ERK1/2 Associates with the c-Met-binding Domain of Growth Factor Receptor-bound Protein 2 (Grb2)-associated Binder-1 (Gab1)

ROLE IN ERK1/2 AND EARLY GROWTH RESPONSE FACTOR-1 (Egr-1) NUCLEAR ACCUMULATION*

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Endothelial cell (EC) migration contributes to reendothelialization after angioplasty or rupture of atherosclerotic plaques. Extracellular signal-regulated kinase (ERK)1/2 translocates to the nucleus and activates transcription factors such as Ets-like transcription factor-1 and early growth response factor-1 (Egr-1) during reendothelialization. Because ERK1/2 does not possess a nuclear localization signal (NLS), its mechanism of translocation and accumulation in the nucleus remains unclear. Because Gab1 has a putative NLS in its N-terminal region, and Gab1 associates with phosphorylated ERK1/2, we hypothesized that Gab1 participates in ERK1/2 and Egr-1 nuclear accumulation. Using regenerating EC as a model system, we found that endogenous growth factor receptor-bound protein 2-associated binder-1 (Gab1) translocates into the nucleus in migrating EC. Wild-type red fluorescent protein-tagged Gab1 could be observed in both nucleus and cytoplasm, whereas the putative NLS deletion mutant (ΔNLS-Gab1) specifically localized in the cytoplasm. In addition, reduction of Gab1 expression by antisense Gab1 oligos or overexpression of ANLS-Gab1 inhibited serum-induced ERK1/2 and Egr-1 nuclear accumulation, suggesting a functional role for the NLS of Gab1 and a role for Gab1-ERK1/2 interactions in ERK1/2-Egr-1 nuclear accumulation. To investigate whether Gab1-ERK1/2 interaction is critical for ERK1/2 and Egr-1 nuclear accumulation, we created a dominant-negative Gab1 construct that consisted of the c-Met binding domain (amino acids 442–536) of Gab1. We found that overexpression of the c-Met binding domain of Gab1 disrupted serum-induced Gab1-ERK1 interaction and inhibited ERK1 and Egr-1 nuclear accumulation. These data suggest that Gab1-ERK1/2 binding and their nuclear translocation play a crucial role in Egr-1 nuclear accumulation.

Endothelial cell (EC) migration is important for angiogenesis, wound repair, tumor growth, and re-endothelialization after angioplasty or rupture of atherosclerotic plaques. Wound repair after arterial injury involves migration and proliferation of EC and is associated with increased expression of platelet-derived growth factor (PDGF)-A and -B, tissue factor, and fibroblast growth factor-2 (1–3). It has been reported that extracellular signal-regulated kinase (ERK1/2) and its downstream transcription factor early growth response (Egr-1) regulate expression of PDGF-A and PDGF-B as well as fibroblast growth factor-2 and tissue factor (1–3). Khachigian et al. (2) demonstrated an immediate and transient increase in the expression of the transcription factor Egr-1 exclusively in EC at a wound edge after gentle injury of the rat aorta. Because Egr-1 expression and its nuclear translocation are dependent upon ERK1/2 activation (4–6), the activation of ERK1/2 and Egr-1 seems to be critical for regulating the expression of several growth factors, cytokines, and coagulation factors in ECs at a wound edge (7).

Although ERK1/2 is widely involved in eukaryotic signal transduction, the mechanism by which ERK1/2 nuclear translocation is regulated remains unclear. Adachi et al. (8, 9) have reported that mitogen-activated protein kinase kinase 1/2 acts as an anchoring molecule for the cytoplasmic localization of ERK1/2. Inactive mitogen-activated protein kinase kinase 1/2 containing the nuclear-exclusion signal sequence associates with ERK1/2, and after mitogen-activated protein kinase kinase 1/2 is activated, ERK1/2 is released and activated by phosphorylation. Khokhlatchev et al. (10, 11) have reported that the nuclear accumulation of ERK1/2 depends upon its phosphorylation state rather than upon its activity, but they also found that the phosphorylation of ERK1/2 induces its dimerization, which is required for its nuclear location. Because ERK1/2 does not possess a nuclear localization signal (NLS), it remains unknown how ERK1/2 translocates into the nucleus after its phosphorylation. Because proteins larger than ~40–50 kDa cannot pass through the nuclear pore by simple diffusion (12, 13), it is most likely that other mechanisms are involved in the nuclear translocation of dimeric ERK1/2. Whitehurst et al. (14) have suggested that ERK1/2 associates directly with components of the nuclear pore. It is also possible that ERK1/2 interacts with other molecules containing an NLS to translocate into the nucleus.

