A Dual Signaling Cascade That Regulates the Ectodomain Shedding of Heparin-binding Epidermal Growth Factor-like Growth Factor*

Received for publication, April 25, 2001, and in revised form, June 5, 2001
Published, JBC Papers in Press, June 11, 2001, DOI 10.1074/jbc.M103673200

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Ectodomain shedding is an important mechanism to regulate the biological activities of membrane proteins. We focus here on the signaling mechanism of the ectodomain shedding of heparin-binding epidermal growth factor (EGF)-like growth factor (pro HB-EGF). Lysophosphatidic acid (LPA), a ligand for seven-transmembrane G protein-coupled receptors, stimulates the shedding of pro HB-EGF, which constitutes a G protein-coupled receptor-mediated transactivation of the EGF receptor. Experiments using a series of inhibitors and overexpression of mutant forms of signaling molecules revealed that the Ras-Raf-MEK signal is essential for the LPA-induced shedding. In addition, the small GTPase Rac is involved in the LPA-induced shedding, possibly to promote MEK activation. 12-O-Tetradecanoylphorbol-13-acetate is another potent inducer of pro HB-EGF shedding. We also demonstrate that the LPA-induced pathway is distinct from the 12-O-tetradecanoylphorbol-13-acetate-induced pathway and that these pathways constitute a dual signaling cascade that regulates the shedding of pro HB-EGF.

The extracellular domain of a number of membrane proteins can be proteolytically cleaved (1, 2). This proteolytic processing, also referred to as “ectodomain shedding,” is observed in growth factors (1), receptors of growth factors and cytokines (3, 4), cell adhesion molecules (5), extracellular matrix proteins (6), and other membrane proteins such as the β-amyloid precursor protein (7). The ectodomain shedding of membrane protein changes their fate, location, and mode of action; thus, it affects the biological activities of membrane proteins (1, 2, 8–11).

Heparin-binding epidermal growth factor (EGF)-like growth factor (HB-EGF) is a member of the EGF family (12). Like EGF, transforming growth factor-α, and amphiregulin, HB-EGF binds to and stimulates the phosphorylation of the EGF receptor (EGFR). HB-EGF is synthesized as a membrane-anchored precursor protein of 208 amino acids composed of signal peptide, heparin-binding, EGF-like, transmembrane, and cytoplasmic domains. Although the membrane-anchored form of HB-EGF (pro HB-EGF) is cleaved on the cell surface to yield a soluble growth factor of 75–86 amino acids, a considerable amount of pro HB-EGF remains uncleaved on the cell surface (13). Pro HB-EGF is not merely a precursor of the soluble form; it is also biologically active such that it forms complexes with both CD9 (14) and integrin α5β1 (15) and transduces biological signals to neighboring cells in a non-diffusible manner (16). Moreover, although secreted mature HB-EGF (sHB-EGF) is a potent mitogen for a number of cell types (12), pro HB-EGF may act as a negative regulator of cell proliferation (17). Thus, the processing of the juxtamembrane domain of pro HB-EGF into sHB-EGF means that the mode of action of this growth factor is switched from juxtacrine to paracrine, or that its activity with respect to cell growth is inverse.

12-O-Tetradecanoylphorbol-13-acetate (TPA), a potent activator of protein kinase C (PKC), induces ectodomain shedding of a number of membrane proteins (18–20), suggesting that PKC is involved in a cellular signaling pathway for regulated shedding. Ectodomain shedding of pro HB-EGF is also accelerated by TPA (13). As demonstrated for other transmembrane proteins, TPA-induced ectodomain shedding of pro HB-EGF is blocked by hydroxamate metalloprotease inhibitors, indicating that a metalloprotease is involved in TPA-induced shedding (21). We have shown, using monkey kidney Vero-H cells (stable transfectants of Vero cells overexpressing human HB-EGF), that the presence of a constitutively active form of PKCε results in the shedding of pro HB-EGF, whereas kinase-negative PKCδ suppresses TPA-induced shedding (21). MDC9 (ADAM9), a member of the metalloprotease disintegrin family (22), binds PKCδ to the cytoplasmic domain of MDC9 in vivo and in vitro. Overexpression of MDC9 results in the shedding of pro HB-EGF, whereas MDC9 mutants lacking the entire metalloprotease domain or the conserved histidine residues in the metalloprotease domain inhibit the TPA-induced cleavage. These results indicate that PKCδ and MDC9 are involved in the TPA-induced ectodomain shedding of pro HB-EGF (21).

