly provided by A. Minden [23]. pGEX-c-Jun79, pCDNA3-Rac1N17 and pCDNA3-Cdc42N17-HA were kindly provided by G. Scita (European Institute of Oncology, Milan, Italy). pSG5-p85αΔSH2-C was a gift from J. Downward [24]. p44-HA was kindly provided by Pouyssegur [25]. pFos-luciferase was previously described [13].

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2.2. Cell cultures and transfections

NIH-3T3 and NIH-RG7 cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% newborn calf serum. Cos-7 cells were cultured in DMEM supplemented with 10% fetal bovine serum (FCS).

NIH-RG7 fibroblasts, grown in 60-mm dishes, were transfected by calcium phosphate method [13] keeping total DNA at 7 μg per plate. NIH-3T3 cells were cultured in 35-mm dishes and transfected using the lipofectamine (GIBCO) method according to the protocol of the manufacturer, keeping total DNA to 1.5 μg per plate.

Subconfluent Cos-7 cells were transfected by a standard DEAE-dextran technique keeping total DNA to 5 μg per 60-mm dish. In all cases the day after transfection cells were serum starved for 24 h.

2.3. Luciferase assay

This assay was performed as described in [13], using Promega luciferase assay system and the LUMAT LB 9501 E4030 (Berthold).

2.4. ERK kinase assays

Cells, transfected with p44-HA and with different constructs (as described in the legends of the figures), were stimulated with α-LPA (Sigma) or left untreated, washed with ice-cold PBS and lysed in lysis buffer as described by Crespo et al. [26]. The HA-tagged kinase was immunoprecipitated from the cleared lysate with monoclonal anti-HA-11 antibody (Babco). Immunocomplexes were recovered with protein G-Sepharose (Sigma) and the MAPK activity was determined in an in vitro kinase assay using myelin basic protein (MBP) as substrate [26]. Aliquots of the in vitro assay were spotted on P81 paper (Whatman) and the radioactivity was measured.

2.5. JNK kinase assay

Serum starved Cos-7 cells transfected with pSRα-HA-JNK1 and pDP-VSV or pDH-Myc, were stimulated with α-LPA for 10 min and lysed in the lysis buffer previously described [26]. The HA-tagged kinase was immunoprecipitated with anti-HA-11 antibodies and the JNK1 activity was measured using 2 μg of purified GST-c-Jun (1–79) fusion protein as substrate [27].

2.6. Detection of the proteins coded by the different constructs

Cells transfected with different constructs of Ras-GRF1 were lysed as described above. Proteins were immunoprecipitated with Ras-GRF antibodies and detected by Western blotting with the same antibodies. Otherwise 25 μg of total cell lysates or 1/50 of cleared cell lysates were directly analyzed by Western blotting, using the different antibodies as described in the legends of the figures. Bound antibodies were visualized with horseradish peroxidase-conjugated anti-rabbit or anti-mouse antibodies using a chemiluminescent substrate (ECL, Amersham).

2.7. Antibodies

Anti-HA (11) and anti-myc (9E10) monoclonal antibodies (Babco), anti-Ras-GRF (C-20), anti-ERK1 (K-23), and anti-p85α antibodies (Santa Cruz), anti-VSV P5D4 monoclonal antibodies (Sigma), anti-Rac1 antibodies (Transduction Laboratories) were used. Polyclonal antibodies against the PH1 domain of Ras-GRF were raised in our laboratory using the PH1 domain fused to the GST protein.

3. Results

3.1. PH1 and IQ domains of Ras-GRF1 are dispensable for LPA-induced activation of gene transcription but are essential for ERK signaling

In fibroblasts ectopically expressing Ras-GRF1 (NIH-RG7) activation of the c-fos promoter and of ERK1 is largely stimulated by LPA and transient expression of the N-terminal region (aa 1–600) of Ras-GRF1 strongly reduces the activation of the c-fos promoter [13]. To investigate the role of the different domains present in the N-terminal region of Ras-GRF1 (PH1, IQ, DH-PH2 module) we analyzed the ability of several deletion mutants to activate the c-fos promoter and ERK in response to LPA.