Because Gab1 has a putative NLS at its NH2-terminal domain and can associate with phosphorylated ERK1/2 (15), we hypothesized that Gab1 mediates ERK1/2 nuclear transloca-

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The abbreviations used are: EC, endothelial cell; PDGF, platelet-derived growth factor; Grb2, growth factor receptor-bound protein 2; Gab1, Grb2-associated binder-1; ERK, extracellular signal-regulated kinase; Egr-1, early growth response factor-1; NLS, nuclear localization signal; MBD, MET-binding domain; BAEC, bovine aortic endothelial cells; CHO, Chinese hamster ovary; WT, wild-type; Gab1-RFP, Gab1 deletion mutant of amino acids 1–179 tagged with RFP; oligo, oligonucleotide; HA, hemagglutinin; CRIS, cytoplasmic retention signals.
tion. Gab1 is a member of the docking protein family, and has binding motifs such as pleckstrin homology domain at its NH2-terminal region, several proline-rich SH3 domain-binding sequences, as well as tyrosine phosphorylation sites for potential Src homology 2-binding molecules such as Grb2, Src homology 2 domain-containing protein tyrosine phosphatase-2 (SHP-2), and phosphatidylinositol 3-kinase. An important domain in Gab1 is the c-MET-box, rich in Met-binding (MB) domain, which mediates the interaction of Gab1 with the c-MET receptor (16). Interestingly, Roshan et al. (15) have reported that Gab1 can associate directly with phosphorylated ERK2 via the MBD and act as a substrate for ERK1/2. However, the functional consequence of this association remains unknown. In the present study we show that the Gab1-ERK1/2 interaction via the MBD is critical for ERK1/2 nuclear translocation and the subsequent induction of Egr-1 expression and nuclear translocation.

MATERIALS AND METHODS

Cell Culture—Fetal bovine aortic endothelial cells (BAEC) were purchased from Clonetics Corp and maintained in Medium 199 (Cellgro) supplemented with 10% fetal bovine serum as described previously. Chinese hamster ovary (CHO) cells were cultured in Ham’s F-12 (Cellgro) supplemented with 10% fetal bovine serum. BAEC and CHO cells were serum deprived for 3 days before treatment with 10% serum to induce EC migration to reendothelialize injured vessels. Because Gab1 can also translocate to the membrane, as we described previously (18), we tried to decrease the contamination of membrane compartment in nuclei-representative fraction as much as possible. Therefore, although we found contamination of proliferating cell nuclear antigen in the Triton-soluble fraction, we used this fractionation method to separate membrane compartment.

Immunofluorescence Microscopy—Cells were fixed with 3.7% formaldehyde for 10 min followed by permeabilization with 0.05% Triton X-100. They were blocked with 10% normal goat serum (Vector) and treated with primary antibodies, polyclonal anti-ERK1 (Santa Cruz Biotechnology) or monoclonal anti-ERK1 (BD Biosciences), phospho-ERK1/2 (Cell Signaling Technology), Gab1 (Upstate Biotechnology), Gab1 (Santa Cruz Biotechnology), Egr-1 (Santa Cruz Biotechnology), Xpress (Invitrogen), and ERK5 antibody (kindly provided by Dr. Jiing-Dwan Lee; Ref. 20) for 45 min at room temperature. Secondary antibodies labeled with Alexa dye against rabbit IgG or mouse IgG were purchased from Molecular Probes. Images were obtained using a fluorescence microscope (Olympus) equipped with a charge-coupled device camera and an Acroplan Water 40, 60XW lens.