Although TPA is a potent agonist for the ectodomain shedding of pro HB-EGF, physiological ligands also induce ectodomain shedding of pro HB-EGF. G protein-coupled receptor tussis toxin; MAPK, mitogen-activated protein kinase; ERK, extracellular signal-regulated kinase; MEK, MAPK/ERK kinase; CHO, Chinese hamster ovary.
EGF shedding, we studied here the signal transduction mechanism of many GPCR ligands (27, 28). Because of the biological significance of LPA (29) and its marked potency to induce pro HB-EGF shedding, we studied here the signal transduction mechanism through which LPA influences the shedding of pro HB-EGF, using Vero-H cells.

**EXPERIMENTAL PROCEDURES**

**Materials**—TPA, wortmannin, and pertussis toxin were purchased from Nacalai Tesque Co., Ltd. (Kyoto, Japan). LPA was from Cayman Chemical Co., Inc. Ro31-8220 and PD98059 were from Calbiochem Biotechnology and R&D Systems, respectively. Anti-pan-Ras antibody (Ab-3) and anti-Raf antibody (URP26K) were kindly provided by Dr. Yasuhiko Ohashi (Nihon Schering K. K.) and Dr. Motokuni Sugai (Hiroshima University), respectively. KB-R8301 was obtained from Nippon Organon K. K.

**Antibodies**—Goat anti-human HB-EGF carboxy-terminal domain antibody (C-18) and anti-HB-EGF neutralizing antibody were purchased from Santa Cruz Biotechnology and R&D Systems, respectively. Anti-pan-Ras antibody (Ab-3) and anti-Raf antibody (URP26K) were obtained from Calbiochem and Pharmingen, respectively. Anti-FLAG monoclonal antibody (M2), anti-e-Myc monoclonal antibody (9E10), and rabbit anti-hemagglutinin antibody (Y-11) were from Eastman Kodak Co., Oncogene Science Inc., and Santa Cruz Biotechnology, respectively. Horseradish peroxidase-conjugated sheep anti-goat IgG and Cy3-conjugated donkey anti-goat IgG were obtained from Chemicon International, Inc.

**Plasmids**—PKC expression vectors encoding DRKA (dominant-negative (dn) PKC) have been described previously (30, 31). The same antibody to MDC9 expression vectors encoding H347A/H351A (dnMDC9) (21) and FLAG-tagged dnRhoA (N19RhoA), dnRac1 (N17Rac1), and dnCdc42 (N17Cdc42) (29). Hemagglutinin-tagged constitutively active (ca) RhoA (V14RhoA), caRac1 (V12Rac1), and caCdc42 (V12Cdc42) (33) were kindly provided by K. Kaibuchi (Nara Institute of Science and Technology). Ha-Ras, caHa-Ras (V12Ha-Ras), and dnHa-Ras (N17Ha-Ras) were donated by A. Yoshimura and A. Sasaki (Kurume University), and Raf-1, dnRaf-1 (N17Raf-1), and caRaf-1 (RafBXX) were donated by Y. Nagamine (34).

**Cell Culture and Transfection**—Vero-H cells (13) were maintained in modified Eagle’s medium with nonessential amino acids supplemented with heat-inactivated 10% fetal calf serum. All transfections, which used plasmids, were carried out following a previously described method (35).

**Ras and Raf Activation Assays**—Activation of Ras and Raf was assayed using GST-RBD and GST-PAK, respectively, following methods reported previously (36, 37).

**Shedding Assay by Western Blot Analysis**—Vero-H cells (5 × 10⁵ cells) were cultured for 18 h in serum-free modified Eagle’s medium with nonessential amino acids and then treated with the indicated concentrations of LPA or TPA for 30 min. For detection of transmembrane forms and tail fragments of HB-EGF, cells were collected and eluted with SDS gel sample buffer. The eluted materials were subjected to Western blotting and detected with goat anti-HB-EGF neutralizing antibody. The antibodies were visualized with horseradish peroxidase-conjugated anti-goat IgG using ECL-Plus (Amersham Pharmacia Biotech).