We genetically engineered two N-terminal deletion mutants: the DPC mutant (aa 240–1262), lacking the first PH, the coiled coil region and the IQ domain, and the Mm1 mutant (aa 674–1262), lacking in addition the DH-PH2 module (Fig. 1a). We then tested their ability to mediate the LPA-induced c-fos promoter activation in comparison with the entire Ras-GRF1. NIH-3T3 fibroblasts were cotransfected with either Ras-GRF1, or DPC or Mm1 together with the c-fos-luciferase reporter gene and the luciferase activity was assayed both in control and LPA-stimulated cells. The expression level of the three constructs was comparable (Fig. 1b). For the experiments reported in Fig. 1c, transfections were performed with low dosage of plasmids in order not to saturate the system and to maintain it susceptible to further stimulations. As shown in Fig. 1c luciferase activity in unstimulated cells was the same in all the cases. A significant increase

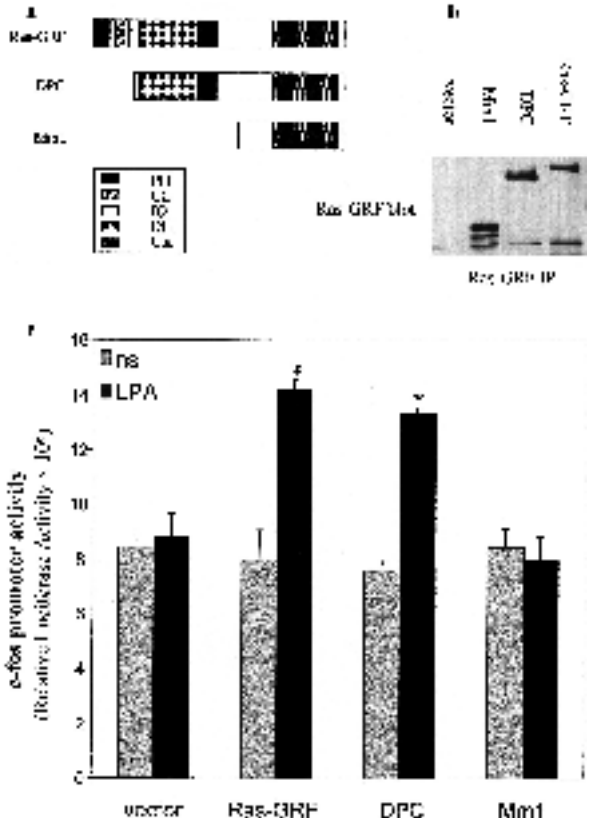

Fig. 1. Effect of Ras-GRF1, DPC and Mm1 expression on the LPA-induced activation of c-fos promoter in NIH-3T3 cells. a: Schematic representation of Ras-GRF1, DPC and Mm1 mutants highlighting functional domains. The plasmids carrying the different constructs are described in Section 2. b: Expression of Mm1, DPC and Ras-GRF1 in NIH-3T3 cells. NIH-3T3 cells were transfected with pMT2-Mm1-HA (Mm1 mutant), pMT2-DPC (DPC mutant), pMT2-GRF-HA (wt Ras-GRF1) or empty vector (as described in Section 2). After 48 h the wild-type and mutants were immunoprecipitated from equal amounts of proteins of cellular lysates with anti-Ras-GRF antibodies; the immunoprecipitates were subjected to SDS-PAGE and immunoblot with anti-Ras-GRF antibodies. c: NIH-3T3 cells grown in 35-mm dishes were cotransfected with 0.2 μg of pMT2-GRF-HA, pMT2-DPC or pMT2-Mm1-HA and 0.33 μg of pFos-luciferase. After serum withdrawal, cells were stimulated with 10 μM LPA or left untreated. After 18 h cellular lysates were prepared and analyzed for luciferase activity as described in Section 2. Each value is the mean ± S.E.M. derived from three independent samples and is expressed as relative luciferase activity normalized for the total protein content in the lysates. This is one of two independent experiments performed in triplicates with similar results ($*P < 0.05$).

in luciferase activity was observed in cells transfected with Ras-GRF1 and with DPC in response to LPA. Instead in cells transfected with Mm1, as well as in control ones, LPA had no effect. Thus DPC, the construct including the DH-PH2 module and the Ras-GEF domain (CDC25), seems to be sufficient to upregulate the c-fos promoter.

