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Hypoxia Induces Activation and Subcellular Translocation of Focal Adhesion Kinase ($p125^{FAK}$) in Cultured Rat Cardiac Myocytes

Yoshinori Seko,*,†,‡ Naoyuki Takahashi,* Hisataka Sabe,§ Kazuyuki Tobe,¶ Takashi Kadowaki,¶ and Ryozo Nagai*

**Department of Cardiovascular Medicine and ¶Department of Metabolic Diseases, Graduate School of Medicine, University of Tokyo, Tokyo; †Department of Immunology, School of Medicine, Juntendo University, Tokyo; ‡Institute for Adult Diseases, Asahi Life Foundation, Tokyo; and §Department of Molecular Biology, Osaka BioScience Institute, Osaka, Japan*

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We previously reported that hypoxia caused rapid activation of RAS/mitogen-activated protein kinase (MAPK) pathway, two other stress-activated MAPK family members, stress-activated protein kinase (SAPK) and p38MAPK, and Src family tyrosine kinases, $p60^{c\text{-}src}$ and $p59^{c\text{-}fyn}$ in cultured rat cardiac myocytes. In this study, to elucidate how hypoxia affects adhesive interaction between cardiac myocytes and extracellular matrix (ECM), we investigated the molecular mechanism of the activation of focal adhesion-associated tyrosine kinases $p125^{FAK}$ and paxillin. Here, we show that hypoxia induced tyrosine phosphorylation of $p125^{FAK}$ and paxillin and that hypoxia-induced activation of $p125^{FAK}$ was accompanied by its increased association with adapter proteins Shc and GRB2, and non-receptor type tyrosine kinase $p60^{c\text{-}src}$. Furthermore, hypoxia caused subcellular translocation of $p125^{FAK}$ from perinuclear sites to the focal adhesions. These results strongly suggest that $p125^{FAK}$ is one of the most important components in hypoxia-induced intracellular signaling in cardiac myocytes and may play a pivotal role in adhesive interaction between cardiac myocytes and ECM.

Cardiac myocytes are known to quickly respond and adapt to environmental stresses such as ischemia, mechanical load, and metabolic changes by expressing a number of various genes. As for ischemic (hypoxic) stresses, we previously investigated the intracellular signaling cascades in cultured rat cardiac myocytes subjected to hypoxia in vitro, and showed that hypoxia causes rapid sequential activation of mitogen-activated protein kinase kinase kinase (MAPKKK) activity of Raf-1, MAP kinase kinase (MAPKK), MAPKs (also called extracellular signal-regulated protein kinases [ERKs]), and S6 kinase ($p90^{rsk}$) as well as $p21^{ras}$ and Src family tyrosine kinases, $p60^{c\text{-}src}$ and $p59^{c\text{-}fyn}$ (1, 2). In addition to the classical MAPK pathway, we also reported that hypoxia causes two other MAPK family members, stress-activated protein kinase (SAPK) (also called Jun N-terminal kinase [JNK]) and p38MAPK, which are known to exist in the signal transduction pathways, especially in response to pro-inflammatory cytokines and environmental stresses (3).

Adhesive interactions between cells and the extracellular matrix (ECM) play a pivotal role in the cell morphology, motility, and growth as well as gene expression in a variety of cell types, especially in the immune system (4, 5). Integrins are heterodimeric transmembrane proteins composed of α and β subunits. It has been known that members of $\beta 1$ or $\beta 3$ integrin family act as cell surface receptors for ECM protein ligands including fibronectin, laminin, and collagens, to link between the ECM with the actin cytoskeleton and also transduce extracellular signals to the cell interior. For these purposes, integrins can be clustered on the ventral surfaces of adherent cells to form structures known as "focal adhesions". Several studies demonstrated that integrin-mediated cell adhesion causes increased tyrosine phosphorylation of a protein localized to focal adhesions, termed focal adhesion kinase ($p125^{FAK}$) (5). It was shown that tyrosine phosphorylation of $p125^{FAK}$ can be increased by integrin-mediated cell adhesion and $pp60^{v\text{-}src}$ transfor-

Abbreviations used: DMEM, Dulbecco's modified Eagle's medium; ECM, extracellular matrix; ERK, extracellular signal-regulated protein kinase; FAK, focal adhesion kinase; JNK, Jun N-terminal kinase; mAb, monoclonal antibody; MAPK, mitogen-activated protein kinase; MAPKK, mitogen-activated protein kinase kinase; MAPKKK, mitogen-activated protein kinase kinase kinase; PAGE, polyacrylamide gel electrophoresis; PDGF, platelet-derived growth factor; PMSF, phenylmethylsulflonyl fluoride; PVDF, polyvinylidene difluoride; SAPK, stress-activated protein kinase; SH, Src homology; VEGF, vascular endothelial growth factor.