**Immunofluorescent Detection of Shedding of the Pro HB-EGF Ectodomain**—Immunostaining of pro HB-EGF was performed with goat anti-HB-EGF neutralizing antibody and Cy3-conjugated goat anti-HB-EGF carboxyl-terminal domain antibody. For detection of shed ectodomain of pro HB-EGF, cells were incubated with 20 ng/ml KB-R8301 for 30 min or untreated) were harvested, and the ectodomain shedding of pro HB-EGF was examined by Western blot analysis using anti-pro HB-EGF carboxy-terminal domain antibody (upper panel). sHB-EGF secreted in the culture medium was also detected by Western blot analysis using anti-pro HB-EGF antibody specific to the EGF-like domain (lower panel).

**RESULTS**

**LPA Is a Potent Inducer of Pro HB-EGF Shedding in Vero-H Cells**—At the beginning of this study, we confirmed the LPA-induced shedding in Vero-H cells by Western blot analysis. Fig. 1 shows the immunoblots of the cell lysates (upper panel) and culture medium (lower panel) of Vero-H cells incubated with either LPA or TPA and detected with anti-pro HB-EGF antibodies that recognize the cytoplasmic domain and the EGF-like domain, respectively. In the cell lysates, bands ranging from 20 to 31 kDa correspond to the transmembrane forms of pro HB-EGF, and the smallest band shown at the position between the 18.7- and 7.2-kDa molecular markers is the proteolytically cleaved fragment comprising the cytoplasmic and transmembrane domains (tail fragment). The addition of LPA strongly induced cleavage and generated a large amount of the tail fragment in the cell lysate (Fig. 1, lane 2). Consistent with the appearance of the tail fragment, sHB-EGF was secreted in the culture medium. The levels of pro HB-EGF shedding induced by LPA and by TPA were similar (Fig. 1, lane 3). A hydroxamic acid-based metalloprotease inhibitor, KB-R8301, completely inhibited both TPA- and LPA-induced cleavage of pro HB-EGF (Fig. 1, lanes 5 and 6, respectively), suggesting that a metalloprotease is involved in both cases.

**Ras-Raf-MEK Cascade Is Involved in LPA-induced Pro HB-EGF Shedding**—LPA activates the small GTPase Ras through the mediation of the βγ-subunits of heterotrimeric G proteins (38). The pull-down assay for Ras activation indicated that LPA

**FIG. 1. Western blot analysis of ectodomain shedding of pro HB-EGF in Vero-H cells.** Vero-H cells were incubated in modified Eagle’s medium with nonessential amino acids for 18 h at 37 °C. Cells (either pretreated with 10 μM KB-R8301 for 30 min or untreated) were incubated with 20 ng/ml LPA or 64 nM TPA for 30 min. Then, the cells were harvested, and the ectodomain shedding of pro HB-EGF was examined by Western blot analysis using anti-pro HB-EGF carboxy-terminal domain antibody (upper panel). sHB-EGF secreted in the culture medium was also detected by Western blot analysis using anti-pro HB-EGF antibody specific to the EGF-like domain (lower panel).
activated Ras endogenous to Vero-H cells 0.5 min after the addition of LPA (Fig. 2A). To investigate whether Ras is involved in the LPA-induced shedding, we examined the effects of the dominant-negative form of Ras. For this assay, Vero-H cells were transfected with plasmids encoding dnHa-Ras (N17Ha-Ras), and then LPA-induced shedding of pro HB-EGF was examined by measuring the immunoreactivity of the cell surface with respect to anti-HB-EGF antibody targeted to the ectodomain of pro HB-EGF (red). The expression of dnRas was detected by anti-pan-Ras antibody (green). The arrows in each image show cells expressing dnHa-Ras or GFP, C, percentage of pro HB-EGF-positive cells among the transfected cells shown in B. The effects of the dominant-negative mutants in LPA-untreated cells are also shown. D, overexpression of Ha-Ras, ca-Raf-1, and caMEK1. Vero-H cells were transfected with plasmids (2 μg) encoding wtHa-Ras (panels a and b), caRaf-1 (panels c and d), Myc-tagged caMEK1 (panels e and f), wtHa-Ras (2 μg) plus dnRaf-1 (8 μg) (panels g and h), caRaf-1 (2 μg) plus dnHa-Ras (8 μg) (panels i and j), and Myc-tagged caMEK1 (5 μg) plus dnRaf-1 (8 μg) (panels k and l). After 48 h of transfection, the cells were double-stained with anti-HB-EGF antibody to detect the pro HB-EGF ectodomain (red) and with anti-pan-Ras antibody to detect cells expressing wtHa-Ras, anti-Raf antibody to detect cells expressing caRaf-1, and anti-Myc antibody to detect cells expressing caMEK1 (green). E, percentage of pro HB-EGF-positive cells among the transfected cells shown in D.

