Vinexin β Regulates the Anchorage Dependence of ERK2 Activation Stimulated by Epidermal Growth Factor*

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ERK is activated by soluble growth factors in adherent cells. However, activation of ERK is barely detectable and not sufficient for cell proliferation in non-adherent cells. Here, we show that exogenous expression of vinexin β, a novel focal adhesion protein, allows anchorage-independent ERK2 activation. The second and third SH3 domains of vinexin β, but not the SH3 domains, are required for this function of vinexin β. To evaluate the pathway regulating the anchorage dependence of ERK2 activation, we used a dominant-negative mutant of p21-activated kinase (PAK) because PAK and PKA are known to regulate the anchorage dependence of ERK2 activation. Analyses using deletion mutants demonstrated that a linker region between the second and third SH3 domains of vinexin β is important for regulating the anchorage dependence of ERK2 activation. Together, these observations indicate that vinexin β plays a key role in regulating the anchorage dependence of ERK2 activation by the PKA-PAK signaling pathway.

Cell attachment to the extracellular matrix and stimulation by soluble growth factors coordinately regulate numerous cellular events, including cell proliferation. Normal cells require both cell adhesion to the appropriate extracellular matrix and stimulation by growth factors for cell proliferation. Loss of this anchorage dependence of cell proliferation leads to cell malignancy transformation. Integrins and downstream molecules have been reported to be involved in the anchorage dependence of ERK activation. Cell attachment to a matrix, in the absence of growth factors, is sufficient for Ras and Rac (27). Expression of vinexin enhances actin cytoskeletal organization and cell spreading as well as growth factor-induced c-Jun N-terminal kinase (JNK) activation, whereas it does not affect ERK activation in adherent cells (27). These observations suggest that vinexin β plays a key role in regulating the anchorage dependence of ERK activation, the detailed mechanisms of this regulation remain to be determined.

Vinexin is localized at focal adhesions and cell-cell junctions and belongs to a novel vinculin-interacting protein family. Vinexin is transcribed into at least two isoforms, vinexin α and β, of which contain a common carboxy-terminal sequence containing three SH3 domains. The first and second SH3 domains bind to vinculin (19), and the third SH3 domain binds to SoS, a guanine nucleotide exchange factor for Ras and Rac (27). Expression of vinexin enhances actin cytoskeletal organization and cell spreading as well as growth factor-induced c-Jun N-terminal kinase (JNK) activation, whereas it does not affect ERK activation in adherent cells (27). These observations suggest that vinexin β plays a role in regulating both cell adhesion and growth factor signaling, although whether vinexin β is involved in the coordinated regulation of cell adhesion and growth factor signaling or the anchorage dependence of signal transduction is unknown.

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Vinexin, the binding partner of vinexin, has also been reported to be involved in the anchorage dependence of cell proliferation (28–30). This raises the possibility that vinexin may function at a convergence point of cell adhesion and growth factor signaling and contribute to regulation of the anchorage dependence of signal transduction. Here, we examined the function of vinexin β in regulating the anchorage dependence of cell proliferation. Activation of ERK by growth factor stimuli is a prerequisite for cell proliferation and is also anchorage-dependent (5–11). In adherent cells, soluble growth factors such as epidermal growth factor (EGF) stimulate their receptor tyrosine kinase, leading to GTP loading of Ras, which in turn activates the Raf-MEK-ERK kinase cascade. Activated ERK then stimulates the transcription of growth-related genes such as c-fos through the activation of transcription factors. However, in non-adherent cells, the activation of ERK by growth factor stimuli is barely detectable and is not sufficient for cell proliferation.

The abbreviations used are: ERK, extracellular signal-regulated kinase; MEK, MAPK/ERK kinase; EGF, epidermal growth factor; PAK, p21-activated kinase; JNK, c-Jun N-terminal kinase; GFP, green fluorescent protein; PKA, PKA-dependent protein kinase; RD, regulatory domain.

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ERK activation. Our results indicate that vinexin β plays a role in PAK-mediated anchorage-dependent signaling.