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mation as well as neuropeptides such as bombesin, vasopressin, and endothelin. Another focal adhesion-associated tyrosine kinase, paxillin was also shown to be activated by these stimuli. $p21^{rac1}$ is one of the small GTP-binding proteins, and belongs to the rho subfamily of ras-related proteins. In phagocytic cells, such as neutrophils and monocytes, $p21^{rac1}$ has been shown to activate an NADPH oxidase to generate superoxide (6). In other cell types, such as fibroblasts, $p21^{rac1}$ stimulates actin filament accumulation at the plasma membrane, forming membrane ruffles (7). A new serine/threonine protein kinase $p65^{PAK}$, was identified as a target for $p21^{rac1}$ (8). $p21^{rac1}$ has been shown to regulate p38MAPK through downstream mediator $p65^{PAK}$ (9). Growth factors such as platelet-derived growth factor (PDGF), epidermal growth factor, and insulin, whose receptors are tyrosine kinases, were shown to initially activate $p21^{rac1}$, which stimulates membrane ruffling, and also leads to $p21^{rho}$ activation, which stimulates assembly of focal adhesions and stress fiber formation (7, 10). We previously reported that hypoxia also activates $p65^{PAK}$ in cardiac myocytes, suggesting the involvement of $p21^{rac1}$ and $p21^{rho}$-dependent pathway (3).

In this study, to elucidate in more detail the mechanisms of cardiac response to the hypoxic stresses, especially focused on the adhesive interaction between cardiac myocytes and ECM, we investigated whether hypoxia activates focal adhesion-associated tyrosine kinases, $p125^{FAK}$ and paxillin. Here, we show that hypoxia activates $p125^{FAK}$ and paxillin and also increases the association of $p125^{FAK}$ with adaptor proteins GRB2 and Shc as well as c-Src. Furthermore, hypoxia causes subcellular translocation of $p125^{FAK}$ from the perinuclear region to the focal adhesions, where it works.

MATERIALS AND METHODS

Cell culture. Primary culture of ventricular cardiac myocytes were prepared from the neonatal rats as previously described (1). Briefly, heart ventricles were aseptically removed from neonatal Wistar rats, minced in calcium-free phosphate-buffered saline (PBS), and digested with 0.025% trypsin-EDTA in PBS. The isolated cardiac myocytes were washed in a Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum, dispersed into plastic dishes for 1 h to separate the fibroblasts, and removed to new gelatin-coated culture dishes. They were cultured for two days until they were confluent. After culturing in a serum-free medium (DMEM) for 24 h, the cells were subjected to hypoxia.

Hypoxia. Hypoxic condition (95% N_2, 5% CO_2, and less than 0.1% O_2) was achieved by using an anaerobic jar equipped with a new type AnaeroPack (disposable O_2 absorbing and CO_2 generating agent, Mitsubishi Gas Chemical, Japan) and an indicator to monitor oxygen depletion as described previously (1). By placing flasks, which contain serum-free medium, in an anaerobic jar overnight, the medium was balanced with the hypoxic atmosphere. Cultured cardiac myocytes were serum-starved for 24 h and subjected to a hypoxic condition by immediately replacing the medium with the hypoxic medium in an anaerobic jar. To keep hypoxic conditions, all the procedures were performed in a glove bag filled with 95% N_2 and 5% CO_2.