In contrast to the transfection with the dominant-negative forms, overexpression of wtHa-Ras, the constitutively active form of Raf-1 (Raf BXB), or caMEK1 resulted in the shedding of pro HB-EGF in the absence of LPA (Fig. 2, D and E). Transfection of caHa-Ras (V12Ha-Ras) caused cell death, preventing reliable results from being obtained. Cotransfection with a combination of wtHa-Ras and dnRaf-1 resulted in the inhibition of wtHa-Ras-induced pro HB-EGF shedding, whereas dnHa-Ras did not inhibit caRaf-1-induced shedding. Similarly, dnRaf-1 did not inhibit caMEK1-induced shedding.

C3 Exoenzyme from E. coli, known as an activator of Rho and Rac (39), inhibits actin stress fiber formation in Vero-H cells under the conditions examined here (data not shown), it did not inhibit LPA-induced shedding of pro HB-EGF (Fig. 3A). However, toxin B from C. difficile, known to activate Rho, Rac, and Cdc42 (42), prevented LPA-induced shedding of pro HB-EGF (Fig. 3A). Consistent with the results obtained with the toxin B, CNF2 from a uropathogenic strain of Escherichia coli, known as an activator of Rho and Rac (43), stimulated the shedding of pro HB-EGF in the absence of LPA (Fig. 3B).
Vero-H cells were incubated with CNF2 at the indicated concentrations for 30 min. Pro HB-EGF shedding was assayed by Western blotting.

Rho is activated downstream of the LPAR-mediated signaling pathway in fibroblastic cells (39). However, the present results indicate that Rac, but not Rho, is involved in LPA-induced pro HB-EGF shedding. To obtain direct evidence for the activation of Rac by LPA treatment, a pull-down assay using GST-PAK fusion protein was performed. As shown in Fig. 4E, upon stimulation of Vero-H cells with LPA, activated Rac was detected 2.5 min after stimulation, although the level of Rac activation by LPA seemed to be much lower than for Ras.

Ras and Rac Are Synergistically Involved in LPA-induced Pro HB-EGF Shedding—To clarify the relationship between Ras-Raf-MEK signaling and Rac signaling, we performed co-transfections with a combination of wtRas and dnRac, caRaf and dnRac, caMEK and dnRas, and caRac and dnRaf. Transfection with wtHa-Ras or caRaf-1 resulted in shedding as shown in Fig. 2, but the effect of these molecules was inhibited by cotenzyme with dnRac1 (Fig. 5). Although caMEK1 also induced shedding of pro HB-EGF, dnRac1 did not inhibit caMEK1-induced shedding. Conversely, the effect of caRac1 was inhibited by cotenzyme with either dnHa-Ras or dnRaf-1. PD98059 also inhibited caRac1-induced shedding. These results indicate that the Ras-Raf signal and Rac are synergistically required for the shedding of pro HB-EGF upon LPA stimulation. Since caMEK1-induced shedding was not inhibited by dnRac1, MEK activation is critical for the shedding.

LPA-induced Shedding Cascade Is Distinct from the TPA-induced Shedding Cascade—We have previously shown that PKCβ and MDC9 are involved in TPA-induced shedding of pro HB-EGF (21). To compare the signaling cascades of LPA- and TPA-induced shedding, the effects of various inhibitors on the shedding of pro HB-EGF were studied (Fig. 6A). The PKC inhibitor Ro31-8220 (10 μM) strongly inhibited TPA-induced shedding, but weakly inhibited LPA-induced shedding. Conversely, the MEK inhibitor PD98059 (30 μM) strongly blocked LPA-induced shedding, as mentioned above, but not TPA-induced shedding. Wortmannin (100 nM), a phosphatidylinositol 3-kinase inhibitor, did not inhibit either TPA- or LPA-induced shedding. These results suggest that LPA- and TPA-induced shedding is regulated by different signaling pathways. KB-R8301 completely inhibited both TPA- and LPA-induced pro HB-EGF shedding, consistent with the results shown in Fig. 1, indicating that a metalloprotease is involved in both TPA- and LPA-induced shedding of pro HB-EGF.