We then tested the ability of the same DPC and Mm1 mutants to activate ERK1 in response to LPA. We cotransfected in NIH-3T3 cells either Ras-GRF1, or DPC or Mm1 together with the HA-tagged ERK1 (p44-HA). The kinase was immunoprecipitated from either unstimulated or LPA-treated cells and its activation was then evaluated in an in vitro kinase assay. As shown in Fig. 2, without stimulation the activity of ERK1 was the same in cells transfected with the vector or with the different constructs. However 5 min stimulation with LPA induced a strong activation of ERK1 in Ras-GRF1 transfected cells, but only minor effects in cells transfected with vector, DPC or Mm1. The same results were obtained by probing immunoprecipitated ERK1 with antibodies against phosphorylated MAPKs (not shown). Also in these experiments intentionally low dosage of the different constructs was used to maintain the ERK cascade more susceptible to external stimuli. The data of Fig. 2 demonstrate that the extreme N-terminal region of Ras-GRF1 (PH1, coiled coil, IQ) is essential for the activation of ERK1 by LPA.

An independent confirmation of this finding comes from the following experiment. Different constructs (pHC21-VSV, pPCCQ, pDP-VSV) coding respectively for products spanning aa 1–630, 1–240 and 240–630 of wild-type Ras-GRF1 (Fig. 3a and b) were cotransfected together with p44-HA in NIH-RG7

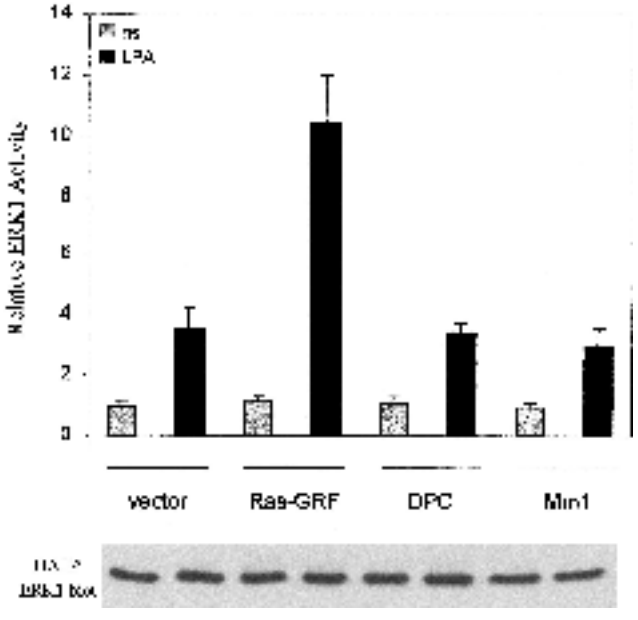

Fig. 2. Effect of Ras-GRF1 and N-terminal deletion mutants on LPA-induced ERK1 activation in NIH-3T3 cells. NIH-3T3 cells grown in 35-mm dishes were cotransfected with 0.2 μg pMT2-GRF-HA, pMT2-DPC or pMT2-Mm1-HA and 0.5 μg of p44-HA (HA-tagged ERK1). After serum withdrawal, cells were stimulated with 3 μM LPA for 5 min or left untreated. ERK1 was immunoprecipitated from cellular lysates and ERK activity tested in an in vitro assay. Results represent the mean ± S.E.M. from four independent samples. ERK1 immunoprecipitated from parallel plates was separated by SDS-PAGE and immunoblotted with anti-ERK1 antibodies.