Western blot analyses for phosphorylation of $p125^{FAK}$ and paxillin. Cardiac myocytes were subjected to hypoxia for the indicated time periods, then they were frozen in liquid nitrogen and lysed on ice with buffer A (25 mmol/L Tris/HCl [pH 7.6], 25 mmol/L NaCl, 1 mmol/L Na_3VO_4, 10 mmol/L sodium pyrophosphate, 10 nmol/L okadaic acid, 0.5 mmol/L EGTA, 1 mmol/L phenylmethylsulfonyl fluoride [PMSF]) and 1% Nonidet P-40. The cell lysates were centrifuged and the supernatants containing detergent soluble proteins were collected. The cell lysates were immunoprecipitated with a rabbit anti-$p125^{FAK}$ polyclonal antibody (11) or a mouse anti-paxillin monoclonal antibody (mAb) (349; Transduction Laboratories, KY, USA) along with protein G-Sepharose (Pharmacia LKB) at 4°C over night. The immunoprecipitates were washed once with lysis buffer and eluted by boiling in Lemli's sample buffer for 5 min. The eluted proteins were subjected to SDS-polyacrylamide gel electrophoresis (PAGE) and transferred onto polyvinylidene difluride (PVDF) transfer membranes (NEN Research Products, MA, USA), then blotted with a mouse anti-phosphotyrosine mAb. (4G10; Upstate Biotechnology Inc., NY, USA). To confirm that equal amounts of $p125^{FAK}$ or paxillin protein were immunoprecipitated in each reaction, the same membranes were stripped by incubating in a solution containing 100 mmol/L 2-mercaptoethanol, 2% SDS, and 62.5 mmol/L Tris-HCl (pH 6.8) for 30 min at 50°C, then blotted with the anti-$p125^{FAK}$ polyclonal antibody or the anti-paxillin mAb. The antibody-antigen complexes were developed with chemiluminescence using alkalinephosphatase (Phototope-Star Detection Kit, New England Biolabs, Inc., Beverly, MA, USA).

Western blot analyses for the association of $p125^{FAK}$ with adaptor proteins GRB2 and Shc as well as c-Src. Cardiac myocytes were subjected to hypoxia for the indicated time periods and lysed with buffer A and 1% Nonidet P-40. Then, the cell lysates were immunoprecipitated with a rabbit anti-GRB2 polyclonal antibody (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA), or a mouse anti-Shc mAb (PG-797; Santa Cruz Biotechnology, Inc.), or a mouse anti-v-Src mAb (2-17; Quality Biotech, Camden, NJ, USA) along with protein G-Sepharose (Pharmacia LKB) at 4°C over night. The immunoprecipitates were washed once with lysis buffer and eluted by boiling in Lemli's sample buffer. The eluted proteins were subjected to SDS-PAGE and transferred onto PVDF transfer membranes, then blotted with the anti-$p125^{FAK}$ polyclonal antibody. The antibody-antigen complexes were developed with chemiluminescence using alkalinephosphatase.

Immunocytochemistry. To investigate the subcellular distribution of $p125^{FAK}$ in cardiac myocytes and fibroblasts, we prepared cultured cardiac myocytes without separating the contaminating fibroblasts. Cardiac myocytes were subjected to hypoxia for 20 min, then the culture media were aspirated immediately and fixed in 3.7% paraformaldehyde in PBS for 5 min at room temperature, and then permeabilized with 0.5% Triton X-100 in Hepes buffer (20 mmol/L Hepes [pH 7.4], 50 mmol/L NaCl, 3 mmol/L $MgCl_2$) for 5 min at 4°C. We performed double staining for $p125^{FAK}$ and F-actin. The cells were incubated first with rabbit anti-$p125^{FAK}$ polyclonal antibody for 1 h at 37°C, then incubated sequentially with biotinylated anti-rabbit IgG (Vector Laboratories, Inc., Burlingame, CA, USA) for 1 h at 37°C and fluorescein isothiocyanate-conjugated avidin D (Vector Laboratories, Inc.) for 30 min at 37°C. For double staining of F-actin, the cells were then incubated with rhodamine-conjugated phalloidin (Molecular Probes, Inc., Eugene, OR, USA) for 1 h at 37°C. The sections were examined and photographed under a fluorescence microscope (Microphoto-FX, Nikon, Tokyo, Japan).

Subcellular fractionation. Cardiac myocytes were subjected to hypoxia for 60 min. Cytosolic and membrane fractions were prepared from control cardiac myocytes and hypoxia-treated cardiac myocytes according to a modification of previously published methods (12, 13). Briefly, cells were washed twice with PBS and then harvested in the buffer containing 20 mmol/L Hepes and 250 mmol/L sucrose (pH 7.4) followed by centrifuging at 1000 × g for 3 min. The supernatants were discarded and the pellets were dissolved in hypotonic Tris buffer containing 10 mmol/L Tris (pH 7.5), 1 mmol/L $MgCl_2$, 50