Next, we examined whether PKCβ and MDC9 were involved in LPA-induced shedding of pro HB-EGF and whether Rac and Ras were involved in TPA-induced shedding. Although both dnPKCβ and dnMDC9 blocked TPA-induced shedding of pro HB-EGF as described previously (21), neither dnRac1 (Fig. 6B) nor dnHa-Ras (data not shown) did. On the other hand, LPA-induced shedding of pro HB-EGF was blocked by dnRac1, but not by dnPKCβ or dnMDC9 (Fig. 6B). These results indicate that the LPA-induced shedding pathway of pro HB-EGF is
distinct from the TPA-induced shedding pathway in Vero-H cells.

**DISCUSSION**

We have investigated the LPA-induced shedding pathway. Fig. 7 depicts a proposed model for the pathways of LPA- and TPA-induced shedding of pro HB-EGF in Vero-H cells. As supported by the expression of LPARs in Vero-H cells and the inhibitory effect of suramin (24), the LPA signal would be mediated by LPARs (of the G protein-coupled seven-transmembrane protein family), followed by the activation of some G proteins in the heterotrimeric G protein family (29, 45). Pseudomonas aeruginosa (PTX) did not inhibit LPA-induced pro HB-EGF shedding (see Fig. 3A), suggesting that PTX-insensitive G proteins are involved in LPA-induced shedding of pro HB-EGF in Vero-H cells. Consistently, LPA-induced PTX-insensitive transactivation was observed previously (46). It was reported that GPCR signals can result in the activation of the small G protein Ras (38). Pull-down assays confirmed that LPA induces Ras activation in Vero-H cells. Using the transfection of mutant forms of Ha-Ras or Raf-1, we demonstrate here that Ras and Raf, situated downstream, are involved in LPA-induced shedding. MEK, a well known downstream component in the Ras-Raf cascade, is also involved in LPA-induced shedding, as was demonstrated by the experiments using caMEK1 and the MEK inhibitor PD98059. In addition to the Ras-Raf-MEK cascade, Rac also contributes to LPA-induced shedding, as it was shown by using inhibitors and a stimulator of the Rac family of GTPases or by transfection with dominant-negative and constitutively active mutants of Rac1. A requirement of both Rac and Ras for the proliferative effects of GPCRs was reported (47), consistent with the present results. We show here that dnRac1 inhibited the effects of both wtHa-Ras and caRaf-1, whereas both dnHa-Ras and dnRaf-1 inhibited caRac1-induced shedding. However, dnRac1 did not suppress the action of caMEK1. These results suggest that activation of MEK is critical for the shedding of pro HB-EGF and that the Ras-Raf signal and Rac are synergistically required to promote MEK activation, as has been indicated previously (48). MEK activates ERK, a member of the MAPK family. We actually observed that ERK activation occurs in Vero-H cells upon LPA stimulation. Therefore, although neither dnERK nor caERK is available, ERK is also postulated to contribute to LPA-induced shedding downstream of MEK.

This study also reveals that the signaling pathway of LPA-induced shedding is distinct from that of TPA-induced shedding. Following from our previous work, where we showed that TPA-induced pro HB-EGF shedding in Vero-H cells is mediated by PKCδ and MDC9 (21), we demonstrate here that LPA-induced shedding was not inhibited by dnPKCδ or dnMDC9, whereas TPA-induced shedding was not inhibited by dnRas, dnRac1, or PD98059. This indicates the different requirements of the signaling molecules of TPA- and LPA-induced shedding.
is possibly regulated by multiple signaling mechanisms. In this regard, identification of the physiological stimulus that activates the TPA-induced pathway would be important in clarifying how the two pathways are used.

This study reveals that GPCR-triggered shedding of pro-HB-EGF is mediated also by the MAPK cascade, as has been previously observed for receptor tyrosine kinase/TPA-induced shedding (49, 50). Receptor tyrosine kinase activation causes stimulation of the MAPK cascade; thus, ligands for receptor tyrosine kinases could also induce the ectodomain shedding of pro HB-EGF in a PKC- and MDC9-independent manner. Actually, we observed that EGF, sHB-EGF, and platelet-derived growth factor weakly induced shedding of pro HB-EGF in Vero-H cells, although the effects were much lower than with LPA (data not shown). EGFR is down-regulated in Vero-H cells, and therefore, EGF ligands may not efficiently induce the ectodomain shedding of pro HB-EGF.