RESULTS

Gab1 Translocates to the Nucleus with ERK1/2 and Egr-1 in BAEC—Because ERK1/2 and Egr-1 activation are critical for the expression of growth factors and inflammatory mediators (2, 7, 21), we determined whether ERK1/2 and Egr-1 are activated during endothelial cell migration by detecting ERK1 and Egr-1 localization. At 24 h after scratch wound assay, we found that both ERK1 and Egr-1 had translocated to cell nuclei at the wound edge (Fig. 1a). We then surveyed the wound margin area with antibodies to several different signaling molecules, including SHP-2, Gab1, Src, and PECAM-1, which are known to regulate ERK1/2-Egr-1 activation (18, 22, 23). Among these molecules, we found that Gab1, ERK1, and Egr-1 accumulated in a similar manner in the nucleus of BAEC at the wound edge area (Fig. 1, a and b). No signal was detected when only secondary antibody was used (data not shown). Because Roshan et al. (15) have reported that Gab1 associates with activated ERK1/2, we hypothesized that Gab1 nuclear translocation may be needed for ERK1/2 and Egr-1 nuclear accumulation in migrating cells.

Nuclear Translocation of Gab1, ERK1/2, and Egr-1—Because EC migration to reendothelialize injured vessels occurs in the presence of platelet aggregation and degranulation, we studied the effect of serum on Gab1 nuclear translocation. As shown in Fig. 2a, Gab1 translocates to the nucleus after 1 h of 10% serum stimulation and maintains its nuclear localization for 24 h. ERK1 and Egr-1 show similar nuclear accumulations after serum stimulation (Fig. 2, b and c). Both ERK1 and Egr-1 appear in the nucleus after 30 min, peak at 1 h, and are sustained after 24 h of serum stimulation. These data suggest that nuclear translocation of Gab1, ERK1, and Egr-1 induced by serum is similar at the wound edge area in migrating endothelial cells.

To determine whether Gab1 nuclear translocation in proliferating cells is a universal characteristic, we determined the nuclear translocation and accumulation of Gab1, ERK1, and Egr-1 in CHO cells. Gab1 localized in cytoplasm in serum-deprived CHO cells. After 10% serum stimulation, Gab1 translocated to the membrane as described previously (24). We also found significant Gab1 nuclear accumulation after 10 min of serum stimulation, which was sustained for more than 4 h after stimulation (Fig. 2d and data not shown). To confirm the specificity of this Gab1 antibody (Upstate Biotechnology), which
was produced with a synthetic C-terminal peptide corresponding to residues 664–694 of human Gab1, we compared endogenous Gab1 staining and translocation with another Gab1 antibody (Santa Cruz Biotechnology), which was produced from a different epitope corresponding to amino acids 119–316 of human Gab1. We found similar staining patterns of Gab1 with these two different antibodies (data not shown). To further confirm the specificity of these Gab1 antibodies for endogenous Gab1, we used antisense Gab1 oligos and determined whether these antisense Gab1 oligos, which were specifically designed to inhibit Gab1 expression, could reduce immunostaining detected by the Gab1 antibodies used in this study (Fig. 2g). We found that antisense Gab1 oligos could significantly decrease Gab1 immunostaining, compared with random oligo-transfected cells as shown in Fig. 2g. These data support the specificity of anti-Gab1 antibodies, which we used in this study, to detect endogenous Gab1 expression and localization. As shown in Fig. 2i, we found that Gab1 and ERK1 expression were increased in Triton-insoluble fractions by serum stimulation, which also supports Gab1 and ERK1 nuclear translocation into the nucleus.

Finally, we found that ERK1 and Egr-1 nuclear translocation and accumulation in CHO cells was accompanied with Gab1 in response to 10% serum as shown in Fig. 2, e and f. These data suggest that Gab1, ERK1, and Egr-1 nuclear translocation and accumulation is a universal phenomenon in migrating and proliferating cells and is not specific to EC. ERK1 and Egr-1 nuclear translocation and accumulation were faster in CHO cells than in BAEC (10 versus 30 min). The faster nuclear translocation of Gab1 in CHO cells may be responsible for faster nuclear translocation and accumulation of ERK1 and Egr-1 (see Fig. 6).