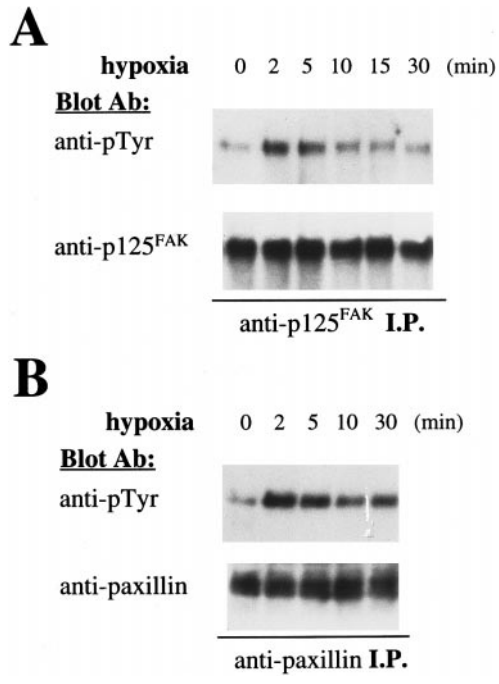

FIG. 1. Hypoxia phosphorylates p125^{FAK} and paxillin. Serum-starved cardiac myocytes were subjected to hypoxia for the indicated time periods, and lysed in buffer A and 1% Nonidet P-40. Cell extracts were immunoprecipitated with anti-p125^{FAK} antibody (A) or anti-paxillin mAb (B), and the immunoprecipitates were electrophoresed on SDS-polyacrylamide gels, transferred to a membrane, and Western blotted with an anti-phosphotyrosine mAb (A and B, upper panels), or with the anti-p125^{FAK} antibody (A, lower panel) or the anti-paxillin mAb (B, lower panel). The antibody-antigen complexes were visualized by alkalinephosphatase reaction. Each result shown represents one typical experiment.

μg/mL leupeptin, 1 mmol/L PMSF, and 1 mmol/L Na_3VO_4. They were homogenized by 80 strokes of Dounce homogenizer, and were centrifuged at 1000 $\times$ g for 15 min. The resulting pellets were suspended in hypotonic Tris buffer with 1% Nonidet P-40 and stored as nucleic fractions. And, the supernatants were centrifuged at 48,000 $\times$ g for 30 min. Then, the resulting pellets were suspended in hypotonic Tris buffer with 1% Nonidet P-40 and stored as plasma membrane fractions. The resulting supernatants were recentrifuged at 246,000 $\times$ g for 90 min to separate microsome fractions. Then, the resulting supernatants were collected as cytosolic fractions. Nucleic, cytosolic, and plasma membrane fractions were also immunoprecipitated with anti-p125^{FAK} polyclonal antibody and protein G-Sepharose, then subjected to SDS-PAGE followed by immunoblotting with anti-p125^{FAK} polyclonal antibody. All steps above were carried out at 4°C unless otherwise indicated. The intensity of p125^{FAK} at the 125 kD bands was quantified by scanning densitometry.

Statistics. Statistical comparisons of control group with treated groups were carried out using the unpaired t-test with p values corrected by the Bonferroni method. Values of $p < 0.05$ were considered significant.

RESULTS

Hypoxia Activates p125^{FAK} and Paxillin

As shown in Fig. 1A (upper panel), hypoxia significantly increased tyrosine phosphorylation of p125^{FAK}. The maximal phosphorylation of p125^{FAK} occurred at 2 to 5 min of hypoxia. We confirmed that almost equal amounts of p125^{FAK} protein were electrophoresed in each reaction (Fig. 1A, lower panel). As shown in Fig. 1B (upper panel), hypoxia also significantly increased tyrosine phosphorylation of paxillin. The maximal phosphorylation of paxillin occurred at 2 to 5 min of hypoxia. We confirmed that almost equal amounts of paxillin protein were electrophoresed in each reaction (Fig. 1B, lower panel). Thus, hypoxia rapidly activated both p125^{FAK} and paxillin as early as 2 min of stimulation.

Hypoxia Increases Association of p125^{FAK} with c-Src, and Adaptor Proteins Shc and GRB2

The phosphorylation of p125^{FAK} has been shown to be stimulated by integrin-mediated cell adhesion as well as in Rous sarcoma virus (pp60$^{v\text{-}src}$)-transformed cells. Recent studies have demonstrated that tyrosine-autophosphorylated pp125^{FAK} directly interacts with pp60$^{c\text{-}src}$ and pp59$^{c\text{-}fyn}$ as one of their major substrates (14, 15). To determine whether c-Src plays a role in hypoxia-induced tyrosine phosphorylation of p125^{FAK}, we examined the effect of hypoxia on the association of p125^{FAK} with c-Src. As shown in Fig. 2 (upper panel), hypoxia caused significant increase in the association of p125^{FAK} with c-Src, which reached a maximal level at 10 min of hypoxia.