EXPERIMENTAL PROCEDURES

Plasmids—The expression plasmids for FLAG-tagged vinexin β and the mutant of the third SH3 domain of FLAG-tagged vinexin β (3rdSH3WF) were described previously (19, 27). Mutations of the first SH3 domain (substitution of tryptophan 76 with phenylalanine, 1stSH3WF) and of the second SH3 domain (substitution of tryptophan 150 with phenylalanine, 2ndSH3WF) of vinexin β were introduced by PCR-based methods, respectively. The first and second SH3 domain containing 25 mM HEPES (pH 8.0), 10 mM MgCl₂, 1 mM dithiothreitol, 150 with phenylalanine, 2ndSH3WF was digested with EcoRI and NotI, and 2ndSH3WF was digested with NotI and XhoI. The two digested fragments were ligated into the EcoRI/XhoI sites of p401F (19). To make ΔSH3 (amino acids 1–271), N-Half (amino acids 1–172), C-Half (amino acids 173–283), and ΔLinker (amino acids 1–172, 252–328), the corresponding regions of vinexin β were amplified by PCR and then subcloned into p401F. All plasmids generated by PCR were confirmed by DNA sequencing. Plasmids containing N17Cdc42 (31) were generous gifts from Drs. H. Teramoto and J. S. Gutkind. (NIDCR, National Institutes of Health). The plasmid containing PAK/RD (32) was a generous gift from Drs. S. Hirai and S. Ohno (Yokohama City University, Yokohama, Japan). The GFP-tagged ERK2 expression plasmid was constructed by subcloning ERK2 cDNA from pCG-HA-ERK2 (33), provided by Dr. K. Nose (Showa University, Tokyo, Japan), into pGZ21 (19).

Antibodies—Anti-FLAG epitope monoclonal antibody M2, anti-phospho-ERK MAPK antibody, and anti-ERK2 antibody were obtained from Sigma, New England Biolabs Inc., and Santa Cruz Biotechnology, respectively.

Cell Culture and Transfection—NIH3T3 cells were cultured with Dulbecco’s modified Eagle’s medium supplemented with 10% calf serum. NIH3T3 cells were transfected with GFP-ERK2 and other expression plasmids using LipofectAMINE Plus (Invitrogen). Transfected cells were then serum-starved by incubation with medium containing 0.5% calf serum for 20 h. For experiments in cell suspension, cells were detached by trypsinization, washed twice with Dulbecco’s modified Eagle’s medium containing 2 mg/ml soybean trypsin inhibitor, and resuspended in OPTI-MEM (Invitrogen) containing 0.2% bovine serum albumin. In some experiments, the PKA inhibitor H89 (Sigma) was added to the media. Cells were incubated in suspension with gentle rocking for 3 h. Adherent and suspended cells were then serum-starved and stimulated with the indicated concentrations of EGF for 5 min. NIH3T3 cells cotransfected with GFP-ERK2 and vector or vinexin β were serum-starved and stimulated with the indicated concentrations of EGF for 5 min. The activity of GFP-ERK2 was measured as described for A, B, and C. GFP-ERK2 was cotransfected with GFP-ERK2 and vector or vinexin β were serum-starved and placed in suspension for the indicated times. After stimulation with 100 ng/ml EGF for 5 min, the activity of GFP-ERK2 was measured as described for A.

RESULTS

Expression of Vinexin β Induces Anchorage-independent ERK2 Activation—To determine whether vinexin β is involved in the anchorage dependence of ERK2 activation, we first generated GFP-tagged ERK2 to allow detection of activated ERK2 in cotransfection experiments. Activated GFP-ERK2 can be separated from endogenous ERK2 by SDS-PAGE without immunoprecipitation and detected using anti-active ERK antibody because GFP-ERK2 migrates slower on SDS-polyacrylamide gel than endogenous ERK2. Expressed GFP-ERK2 was strongly activated by EGF stimulation in adherent NIH3T3 cells, but was barely activated in suspended cells, similar to endogenous or hemagglutinin-tagged ERK2 (Fig. 1, A, C, and D; and data not shown). An immune complex kinase assay for GFP-ERK2 verified the results obtained by Western blotting using anti-active ERK antibody (see Fig. 2A and data not shown). These observations indicate that both GFP-ERK2 and endogenous ERK2 respond to EGF in an anchorage-dependent manner in NIH3T3 cells and that GFP-ERK2 behaves naturally.