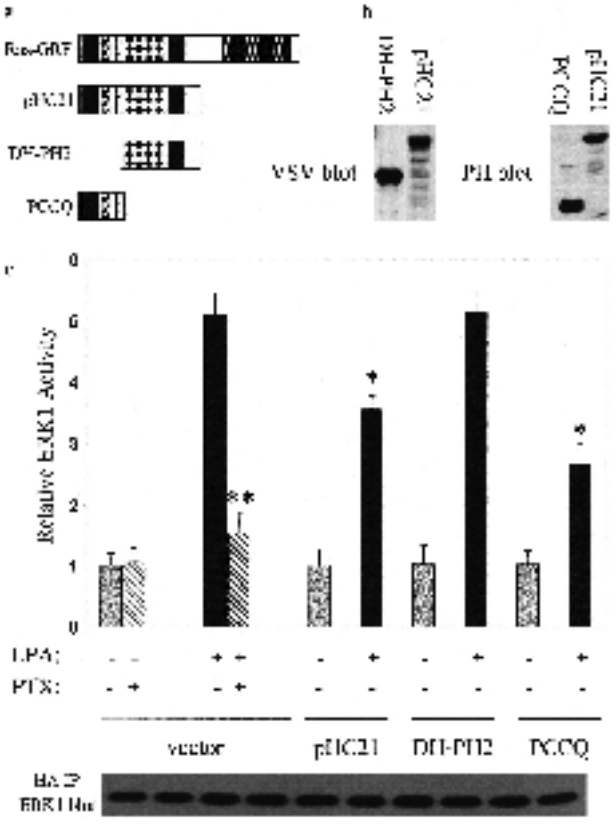

Fig. 3. Effect of pertussis toxin and of the expression of N-terminal regions of the Ras-GRF1 on the LPA-induced ERK1 activation in fibroblasts overexpressing Ras-GRF1. a: Schematic diagram of N-terminal mutants depicted as in Fig. 1a. b: Expression of the mutants of Ras-GRF1 described in a. Equal amounts of proteins from total lysates of NIH-3T3 cells transiently transfected with pHC21-VSV, pDP-VSV or pPCCQ (see Section 2 for the details) were separated by SDS-PAGE and immunoblotted with anti-VSV or anti-PH1 of Ras-GRF antibodies. c: NIH-RG7 cells that stably overexpress Ras-GRF1 were transfected with 5 μg of the constructs described above together with 2 μg of HA-tagged ERK1 (see Section 2). After serum starvation cells were stimulated with 10 μM LPA for 5 min or left untreated. When indicated NIH-RG7 transfected with vector alone were treated for 5 h with pertussis toxin (100 ng/ml) and then stimulated as above with LPA. ERK1 was immunoprecipitated from cell lysates and its activity assayed with myelin basic protein as substrate. The kinase activity of cells transfected with an empty vector (marked vector) and not stimulated was set to 1. Results represent the mean ± S.E.M. from four to six independent samples ($^*P < 0.01$ with respect to vector plus LPA). Immunoprecipitated ERK1 from parallel plates was separated by SDS-PAGE and immunoblotted with anti-ERK1 antibodies.

fibroblasts which overexpress Ras-GRF1. Following a 5 min stimulation with LPA the kinase was immunoprecipitated and its activity was determined. As shown in Fig. 3c, the ERK1 activity measured in the absence of LPA was not modified by the expression of the three mutants. Instead both the entire N-terminal region (aa 1–630) and the PCCQ protein, which contains the PH1, the coiled coil and the IQ domain, reduced the LPA-induced ERK1 activation in NIH-RG7 cells, thus acting as dominant negative mutants. On the contrary the DH-PH2 module (aa 240–630) did not exert negative effects. The mutants were expressed in fibroblasts at similar level (Fig. 3b) and, as Ras-GRF1, all of them were preferentially localized in the membrane fraction (data not shown). In NIH-3T3 cells these mutants did not interfere with the basal or the PDGF-