Next, to determine whether hypoxia-activated p125^{FAK} signaling complexes contained other known Src homology (SH) 2-containing proteins, such as SH2/SH3 adapter proteins GRB2 and Shc, we also examined hypoxia-induced association of p125^{FAK} with these

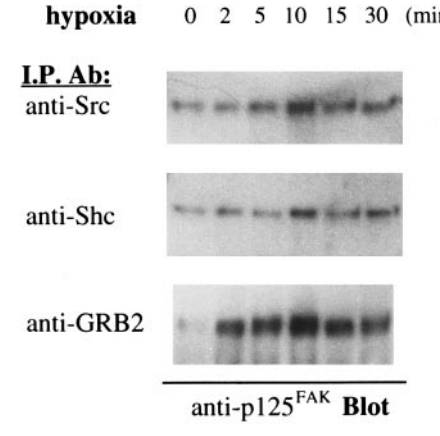

FIG. 2. Hypoxia induces association of p125^{FAK} with p60$^{c\text{-}src}$, Shc, or GRB2. Serum-starved cardiac myocytes were subjected to hypoxia for the indicated periods. Cells were subsequently lysed with buffer A and 1% Nonidet P-40, and immunoprecipitated with anti-v-Src mAb (upper panel), anti-Shc mAb (middle panel), or anti-GRB2 antibody (lower panel). The immunoprecipitates were Western blotted with anti-p125^{FAK} antibody. The results shown are representative of three independent experiments.