Using GFP-ERK2, we determined the effects of vinexin β on the anchorage dependence of ERK2 activation in NIH3T3 cells. GFP-ERK2 was efficiently activated by EGF stimulation in adherent cells, but was barely activated in suspended cells, as described above. However, expression of vinexin β permitted ERK2 activation in suspended cells to 50% of the levels in adherent cells (Fig. 1, A and B). Interestingly, expression of vinexin β did not activate ERK2 without EGF stimulation or alter ERK2 activity in adherent cells (Fig. 1, A and B). Consistent with our previous report (27). To confirm the results obtained with anti-active ERK antibody, we performed an immune complex kinase assay for detecting GFP-ERK2 activity (Fig. 2). Again, expression of vinexin β increased the GFP-ERK2 activity in suspended cells (p < 0.05), but had no effect in adherent cells (Fig. 2). These observations suggest that vinexin β is involved in the anchorage dependence of ERK2 activation. To verify that vinexin β does not activate ERK2 directly, various concentrations of EGF were used to stimulate NIH3T3 cells transfected with vector alone or FLAG-tagged vinexin β under adherent conditions (Fig. 1C). Vinexin β-transfected cells showed the same ability to activate ERK2 as vector-
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Vinexin induces the anchorage-independent activation of GFP-ERK2 activity. A, GFP-ERK2 was cotransfected with vector alone or FLAG-tagged vinexin β into NIH3T3 cells. Cells were then serum-starved and maintained on tissue culture plates (adherent (Adh)) or were placed in suspension (Sus) for 3 h before stimulation with 100 ng/ml EGF for 5 min as indicated. Two µg of lysates were directly immunoblotted (IB) with anti-ERK2 antibody (lower panel) to verify expression of GFP-ERK2. The remaining lysates (200 µg) were immunoprecipitated with anti-GFP antibody, and the immune complexes were subjected to ERK2 kinase assay using myelin basic protein as a substrate. The phosphorylated substrates were resolved by SDS-PAGE and visualized by autoradiography (upper panel). B, quantification of the GFP-ERK2 kinase assays is shown graphically. Values are expressed as -fold increase with respect to EGF-stimulated vector-transfected adherent cells and represent the means ± S.D. from four independent experiments.

transfected cells at any concentration of EGF under adherent conditions. These results suggest that vinexin β is involved in the anchorage dependence of ERK2 activation rather than in the direct activation of ERK2.

We examined the duration of the anchorage-independent ERK2 activation induced by expression of vinexin β. NIH3T3 cells transfected with FLAG-tagged vinexin β were incubated in suspension for various times and then stimulated with EGF. As shown in Fig. 1D, ERK2 could not be activated in the vector-transfected cells after incubation in suspension for 5 h in contrast, ERK2 was activated to some degree in vinexin β-transfected cells even after incubation in suspension for 5 h. This observation indicates that expression of vinexin β can sustain the capacity for EGF-induced ERK2 activation in suspension. Furthermore, to confirm that cells incubated in suspension for 3 h were still alive and not dead due to anoikis, cells incubated in suspension for 3 h were allowed to re-adhere to tissue culture plates and then stimulated with EGF. These cells showed EGF-induced ERK2 activation equivalent to cells continuously maintained in adherent culture (data not shown), suggesting that vinexin β is involved in the anchorage dependence of ERK2 activation and not in preventing anoikis under these conditions. Altogether, these observations show that expression of vinexin β can substitute for cell adhesion at least partially and that it allows anchorage-independent ERK2 activation in response to EGF.

A Linker Region between the Second and Third SH3 Domains of Vinexin β Is Necessary for Anchorage-independent ERK2 Activation—The first and second SH3 domains of vinexin β bind to vinculin, and the third SH3 domain binds to Sos. Therefore, we first used mutants of each SH3 domain of vinexin β to test whether any of these domains might be involved in the anchorage independence of ERK2 activation (Fig. 3). All mutants were expressed at similar levels compared with wild-type vinexin β in NIH3T3 cells (data not shown). As shown in Fig. 3, expression of vinexin β with a mutation in the third SH3 domain (3rdSH3WF) allowed ERK2 activation induced by EGF stimulation in suspension. We next tested mutants of the first SH3 domain (1stSH3WF), the second SH3 domain (2nd SH3WF), and both domains (1st2ndSH3WF), which show induced vinculin-binding ability (2). All of these mutants also permitted ERK2 activation in an anchorage-independent manner (Fig. 3). These observations suggest that the functions of SH3 domains are dispensable for the anchorage-independent ERK2 activation induced by expression of vinexin β.