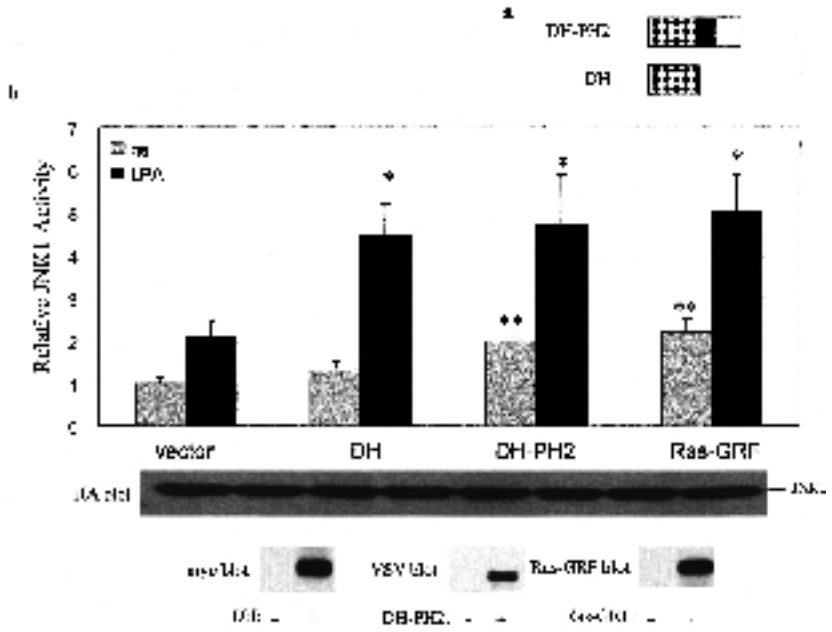

Fig. 4. Effect of Ras-GRF1, DH-PH2 and DH on JNK1 activity. a: Schematic diagram of DH-PH2 and DH mutants depicted as in Fig. 1a. b: Cos-7 cells were cotransfected with 1 μg of pSRα-HA-JNK1 together with 3 μg of pDP-VSV (DH-PH2) or pDH-Myc (DH) or 3 μg of pMT2-GRF-HA. After 18 h serum starvation, cells were stimulated with 10 μM LPA for 10 min or left untreated. The tagged kinase was immunoprecipitated with anti-HA antibodies. JNK1 activity in the immunocomplexes was measured using GST-c-Jun as substrate (as described in Section 2). The basal kinase activity of samples from cells transfected with the empty vector (marked vector) was set to 1. Results represent the mean ± S.E.M. from four independent samples. The experiment was repeated three times with similar results (**$P < 0.01$ with respect to vector; *$P < 0.05$ with respect to vector plus LPA). Aliquots of lysates and immunoprecipitates were subjected to SDS-PAGE and Western blotting with anti-Ras-GRF, anti-myc (for DH expression), anti-VSV (for DH-PH2 expression) or anti-HA (for JNK1) antibodies.

induced c-fos promoter activity ([13], and data not shown), thus ruling out possible aspecific inhibitory effects.

Taken together these data demonstrate that the ability of Ras-GRF1 to potentiate LPA signaling via ERK activation is dependent on the PCCQ region. On the other hand the DH-PH2 module seems to be essential for activating gene transcription in absence of significant potentiation of ERK signaling.

In the same figure it is also shown that in NIH-RG7 cells the effect of LPA on ERK1 activation is inhibited by pretreatment with pertussis toxin, indicating the involvement of Gi protein-coupled receptors, as already reported for other responses to LPA in these cells [13].

3.2. *The isolated DH-PH2 module of Ras-GRF1 activates in vivo JNK1 and the c-fos promoter*

Activation of Rac/Cdc42 members of the Rho family of small GTPases by Dbl-like GEFs can lead to induction of the c-Jun NH_2-terminal kinase (JNK1) activity [16]. We thus considered the hypothesis that the DH domain might activate JNK1 and thus cooperate in the induction of gene expression. Therefore we cotransfected an epitope-tagged JNK1 together with Ras-GRF1 in Cos-7 cells. The activity of JNK was then tested in vitro using as substrate a glutathione S-transferase-c-Jun fusion protein (GST-c-Jun).