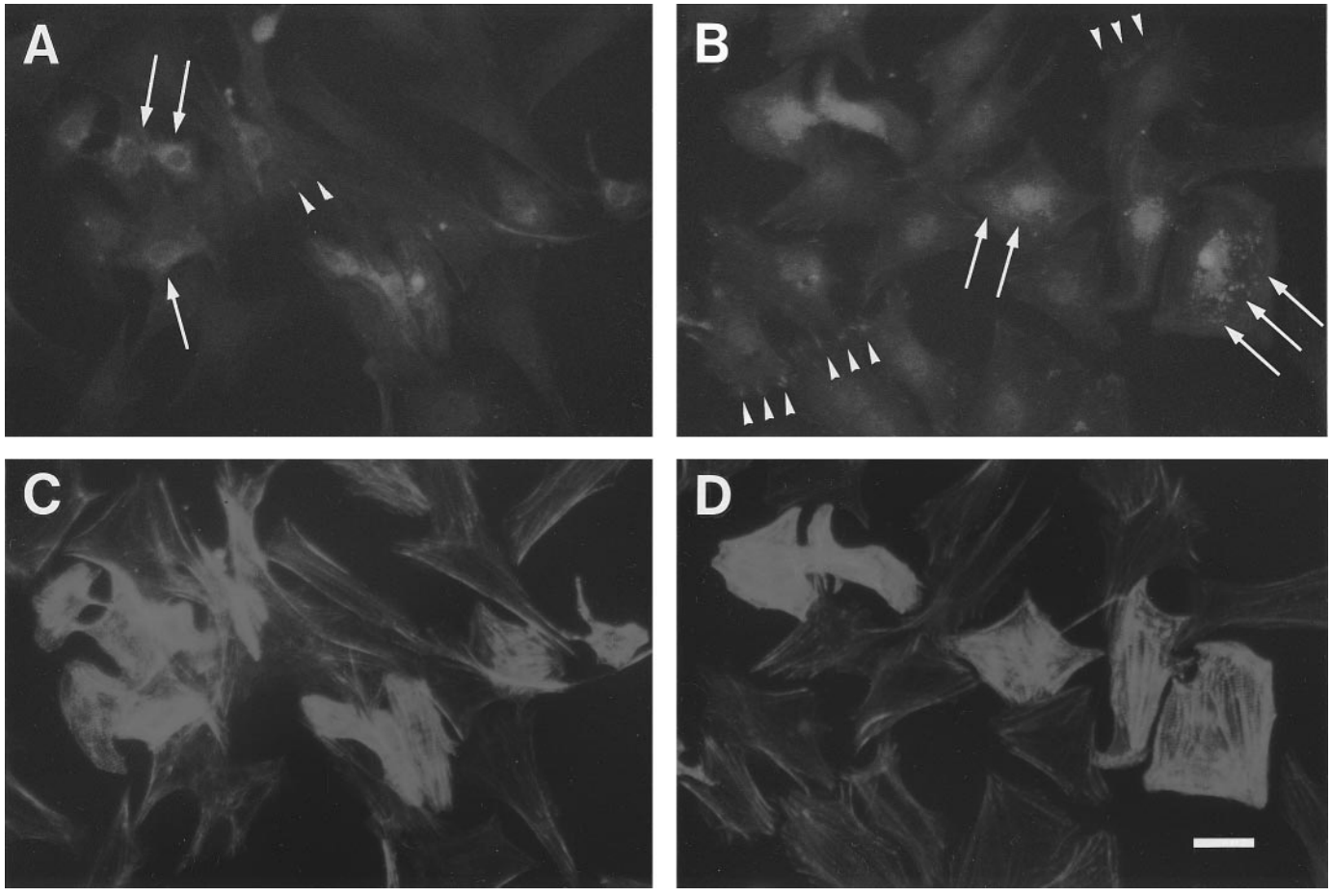

FIG. 3. Hypoxia alters the subcellular localization of $p125^{FAK}$ in cardiac myocytes and fibroblasts. Serum-starved cardiac myocytes and fibroblasts were either not subjected (A and C) or subjected to hypoxia for 20 min (B and D), fixed with paraformaldehyde, permeabilized, and incubated with anti-$p125^{FAK}$ antibody, then sequentially incubated with biotinylated anti-rabbit IgG and fluorescein isothiocyanate-conjugated avidin-D (A and B). The same cells were double-stained for F-actin with rhodamine-phalloidin (C and D, respectively). Arrows and arrowheads indicate the localization of $p125^{FAK}$ in cardiac myocytes and fibroblasts, respectively, either not subjected (A) or subjected to hypoxia for 20 min (B). Scale bar, 10 μm.

adapter proteins. As shown in Fig. 2 (middle and lower panels), hypoxia again caused significant increase in the association of $p125^{FAK}$ with Shc and GRB2, respectively. Both of the association of $p125^{FAK}$ with Shc and GRB2 reached a maximal level at 10 min of hypoxia.

Hypoxia Induces Subcellular Translocation of $p125^{FAK}$ in Cardiac Myocytes and Fibroblasts

It has been shown that cell adhesion to ECM, such as fibronectin, through integrins causes increased tyrosine phosphorylation of $p125^{FAK}$ as well as accumulation of $p125^{FAK}$ in focal adhesions. Furthermore, structural analyses revealed $p125^{FAK}$ to be purely a cytoplasmic protein because it does not contain a transmembrane domain or any obvious sites for conjugation of membrane lipid anchors (16–18). Therefore, we examined the effects of hypoxia on the subcellular localization of $p125^{FAK}$ in cardiac myocytes and fibroblasts. To distinguish cardiac myocytes from fibroblasts and to investigate the relationship between the organization of myofibrils or actin stress fibers and $p125^{FAK}$, we did double-staining for F-actin and $p125^{FAK}$ by immunofluorescence. Figure 3 (C and D) shows that myofibrils of cardiac myocytes were strongly stained, whereas actin stress fibers of fibroblasts were weakly stained with rhodamine-phalloidin, making it easy to distinguish cardiac myocytes from fibroblasts. As shown in Fig. 3A, $p125^{FAK}$ predominantly localized in the perinuclear region in non-stimulated cardiac myocytes (Fig. 3A, arrows). Only few of weak fluorescent dots of $p125^{FAK}$ were seen at the ends of the actin stress fibers in some of non-stimulated fibroblasts (Fig. 3A, arrowheads). In contrast, fluorescent dots of $p125^{FAK}$, which mostly localized in the central regions in a higher density, appeared to scatter to the peripheral cytoplasm of cardiac myocytes subjected to hypoxia for 20 min (Fig. 3B, arrows). In fibroblasts subjected