**NLS and Cytoplasmic Retention Signals (CRS) in Gab1**—Because a putative NLS (amino acids 15–23) was observed in the Gab1 sequence, we evaluated its function by generating an NLS deletion mutant of Gab1 (ΔNLS-Gab1) (Fig. 3a) and transfected it into CHO cells. Wild-type Gab1 tagged with RFP (WT-Gab1-RFP) localized to the nucleus, cytoplasm, and plasma membrane (Fig. 3b). In contrast, in cells transfected with ΔNLS-Gab1-RFP, we could not detect any fluorescent signal in the nucleus, and the signal was mainly localized in cytoplasm (Fig. 3c). These results suggest that the NLS domain of Gab1 is functional. To identify the CRS in Gab1, we generated several deletion mutants of Gab1 containing NLS (Fig. 3a). Gab1 (amino acids 1–99) localized only in the nucleus (Fig. 3d), whereas Gab1 (amino acids 1–422) could be detected in both nucleus and cytoplasm (Fig. 3e), suggesting that the region of amino acids 99–442 of Gab1 contains a CRS. Because the Gab1 fragment (amino acids 1–99) is smaller than 20 kDa, it is possible that Gab1 could translocate to the nucleus independently of the NLS (12, 13). Therefore, we generated two different RFP-tagged Gab1-deletion mutants containing the N-terminal region of Gab1 with or without NLS, Gab1-RFP, and ΔNLS-Gab1-RFP, respectively (Fig. 3a). ΔNLS-Gab1-RFP signals were not found in the nucleus (Fig. 3g). In contrast, Gab1-RFP accumulated in the nucleus in more than 20% of the transfected cells (Fig. 3f). Importantly, Gab1-RFP (amino acids 1–179) is longer than ΔNLS-Gab1-RFP (amino acids 23–179) (Fig. 3a). Of note, because the RFP-tag contains 226 amino acids, the total length of Gab1-RFP is similar to Gab1-Xpress (amino acids 1–422). Therefore, the different localization of Gab1-Xpress and Gab1-RFP is not due to the size of these molecules. These data suggest that Gab1 has functional NLS and CRS domains, which determine Gab1 cellular localization independently of the size of the deletion mutants.

As shown in Fig. 3, when WT-Gab1-RFP transfected cells were cultured at low confluence (<30%), we could detect WT-Gab1-RFP signals in both cytoplasm and nucleus. Because endogenous Gab1 accumulated in the nucleus in migrating and...
serum-stimulated cells, the localization of exogenously overexpressed WT-Gab1-RFP was slightly different from endogenous Gab1. However, when the cells became confluent, WT-Gab1-RFP translocated from the nucleus to the cytoplasm (Fig. 3, b versus h and i). This finding is consistent with endogenous Gab1 localization in confluent areas, as we observed at wound edge (Fig. 1). This cell contact-mediated Gab1 translocation from nucleus to cytosol also suggests a functional role of the CRS domain of Gab1.

**Gab1 Associates and Regulates ERK1/2 Localization**—To de-

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**Fig. 2. Serum-induced nuclear translocation of Gab1, ERK1, and Egr-1 in BAEC and CHO cells.** a–c, BAECs were subjected to serum starvation for 16 h and were stimulated by 10% serum. The time courses for nuclear translocation and accumulation of Gab1 (a), ERK1 (b), and Egr-1 (c) were detected by immunofluorescence. d–f, CHO cells were subjected to serum starvation for 2 days and were stimulated by 10% serum. The time courses for nuclear translocation and accumulation of Gab1 (d), ERK1 (e), and Egr-1 (f) were detected by immunofluorescence. g, antisense Gab1 oligo, but not random S-oligo, reduced Gab1 expression in CHO cells. h, reduction of Gab1 expression by antisense S-oligo Gab1 inhibited ERK1 nuclear translocation in CHO cells. Signals from double-immunofluorescence staining using anti-Gab1 and anti-ERK1 were observed in the same cells. The intensity of immunofluorescence of ERK1 was compared in cytoplasm with that in nucleus, and nuclear accumulations of ERK1 were determined when the intensity in the nucleus was higher than that in cytoplasm. i and j, serum increased Gab1 and ERK1 expression in Triton-insoluble fraction in BAEC (i) and CHO cells (j), and reduction of Gab1 expression decreased serum-induced ERK1 expression in Triton-insoluble fraction. Expressions of proliferating cell nuclear antigen, caveolin, and β-actin in both fractions were determined by Western blot analysis.
etermine whether Gab1 can regulate ERK nuclear translocation, we transfected ΔNLS-Gab1 in CHO cells and determined serum-mediated endogenous ERK1/2 activation and nuclear translocation. To detect the nuclear translocation, the intensities of immunofluorescence of Gab1, ERK1, and Egr-1 were compared in cytoplasm to nucleus by image analysis, and the nuclear accumulation of Gab1, ERK1, and Egr-1 was determined. As shown in Fig. 4a, WT-Gab1-RFP localized in both nucleus and cytoplasm, whereas ΔNLS-Gab1-RFP mainly localized in the cytoplasm as described in Fig. 3.