As shown in Fig. 4b, the expression of Ras-GRF1 significantly increased JNK1 activity, and LPA further stimulated it. Moreover both the isolated DH domain and DH-PH2 module had a similar effect on the LPA-stimulated JNK1 activity, and the DH-PH2 module increases the JNK1 activity also in the absence of exogenous stimuli. Cotransfection with the dominant negative allele of Rac1 (RacN17) greatly reduced the

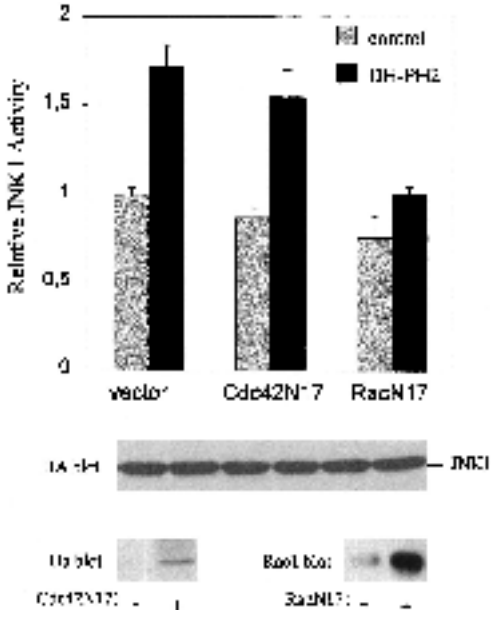

Fig. 5. Effect of RacN17, Cdc42N17 on the DH-PH2-mediated JNK1 activation. Cos-7 cells were cotransfected with 1 μg of pSRα-HA-JNK1, 3 μg of pDP-VSV (DH-PH2) and with 1 μg of pCDNA3-Rac1N17 or pCDNA3-Cdc42N17-HA. After 18 h in the absence of serum the kinase activity was determined as above. The basal kinase activity of samples from cells transfected with an empty vector (marked vector) was set to 1. Results represent the mean ± S.E.M. from three independent samples. Aliquots of lysates and immunoprecipitates were subjected to SDS-PAGE and Western blotting with anti-HA (for Cdc42N17 expression), anti-Rac1 (for Rac1N17 expression) or anti-HA (for JNK1) antibodies.

activation of JNK1 induced by DH-PH2, while Cdc42N17 had no effect on it, even when the plasmid concentration was doubled (not shown) (Fig. 5). This result suggests that the DH-PH2-induced JNK1 activation may be mediated by Rac, in accordance with the report of Kiyono et al. [20].

It was recently shown that interaction of phosphatidylinositol [3,4,5]-Tris-phosphate with the PH domain of DH-PH modules of Sos and Vav, upregulated the Rac-GEF activity of these proteins [17,28]. We cotransfected Cos-7 cells with JNK1, the DH-PH2 module of Ras-GRF1 and a dominant negative PI3-K p85 adaptor subunit, which abrogates the activity of PI3-K [24]. We found (Fig. 6) that the basal activity of control and DH-PH2 transfected cells was not affected by p85 dominant negative (p85DN) and similar results were obtained with wortmannin (not shown). Instead p85DN reduced LPA-induced JNK1 activity, both in control cells and in cells transfected with DH-PH2 module. These results indicate that the stimulation of Rac/JNK pathway by DH-PH2 does not require the activity of p85/p110 PI3-K, while the effect of LPA does.

The isolated DH-PH2 module, transfected in NIH-3T3 cells, was able to activate the c-fos promoter in response to LPA, to an extent comparable to the entire Ras-GRF1 molecule, while it had no significant effect on the basal activity (Fig. 7). These results further support a role for the DH-PH2 module of Ras-GRF1 in modulating gene expression.