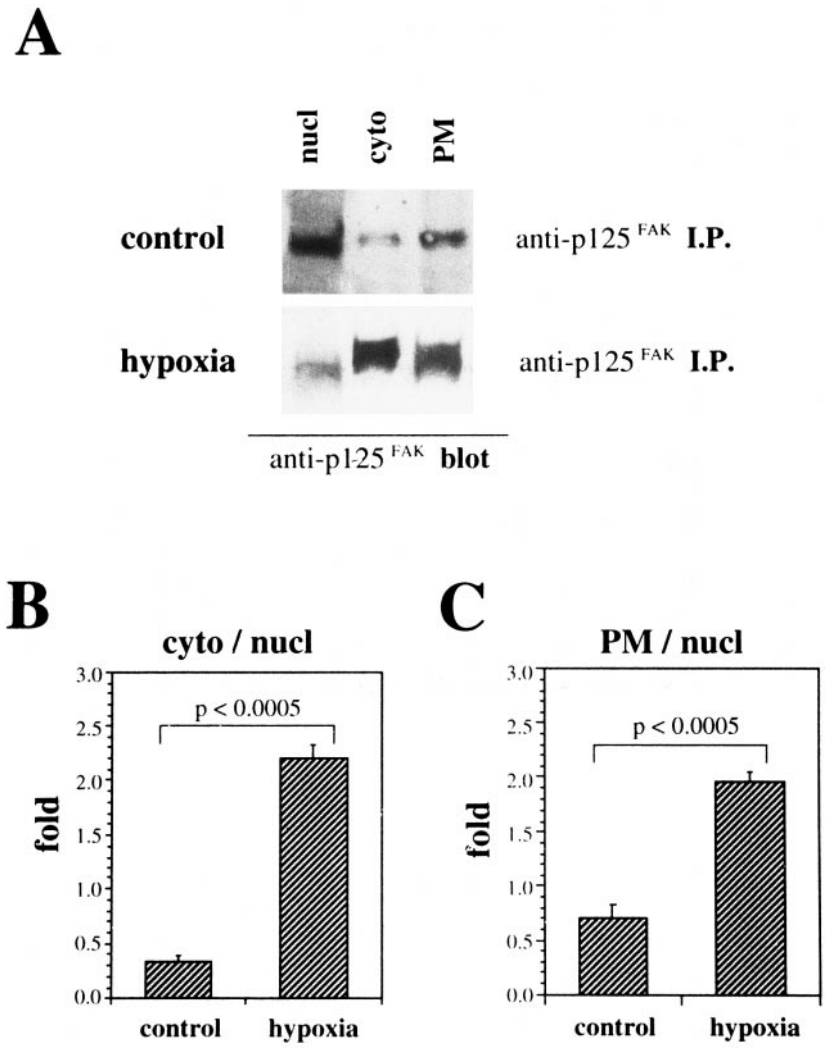

FIG. 4. Effect of hypoxia on the subcellular distribution of $p125^{FAK}$. Serum-starved cardiac myocytes were either not subjected or subjected to hypoxia for 60 min, and nuclear-related, cytosolic, and plasma membrane-rich fractions were prepared from cardiac myocytes as described under Material and Methods. Then, they were immunoprecipitated with anti-$p125^{FAK}$ antibody and Western blotted with anti-$p125^{FAK}$ antibody (A). The bands of $p125^{FAK}$ were quantified by scanning densitometry of the autoradiogram, and the ratio of the amount of $p125^{FAK}$ in cytosolic fraction to that in nuclear-related fraction (cyto/nucl), and the ratio of the amount of $p125^{FAK}$ in plasma membrane-rich fraction to that in nuclear-related fraction (PM/nucl) were shown in panels B and C, respectively.

to hypoxia for 20 min, fluorescent dots of $p125^{FAK}$ were observed to be concentrated in the patchy arrowhead-like structures at the peripheral cytoplasm reminiscent of focal adhesions, specialized regions of the plasma membrane formed at sites where cells adhere tightly to the substratum (Fig. 3B, arrowheads). Cardiac myocytes stained with nonimmune rabbit sera as a negative control showed no significant level of signals, indicating that nonspecific background was very low (data not shown).

Next, to confirm whether hypoxia really induces subcellular translocation of $p125^{FAK}$, we collected subcellular fractions from control and hypoxia-stimulated cardiac myocytes, and analyzed the content of $p125^{FAK}$ in each fraction by Western blot. Plasma membrane-rich fraction (PM), cytosolic fraction (cyto), and nuclear-related fraction (nucl) were immunoprecipitated with anti-$p125^{FAK}$ antibody and subjected to SDS-PAGE, then immunoblotted with anti-$p125^{FAK}$ antibody. As shown in Fig. 4, the ratio of the amount of $p125^{FAK}$ in cytosolic fraction to that in nuclear-related fraction was significantly ($p < 0.0005$) and markedly increased by hypoxia (Fig. 4, A and B). The ratio of the amount of $p125^{FAK}$ in plasma membrane-rich fraction to that in nuclear-related fraction was also significantly ($p < 0.0005$) increased by hypoxia (Fig. 4, A and C). Thus, these data indicate that hypoxia induced subcellular translocation of $p125^{FAK}$ from the perinuclear region to the cytosolic region and the plasma membrane.