When cells were transfected with WT-Gab1-RFP, ERK1 translocated to the nucleus after 10% serum stimulation (Fig. 4a), and this nuclear translocation was also detected by anti-phospho-ERK1/2 antibody (Fig. 4c), which was similar to the findings in non-transfected cells (Fig. 2). In contrast, serum-induced nuclear translocation of ERK1 and phospho-ERK1/2 was significantly inhibited by the expression of ΔNLS-Gab1 (Fig. 4, b and d). We also counted the number of the cells that were transfected by Gab1 wild-type or mutants and then determined what percentage of the total transfected cells revealed nuclear translocation (Fig. 4, e and f). We found that expression of ΔNLS-Gab1 significantly decreased the ratio of ERK1 or phosphorylated ERK1/2 nuclear-accumulated cells per ΔNLS-Gab1-RFP-transfected cells, compared with those in WT-Gab1-RFP-transfected cells. These results suggest a critical role for Gab1 nuclear translocation in regulating ERK1/2 nuclear translocation, but not for ERK1/2 phosphorylation.

As shown in Fig. 4g, we also found that serum-induced ERK1/2 phosphorylation was not inhibited by ΔNLS-Gab1. In addition, we did not find any significant difference in serum-induced ERK5 nuclear translocation between WT-Gab1-transfected (Fig. 4h) and ΔNLS-Gab1-transfected (Fig. 4i) cells. These data suggest that the regulatory effect of Gab1 is specific for ERK1/2 nuclear translocation, but not for ERK1/2 kinase activation and ERK5 nuclear translocation.