To identify the functional domain of vinexin β involved in anchorage-independent ERK2 activation, we constructed various deletion mutants of vinexin β (Fig. 4A). The mutants were expressed at comparable levels in NIH3T3 cells, except the C-Half mutant, which was significantly expressed at low levels (data not shown). The Δ3SH3 and C-Half mutants lack the third SH3 domain or the first and second SH3 domains, respectively. Both mutants contain a linker region between the second and third SH3 domains (Fig. 4A). These mutants allowed ERK2 activation in suspended cells (Fig. 4B). The effect of Δ3SH3 on the anchorage independence of ERK2 activation was significantly greater than that of wild-type vinexin β. In contrast, the N-Half and ΔLinker mutants, which lack the linker region, failed to activate ERK2 in suspended cells. Furthermore, these deletion mutants slightly but significantly reduced the efficient activation of ERK2 even in adherent cells (Figs. 4B and 5C), possibly by dominant-negative blockade of cell adhesion signals, whereas the mutants containing the linker region (Δ3SH3 and C-Half) permitted increased ERK2 activation under the same conditions (Fig. 4B). These results indicate that the linker region between the second and third SH3 domains plays important roles in supporting anchorage-independent ERK2 activation in response to EGF stimulation and that Δ3SH3 and ΔLinker function as an active and a negative mutant, respectively.

Vinexin β Functions in the PKA-PAK Signaling Pathway Regulating Anchorage-dependent ERK2 Activation—We next searched for proteins that may link vinexin β and the anchorage dependence of ERK2 activation. We first determined the effect of vinexin β or Δ3SH3 on focal adhesion kinase, which has been implicated in the anchorage dependence of ERK activation (18). However, expression of vinexin β did not affect the protein levels and tyrosine phosphorylation of GFP-tagged

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**Fig. 4.** The linker region between the second and third SH3 domains is necessary for activating ERK2 in suspended cells. A, shown is a schematic diagram of deletion mutants of vinexin β. GFP-ERK2 was cotransfected with vector alone, FLAG-tagged wild-type vinexin β (w.t.), or deletion mutants (Δ3SH3, C-Half, N-Half, and ΔLinker) into NIH3T3 cells. Cells were then serum-starved and maintained on tissue culture plates or were placed in suspension for 3 h before stimulation with 100 ng/ml EGF for 5 min. Cell lysates were immunoblotted (IB) using anti-active ERK (phospho-ERK (p-ERK)) antibody to detect the activated forms of GFP-ERK2 and anti-ERK2 antibody to detect total GFP-ERK2 protein. Results representative of at least three independent experiments are shown.

**Fig. 5.** Vinexin β is involved in anchorage-dependent ERK2 activation regulated by the PKA-PAK pathway. A, GFP-ERK2 was cotransfected with vector alone, the active mutant of vinexin β (Δ3SH3), or the dominant-inhibitory mutant of PAK (PAK/RD) into NIH3T3 cells. Cells were incubated for 3 h on tissue culture plates (adherent (Adh)) or in suspension (Sus). Cell lysates were immunoblotted using anti-active ERK (phospho-ERK (p-ERK)) antibody to detect the activated forms of GFP-ERK2 and anti-ERK2 antibody to detect total GFP-ERK2 protein. B, shown are the dose-dependent effects of the PKA-specific inhibitor H89. NIH3T3 cells were incubated for 3 h on tissue culture plates or in suspension with or without the indicated concentrations of H89. Cell lysates were immunoblotted using anti-active ERK and anti-ERK2 antibodies. Results representative of at least three independent experiments are shown.

Vinexin is a novel focal adhesion protein that binds to vinculin and regulates cell adhesion and growth factor-mediated signaling (19, 27). Here, we have shown that vinexin β is involved in the anchorage dependence of ERK2 activation.