DISCUSSION

$p125^{FAK}$ is a widely expressed nonreceptor protein-tyrosine kinase which localizes to the focal adhesion structures. $p125^{FAK}$ is thought to be one of the key elements in signal transduction pathway underlying changes in cell behavior induced by diverse stimuli including integrin engagement, oncogenic transformation, mitogenic neuropeptides such as bombesin, endothelin, vasopressin, angiotensin, platelet-derived growth factor (PDGF) and lysophosphatidic acid (17–23). It was shown that tyrosine-phosphorylated $pp125^{FAK}$ directly interacts with $pp60^{c\text{-}src}$ and $pp59^{fyn}$ as one of their major substrates and that tyrosine phosphorylation of $p125^{FAK}$ by Src family kinases is directly correlated with increased protein tyrosine kinase activity, which is an important step in the formation of an active signaling complex (19). Paxillin is a cytoskeletal protein involved in actin-membrane attachment at sites of cell adhesion to the ECM, which has also been demonstrated to be one of the major substrates of $pp60^{c\text{-}src}$ in Rous sarcoma virus-transformed cells as $p125^{FAK}$ (24). Paxillin becomes tyrosine-phosphorylated concomitantly with $p125^{FAK}$, in response to multiple stimuli including integrin-mediated cell adhesion, several neuropeptide growth factors, and PDGF (21, 25, 26).

In the present study, we have shown that hypoxia stimulated tyrosine phosphorylation of $p125^{FAK}$ and paxillin in cultured cardiac myocytes and that hypoxia also caused increased association of $p125^{FAK}$ with $pp60^{c\text{-}src}$ concomitantly with increased tyrosine phosphorylation of $p125^{FAK}$. This suggests that tyrosine kinases, such as $p60^{c\text{-}src}$ might take some part in hypoxia-induced tyrosine phosphorylation and activation of $p125^{FAK}$ in cardiac myocytes.

Transduction of various mitogenic signals from the cell membrane to the nucleus involves the adapter proteins Shc and GRB2, which mediate activation of the Ras/ERK pathway (27–29). Shc is an immediate substrate of receptor tyrosine kinase activity and serves to physically link activated receptors to downstream signaling components (30, 31). GRB2 is a ubiquitously expressed 24 kD mammalian protein, which directly binds autophosphorylated tyrosine kinase receptors as well as phosphorylated Shc proteins and $p125^{FAK}$ through its SH2 domain. And, it was demonstrated that adhesion of fibroblasts to fibronectin promotes SH2-domain-mediated association of GRB2 and $p60^{c\text{-}src}$ with $p125^{FAK}$ in vivo, resulting in activation of MAPK (27, 28, 32, 33). It was shown that tyrosine kinase-Shc-Grb2-Sos pathway was involved in the signal transduction from Gq protein-coupled angiotensin II receptor leading to activation of $p21^{ras}$ in cardiac myocytes (34). In the present study, we demonstrated hypoxia-induced association of $p125^{FAK}$ with GRB2 and Shc, concomitantly with increased tyrosine phosphorylation of $p125^{FAK}$. This strongly suggests that two ubiquitously expressed adapter proteins, GRB2 and Shc play a role in hypoxia-induced signal transduction involving MAPK pathway in cardiac myocytes as we previously reported (1).

In the present study, we also demonstrated by immunofluorescence that hypoxia significantly altered the subcellular localization of $p125^{FAK}$ from the perinuclear region to the peripheral cytoplasm in cardiac myocytes and increased accumulation of $p125^{FAK}$ in the patchy arrowhead-like structures at the peripheral cytoplasm, that is, focal adhesions, in cardiac myocytes as well as fibroblasts. In addition, we confirmed quantitatively by Western analysis that the amount of $p125^{FAK}$ in plasma membrane-rich fraction and cytosolic fraction as compared with that in nuclear-related fraction significantly increased in response to hypoxia. These results strongly suggest that $p125^{FAK}$ translocates to focal adhesions in response to hypoxia. Taken together, hypoxia causes activation as well as subcellular translocation of $p125^{FAK}$ to focal adhesions, where it works. Activation and accumulation of $p125^{FAK}$ in focal adhesions strongly suggest that adhesive interaction between cardiac myocytes and ECM may be strengthened in response to hypoxia. The signals from integrin receptors are thought to be integrated from those originating from growth factor and cytokine receptors to stimulate various intracellular signaling, organize the cytoskeleton, and rescue the cells from ECM detachment-induced programmed cell death (35). Therefore, we speculate that this may reflect one of the cardiac adaptive responses to the hypoxic stresses and that signals from humoral factors such as vascular endothelial growth factor (VEGF), which is known to be induced in response to hypoxia, may play some role in this situation. However, the precise mechanism of hypoxia-induced interaction between cardiac myocytes and ECM is unknown and remains to be clarified.

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