**Gab1-MBD Disrupts Gab1/ERK Association and Inhibits ERK1/2 and Egr-1 Nuclear Accumulation**—Because Roshan et al. (15) have reported that Gab1 associates with ERK1/2 via the Gab1 MBD domain, we next determined whether the expression of the Gab1-MBD domain fragment can disrupt Gab1 and ERK1 association and the subsequent ERK1 and Egr-1 nuclear accumulation. CHO cells were co-transfected with Xpress-tagged Gab1-MBD or vector alone with HA-tagged ERK2, co-immunoprecipitated with anti-HA antibody, and immunoblotted with anti-Gab1 antibody. As shown in Fig. 5, a and c, overexpression of the Gab1-MBD domain fragment (Fig. 5a), but not the Gab1-ΔMBD fragment (Fig. 5c) containing the N-terminal domain of Gab1 (except the MBD domain) (Fig. 3a), inhibited serum-induced Gab1 and HA-ERK2 association (Fig. 5a). The Gab1-MBD fragment did not inhibit ERK1/2 activation (Fig. 5a), suggesting a specific effect of the Gab1-MBD fragment upon Gab1-ERK1/2 interaction. To examine the role
of Gab1-ERK1/2 interaction for serum-induced ERK1 and Egr-1 nuclear translocation, we expressed the Gab1-MBD-Xpress fragment (Fig. 5, b and e) or Gab1-H9004-MBD-Xpress fragment (Fig. 5, d and f), and determined ERK1 (Fig. 5, b, d, and g) and Egr-1 (Fig. 5, e, f, and h) nuclear accumulation after serum stimulation. ERK1 (Fig. 5, b and g) and Egr-1 (Fig. 5, e and h) nuclear accumulation were significantly inhibited in Gab1-MBD fragment-transfected cells. In contrast, the nuclear accumulation of ERK1 (Fig. 5, d and g) and Egr-1 (Fig. 5, e, f, and h) was not affected by Gab1-H9004-MBD domain expression. Furthermore, we found that reduction of Gab1 expression by antisense Gab1 oligos significantly inhibited serum-induced ERK1 nuclear accumulation compared with random oligos in a high transfection efficiency conditions (60–80%) (Fig. 2h). We also observed a similar inhibition of ERK1 nuclear accumulation by antisense Gab1 under low transfection efficiency conditions (30–40%, and data not shown), suggesting that the inhibition of ERK1 nuclear accumulation by antisense Gab1 is specific to down-regulation of Gab1 expression, and this is not due to the condition of a high dose of antisense transfection. To determine the role of Gab1 on ERK1 translocation by cell fractionation using Western blot analysis, we do need to knock down Gab1 expression by at least 60–70%. Therefore, we used the high transfection efficiency conditions, and we found that 80% of cells were transfected (Fig. 2g) and the Gab1 expression of the culture was decreased by 70% (Fig. 2g). In this case, the serum-induced ERK1 expression in the Triton-insoluble fraction was significantly decreased by the reduction of Gab1 (as shown in Fig. 2j). Taken together, these results suggest that Gab1 and ERK1/2 association are critical for ERK1/2 and Egr-1 nuclear translocation and accumulation.

**DISCUSSION**

In the present study, we found that Gab1 regulates the active import of phosphorylated ERK1/2 into the nucleus. To our knowledge, this is the first report to document the nuclear translocation of Gab1. We determined that Gab1 has functional NLS and CRS domains. Gab1 associates with phosphorylated ERK1/2 by means of the Gab1 MBD. Importantly, disruption of Gab1 and phosphorylated ERK1/2 interaction by transfection of a Gab1-MBD construct prevented endogenous ERK1 and Egr-1 nuclear translocation and accumulation. These data suggest the importance of Gab1-ERK1/2 interaction for ERK1/2 and Egr-1 nuclear accumulation in migrating and proliferating cells (Fig. 6). It remains unclear how Gab1 switches the activity of NLS and CRS. Because Gab1 has many serine, threonine, and tyrosine phosphorylation sites and associates with many regulators of phosphorylation (e.g. SHP-2 and PI-3K), phosphorylation may change Gab1 conformation and switch the activity of NLS and CRS.

Because proteins smaller than 40–50 kDa readily diffuse into the nucleus (12, 13), it has been presumed that monomeric ERKs enter the nucleus by diffusion. However, Khokhlatchev et al. (11) reported that dimerization of ERKs is essential for...
ligand-dependent nuclear accumulation, and nuclear accumulation of ERK2 depends upon its phosphorylation state rather than on its activity. Although active dimeric ERK1/2 is too large for unrestricted diffusion into the nucleus, upon introduction of active dimeric ERK1/2 into the cytoplasm, there is rapid accumulation in the nucleus. These data strongly suggest that ERK1/2 dimers are actively imported (10, 11), despite the fact that ERK1/2 does not contain a nuclear localization signal. Therefore, it has been proposed that dimerization of ERK1/2 may expose a binding site for a protein containing a NLS (11) and be imported by a piggy-back mechanism because of the association with NLS-containing protein(s) (13). Although Whitehurst et al. (14) have shown that ERK2 binds directly to nucleoporins, which are the components of the nuclear pore and mediate the entry and exit of transport factors by an energy- and carrier-independent mechanism, the presence of an active transport system is also suggested (11) by the association of ERK1/2 with one or more proteins that contain an NLS (14). Therefore, we propose that Gab1 is one of the active proteins that transports ERK1/2 into the nucleus.