**DISCUSSION**

We have recently identified vinexin β as a novel focal adhesion protein that binds to vinculin and regulates cell adhesion and growth factor-mediated signaling (19, 27). Here, we have shown that vinexin β is involved in the anchorage dependence...
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Vinexin lacks the binding site for Sos, still supporting anchorage-independent ERK2 activation elicited by inhibition of PKA as well as ERK2 activation in adherent cells. Third, the anchorage-independent ERK2 activation induced by vinexin β was inhibited by a dominant-negative mutant of PAK, which is known to play a role in the anchorage dependence of ERK2 activation (17, 32). These observations indicate that vinexin β is involved in the anchorage dependence of ERK2 activation.

In this study, we demonstrated that vinexin β functions upstream of PAK for regulating the anchorage dependence of ERK2 activation. PAK activation itself has been shown to be anchorage-dependent (17, 39), whereas constitutively active PAK allows anchorage-independent ERK2 activation (17). Therefore, regulation of PAK activity is likely to play a role in the anchorage dependence of ERK2 activation. PAK is activated by binding with GTP-bound Cdc42 or Rac (38, 40) and by binding with other PAK-binding proteins (41, 42). PAK-binding proteins, including Nck and PIX, have been shown to activate PAK through physical interaction and/or recruitment of PAK to the membrane (41, 42). Human vinexin (GenBankTM/EBI accession number AF037261) was also reported to bind to PAK in the GenBankTM/EBI Data Bank. Therefore, one possible mechanism for regulating the anchorage dependence of ERK2 activation by vinexin β is that vinexin β may support the efficient activation of PAK by binding or recruiting it to the membrane under adherent conditions.

We showed that the linker region between the second and third SH3 domains plays a role in regulating the anchorage dependence of ERK2 activation. The function of this region is unclear so far. There are three PXXP sequences, core sequences for SH3 domain binding, in the linker region. Interestingly, CAP (c-Cbl-associated protein/ponsin, another member of the vinexin family, contains PQQP sequence in the linker region between the second and third SH3 domains (22, 24). This region actually binds to the SH3 domains of Grb4/Nckβ, an adaptor molecule that binds to PAK (43). Therefore, it is possible that the linker region of vinexin β functions as a protein-interacting domain and regulates PAK activity indirectly. Indeed, we have isolated several proteins that bind to the linker region using yeast two-hybrid screening.

Vinexin β binds to Sos, a guanine nucleotide exchange factor for Ras and Rac, and enhances EGF-induced JNK activation in NIH3T3 cells (27). However, the vinexin β-Sos interaction is not likely to be involved in the anchorage dependence of ERK2 activation. Vinexin β binds to Sos via its third SH3 domain (27). Mutation of the third SH3 domain disrupts binding to Sos and the ability to enhance JNK (27). Moreover, vinexin β with a mutation in the third SH3 domain inhibits EGF-induced JNK activation in a dominant-negative fashion (27). In contrast, the same mutation of the third SH3 domain did not affect the function of vinexin β in its regulation of the anchorage dependence of ERK2 activation. The vinexin β mutant ΔSH3, which lacks the binding site for Sos, still supported anchorage-independent ERK2 activation in response to EGF stimulation. These observations suggest that vinexin β regulates ERK2 and JNK by different mechanisms.

Vinexin, a vinexin-binding protein, is also involved in the anchorage dependence of cell proliferation (28–30). One hypothesis regarding the role of vinexin-vinulin interaction is that vinexin β is localized at focal adhesions by binding to vinculin and recruits signaling molecules such as PAK under adherent conditions, whereas in suspension culture, vinexin β does not bind to vinculin and does not recruit signaling molecules to the membrane. Indeed, vinculin binding of vinexin is regulated by cell adhesion.4 This model is compatible with the finding that the 1stSH3WF and 2ndSH3WF mutants, which do not bind to vinculin, still induced adhesion signaling. Loss of the membrane-localizing and PAK-activating abilities of Rac1 is overcome by overexpression of Rac1 (39). In our system, overexpression of vinexin mutants may overcome the loss of vinculin-binding and membrane-localizing abilities.

In conclusion, we have demonstrated that vinexin β can regulate the anchorage dependence of ERK2 activation through PAK. Expression of vinexin β allows ERK2 activation stimulated by EGF without cell anchorage.

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