In Chinese hamster ovary cells (CHO), TPA-induced shedding of pro HB-EGF is inhibited by PD98059 (50), suggesting that MEK is implicated in TPA-induced cleavage. However, as shown in this study, TPA-induced shedding of pro HB-EGF in Vero-H cells was not inhibited by PD98059. The discrepancy between the results with CHO cells and Vero-H cells can be explained: in the case of Vero-H cells, PKCδ (among other PKC isotypes) is involved in TPA-induced shedding, whereas in CHO cells, PKCδ does not seem to be involved in TPA-induced shedding because caPKCδ does not induce pro HB-EGF shedding.2 Thus, other isotypes of PKC could be activated by TPA and be involved in TPA-induced shedding in CHO cells. Possibly, the TPA-activated PKC isotype is located upstream of the MEK-mediated shedding pathway in CHO cells, and therefore, the MEK inhibitor blocked the TPA-induced shedding.

Transactivation of EGFR by GPCR ligands is critical for the mitogenic activity of ligands such as LPA, endothelin, carbacol, thrombin, and angiotensin II (45, 46, 51). Recent studies revealed that ectodomain shedding of pro HB-EGF contributes to the transactivation of EGFR by LPA and other GPCR ligands (23). HB-EGF is the only growth factor mediating EGFR transactivation in COS-7 and HEK293 cells when these cell lines are stimulated with LPA or endothelin, suggesting an essential role of HB-EGF among EGFR ligands for transactivation of EGFR. Moreover, not only GPCR ligands, but also insulin-like growth factor-1, which binds to and mediates signals through the insulin-like growth factor-1 receptor, induces HB-EGF-mediated transactivation (26), raising a possibility of

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the common role of HB-EGF in EGFR transactivation. We demonstrate here that MEK activation is critical for LPA-induced ectodomain shedding of pro HB-EGF. Therefore, not only GPCR ligands, but also other factors that activate MEK would have the ability to stimulate the shedding of pro HB-EGF, followed by EGFR transactivation. In fact, EGF and sHB-EGF induce the shedding of pro HB-EGF in Vero-H cells, as mentioned.

It is postulated that shed sHB-EGF causes EGFR activation, which results in activation of the Ras-MEK-ERK pathway. Thus, the Ras-MEK-ERK pathway would be used twice in GPCR-induced transactivation events, i.e. for stimulation of pro HB-EGF shedding and as a consequence of EGFR activation by shed sHB-EGF. Regarding this context, EGFR and ectodomain shedding of pro HB-EGF may constitute a positive feedback loop for the Ras-MAPK signal. A previous report (36) may suggest the presence of such two-cycle signaling cascades in the transactivation events.

GPCR-mediated ectodomain shedding and transactivation of EGFR would be a generally conserved mechanism, from insects to mammals, to efficiently regulate signal transduction through growth factors. SPITZS is the principal ligand of EGFR in embryonic and larval development of Drosophila (8). This protein, like transforming growth factor-α and HB-EGF, is synthesized as a transmembrane protein, but has no activity until the extracellular domain is proteolytically released from the cell surface. SPITZS processing is regulated by two other members of the spitz group, rhomboid and star. RHOMBOID, a seven-transmembrane protein, is thought to regulate the cleavage of SPITZS and to be essential for the efficient activation of the receptor by SPITZS. Moreover, mutations in the genes spitz and rhomboid cause similar pattern alterations in the development process of the Drosophila embryo. The similarity of the SPITZS-RHOMBOID system to the LPAR-HB-EGF system implies the existence of a common mechanism in the signaling pathway.

Acknowledgments—We are grateful to K. Kaibuchi for plasmids encoding constitutively active mutants of RhoA, Rac1, Cdc42, and GST-PAK; A. Yoshimura and A. Sasaki for plasmids encoding wtRas, Ras mutants, and GST-RBD-short; G. Thomas for plasmids encoding Raf-1 and Raf-1 mutants; M. Sugai for CNF2; and K. Yoshino for KB-R8301. We also thank Dr. Y. Ohashi for valuable discussion.

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J. Biol. Chem. 2001, 276:30475-30482.
doi: 10.1074/jbc.M103673200 originally published online June 11, 2001

Access the most updated version of this article at doi: 10.1074/jbc.M103673200

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