Fig. 4. Inhibition of ERK1 nuclear translocation by the expression of Δ-NLS-Gab1-RFP. Wild-type Gab-RFP or Δ-NLS-Gab-RFP was expressed in CHO cells. Transfectants were treated with serum for 15 min. Signals from RFP and immunofluorescence staining using anti-ERK1 (a, b) and anti-phospho-ERK1/2 (c, d) were observed in the same cells. Δ-NLS-Gab-RFP inhibits ERK1 nuclear translocation (b, d) but not ERK1/2 activation in cytoplasm (d). e and f, the ratio of ERK1 (e) or Egr-1 (f) nuclear-accumulated cells per WT-Gab1-RFP or Δ-NLS-Gab1-RFP transfected cells. g and h, Δ-NLS-Gab-RFP does not inhibit ERK2 activation and ERK5 nuclear translocation. g, FLAG-ERK2 was transiently co-expressed with Δ-NLS-Gab1-RFP (Δ-NLS-Gab1) or wild-type Gab1-RFP (WT-Gab1) into serum-starved CHO cells. Transfectants were treated with serum for 15 min. Cell lysates were immunoprecipitated (IP) with anti-FLAG followed by immunoblotting with anti-phospho-ERK1/2 and anti-FLAG. h and i, as described in Fig. 4, a and b, CHO cells expressing WT-Gab-RFP (h) or Δ-NLS-Gab-RFP (i) were treated with 10% serum for 15 min and then stained with anti-ERK5.
Egr-1 promoter contains five serum-response elements, and transcriptional activation by extracellular signals is often mediated by serum-response elements or Ets elements (4, 25). The interaction between serum response factor and ternary complex factors, such as Ets-like transcription factor-1 and sphingolipid activator protein-1, at serum-response elements regulates Egr-1 expression. Because both Ets-like transcription factor-1 and sphingolipid activator protein-1 are phosphorylated and activated by c-Jun NH₂-terminal kinase and ERK1/2 kinase activation in nucleus, ERK1/2 activation and nuclear translocation are clearly two of the critical mediators for Egr-1 transcriptional activity and its expression (2, 6, 7, 26).

Egr-1 plays a positive regulatory role in the inducible expression of many endothelial genes which influence chemotactic, proliferative, and thrombogenic events associated with atherogenesis (7). These include growth factors such as PDGF-A and PDGF-B chains, fibroblast growth factor-2, and transforming growth factor-β (2, 27–30). Egr-1 also regulates the expression of...
cytokine and adhesion molecules, such as tumor necrosis factor-α and intercellular adhesion molecule 1 (31, 32). In addition, Egr-1 regulates tissue factor and urokinase-type plasminogen activator (2, 33). These findings suggest that Egr-1 is an important nuclear mediator in signal transduction to regulate multiple pathophysiologically relevant genes in response to vascular injury. In the present study, we found that Gab1 nuclear translocation is critical for serum-induced ERK1/2 and Egr-1 nuclear translocation and accumulation, suggesting a potential role of Gab1 in endothelial damage and subsequent atherogenesis.

Recent studies have shown that the duration of ERK1/2 signaling is critical for immediate early gene-mediated signaling and subsequent cell-cycle progression. When ERK1/2 activation is transient and not sustained, immediate early gene product c-Fos is unstable and rapidly degraded. In contrast, sustained ERK1/2 activation phosphorylates and stabilizes c-Fos, and promotes c-Fos-mediated cellular transformation (34). We found that Gab1 and ERK1 were still localized to the nucleus up to 4 h after serum stimulation (data not shown). Gab1 and ERK1/2 associate and persistently localize in the nucleus, suggesting that Gab1 may also be important for anchoring ERK1/2 in the nucleus (Fig. 6). Further studies are required to clarify the anchoring mechanism of ERK1/2 in the nucleus.

We found that overexpression of Gab1 seems to “flatten” the cells (Figs. 3 and 4). Because Gab1 is also associated with membrane-localized molecules, including tyrosine kinase receptor and SHP-2, and several focal adhesion molecules, it is reasonable to think that Gab1 overexpression may affect the focal adhesion molecules and change structure proteins and cell shapes. This field is a very interesting one to investigate, and further investigation is necessary to clarify this phenomenon.

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