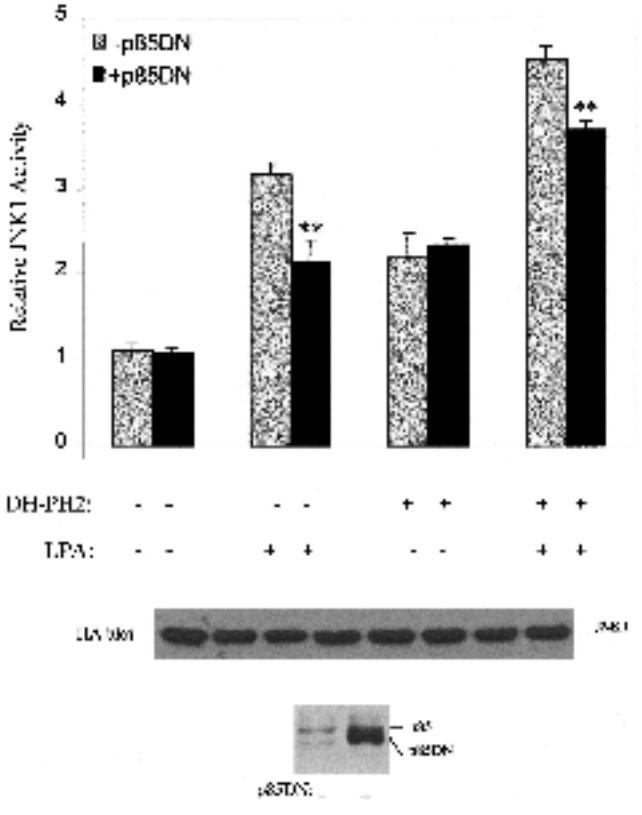

Fig. 6. Effect of p85DN on the DH-PH2-mediated JNK1 activation. Cos-7 cells were cotransfected with 1 μg of pSRα-HA-JNK1, 3 μg of pDP-VSV (DH-PH2) and with 1.5 μg of pSG5-p85αΔSH2-C (p85DN). After 18 h serum starvation, cells were stimulated with 10 μM LPA for 10 min or left untreated. The kinase activity was determined as above. The basal kinase activity of samples from cells transfected with an empty vector (marked vector) was set to 1. Results represent the mean ± S.E.M. from three independent samples and the experiment was repeated two times with similar results. Aliquots of lysates and immunoprecipitates were subjected to SDS-PAGE and Western blotting with anti-p85α (for p85DN expression) or anti-HA (for JNK1) antibodies.

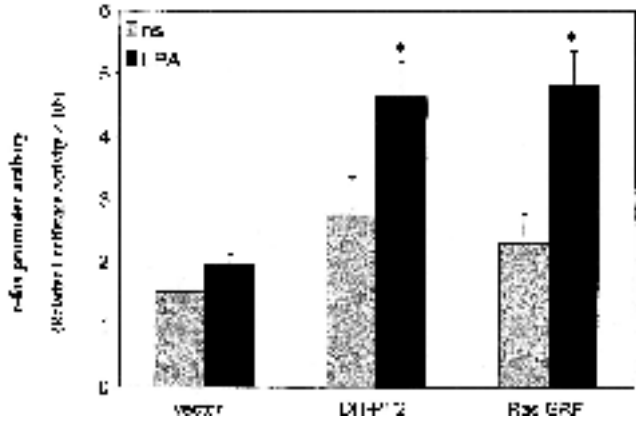

Fig. 7. Effect of DH-PH2 expression on the LPA-induced activation of c-fos promoter in NIH-3T3 cells. NIH-3T3 cells were transfected with the different constructs (0.2 μg of pMT2-GRF-HA or 1 μg pDP-VSV) together with 0.11 μg pFos-luciferase and treated as described in Fig. 1c. Luciferase activity was then determined. Each value is the mean ± S.E.M. derived from three independent samples and is expressed as relative luciferase activity normalized for the total protein content in the lysates. This is one of two independent experiments performed in triplicates with similar results ($*P < 0.05$).

4. Discussion

Ras-GEFs such as Ras-GRF1, Ras-GRF2 and Sos proteins exhibit a modular structure [1,5,7]. In Ras-GRFs, domains localized at the N-terminal moiety of the molecule might play diverse roles such as allowing protein/protein or protein/lipid interactions, providing autoregulatory mechanisms or other catalytic activities in addition to the Ras-GEF activity of the C-terminal domain. However the in vivo functions of such additional domains are not yet fully understood. To investigate the role of these domains we tested different constructs of Ras-GRF1 for their ability to mediate responses to LPA.

We found that the DPC mutant, in which the first PH domain, the coiled coil and the calcium calmodulin-binding IQ domain were deleted (but the DH-PH2 module and the Ras-GEF catalytic domain were maintained), was impaired in the LPA-induced activation of ERK1. Moreover the PCCQ mutant (PH1 through IQ), when expressed in *trans*, was found to inhibit the LPA-induced activation of ERK1 in fibroblasts stably overexpressing Ras-GRF1. This effect might be due to competition for signaling molecules activated by LPA: the calcium-calmodulin complex [11] and G protein βγ subunits [20] are the most likely candidates. In fact pertussis toxin, inhibitor of G1 proteins, abolishes the effect of LPA on ERK activation in NIH-RG7 cells. Moreover Kiyono et al. [20] recently reported that βγ complex potentiates Ras-GRF1 effect on ERK activity, the first PH domain being essential for this effect.

Others [14] suggest that activation of Ras-GRF1 by LPA requires an increase of calcium and a phosphorylation event. Thus the effect of LPA on the Ras-GRF1-dependent activation of ERK should be the result of βγ release and possibly interaction with Ras-GRF1 [29], increase in calcium (acting

on Ras-GRF1 in complex with calmodulin (Zippel et al., submitted)) and a phosphorylation. With regard to the effect of calcium ionophores on ERK, it has been reported that intact DH and PH2 domains are essential for this response [19,30] while phosphorylation of Ras-GRF1 does not occur [14].

On the other hand the DPC mutant, unable to mediate LPA signaling on ERK, behaves in the same way as the entire molecule in activating the c-fos promoter, a downstream target of different signaling pathways [31], in response to stimulation with LPA. The Mm1 construct (which in addition to the PCCQ also lacks the DH-PH2 module) is unable to do so. Thus the Ras-GRF1-mediated long term effects of LPA require the presence of DH-PH2 module (but do not need the PCCQ region), and in fact the isolated DH-PH2 module is able to activate c-fos in response to LPA.

These observations suggest that activation of the c-fos promoter can occur in the absence of hyperactivation Ras-ERK pathway. Anyway in NIH-3T3 cells even in the absence of Ras-GRF1, LPA induces a certain activation of ERK possibly through the Sos exchange factor [3] and this might contribute to the observed effects.

The effect of isolated DH-PH2 module on gene transcription may be explained by the finding that the same module activates JNK1, as also does the entire Ras-GRF1 molecule, and this effect is further stimulated by LPA. In accordance with this finding it has very recently been reported that Ras-GRF1 activates JNK1, that this effect is potentiated by βγ and that the DH domain is essential for this effect [20].

The activation of JNK1 by the isolated DH-PH2 appears to be mediated by the small GTPase Rac, does not need the activity of PI3K and, since it takes place in the absence of the catalytic domain of Ras-GRF1 and without stimuli, does not seem to require an activated Ras. We found that while the effect of both the isolated DH domain and the DH-PH2 module can be potentiated by LPA, isolated DH displays no constitutive activity. Thus regulation of the DH domain of Ras-GRF1 seems rather different from that of the DH of Sos1 [17]. Moreover the data suggest that the effect of LPA on Ras-GRF1-mediated JNK1 activation passes through a regulation of the DH domain.

In conclusion this work supports the view that, in response to LPA, Ras-GRF1 acts as a bifunctional exchange factor activating Ras/ERK pathway through its Ras-GEF domain and Rac/JNK pathway through DH-PH2 module.

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