DOK1 Mediates SHP-2 Binding to the αVβ3 Integrin and Thereby Regulates Insulin-like Growth Factor I Signaling in Cultured Vascular Smooth Muscle Cells*

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Recruitment of the Src homology 2 domain tyrosine phosphatase (SHP-2) to the phosphorylated β3 subunit of the αVβ3 integrin is required for insulin-like growth factor I (IGF-I)-stimulated cell migration and proliferation in vascular smooth muscle cells. Because SHP-2 does not bind directly to β3, we attempted to identify a linker protein that could mediate SHP-2/β3 association. DOK1 is a member of the insulin receptor substrate protein family that binds β3 and contains XXXI/I motifs that are potential binding sites for SHP-2. Our results show that IGF-I induces DOK1 binding to β3 and to SHP-2. Preculture of cells with synthetic peptides that blocked either DOK1/β3 or DOK1/SHP-2 association inhibited SHP-2 recruitment to β3. Expression of a DOK1 mutant that does not bind to β3 also disrupts SHP-2/β3 association. As a result of SHP-2/β3 disruption, IGF-I-dependent phosphorylation of Akt and p44/p42 mitogen-activated protein kinase and its ability to stimulate cell migration and proliferation were significantly impaired. These results demonstrate that DOK1 mediates SHP-2/β3 association in response to IGF-I thereby mediating the effect of integrin ligand occupancy on IGF-IR-linked signaling in smooth muscle cells.

Vascular smooth muscle cell (SMC) migration and proliferation play significant roles in atherosclerotic plaque formation (1). Insulin-like growth factor I (IGF-I) is a potent stimulant of SMC migration and proliferation responses (2). We have shown previously that ligand occupancy of the αVβ3 integrin is required for SMC to respond appropriately to IGF-I (3). Blocking the ligand occupancy of αVβ3 inhibits IGF-I-dependent downstream signaling including phosphorylation of IRS-1 (3) and the trans-membrane, scaffolding protein Src homology 2 domain containing protein-tyrosine phosphatase substrate-1 (SHPS-1), as well as cell migration and proliferation (4). One important event that occurs in response to ligand occupancy of αVβ3 is the phosphorylation of the β3 subunit, and previous studies have shown that this is required for IGF-I-dependent signaling and biologic actions (5). SMCs expressing a mutant form of β3 in which the two tyrosines in the cytoplasmic domain of β3 were substituted with phenylalanines did not respond to IGF-1 with an increase in DNA synthesis (5). Therefore in SMC phosphorylation of the β3 subunit of αVβ3 integrin plays a key role in regulating IGF-I dependent cellular responses.

Our prior studies have shown that ligand occupancy of αVβ3 regulates IGF-IR signaling by regulating the transfer of the protein-tyrosine phosphatase SHP-2 (4, 5). In high density cultures, the β3 subunit is constitutively tyrosine phosphorylated and Src homology 2 domain tyrosine phosphatase (SHP-2) can be co-immunoprecipitated with phosphorylated β3 (5). This association correlates with membrane localization of SHP-2 and is required for the subsequent transfer of SHP-2 to its membrane substrate protein SHPS-1 following IGF-1 stimulation (5). The disruption of SHP-2 and β3 association results in the elimination of SHP-2 transfer to SHPS-1 (5). As a result, IGF-I-dependent cell migration and DNA synthesis are both decreased (5, 6). These results suggest that the association of SHP-2 and the β3 subunit is a prerequisite for proper SHP-2 transfer and that this is required for IGF-I-stimulated biologic actions.

We have previously shown that the addition of IGF-I to subconfluent cultures induces an increase in β3 phosphorylation and a corresponding increase of SHP-2 association (5). The incubation of SMC cultures with a Src-family kinase inhibitor PP2 inhibits β3 phosphorylation and blocks SHP-2 association with β3 (5), suggesting a phosphorylation-dependent association between SHP-2 and the β3 subunit. The β3 cytoplasmic domain contains one NPXY motif. This motif has been shown to interact with proteins containing phosphoryrosine binding (PTB) domains (7). SHP-2 does not contain a PTB domain, but it has two SH2 domains, which have been shown to mediate binding to phosphorylated tyrosine residues that are followed by a specific motif YXXL/I (8). Therefore it is likely that a linker protein containing both PTB domain and YXXL/I motif(s) modulates SHP-2 binding to β3. The adaptor proteins insulin receptor substrates 1 and 2 (IRS-1, IRS-2) contain both motifs, and IRS-1 has been shown to bind SHP-2 upon insulin receptor activation (9). In addition, IRS-1 can be co-immunoprecipitated with β3 in response to insulin in rat fibroblasts that overexpress insulin receptors (10). However, although both IRS-1 and IRS-2 are expressed in primary SMC cultures, we could not detect co-immunoprecipitation of IRS-1 or IRS-2 with β3, excluding them as linker proteins for SHP-2 and β3 association.

DOK1 is a member of the IRS family of proteins that contains a PH domain followed by a PTB domain in its N terminus. It has multiple tyrosine residues in its C-terminal sequence that undergo phosphorylation upon tyrosine kinase activation.
(11, 12). DOK1 has been shown to function as a scaffolding protein that recruits key signaling molecules such as, the Ras-GTP-activating protein (Ras-GAP) and the adaptor protein Nck (13, 14) following its tyrosine phosphorylation. These associations have suggested that DOK1 plays a role in regulating cell functions such as migration, proliferation, and transformation (14–16). It is not known whether SHP-2 binds DOK1; however, tyrosines 203 and 337 of DOK1 reside in YXXL motifs and therefore have the potential to bind to the SH2 domains of SHP-2. The PTB domain of DOK1 has been shown to be necessary for its regulatory role in cell transformation (15). Recently, DOK1 has been shown to bind to the NPXY motif of β3 via its PTB domain (17). However, the functional significance of the DOK1-β3 interaction has yet to be determined. Because of these properties DOK1 seemed a likely candidate for mediating phosphorytrosine-dependent binding of SHP-2 to β3.

In the current studies, we determined whether DOK1 mediated the association of SHP-2 with β3 and analyzed the functional consequences of disrupting this association on SHP-2 transfer to downstream signaling molecules. In addition, we further determined whether disruption of this interaction was associated with a change in IGFR-linked signaling and biologic actions.

**EXPERIMENTAL PROCEDURES**

Human IGFR-1 was a gift from Genentech (San Francisco, CA). Immobilon-P membranes were purchased from Millipore Corp. (Bedford, MA). DMEM containing 4500 μg of glucose/liter (DMEM-H) was purchased from Invitrogen. Streptomyacin and penicillin were purchased from Invitrogen. Streptomycin and penicillin were purchased previously (19). The cells were maintained in DMEM-H with 10% dialyzed horse serum at 37 °C. On the day before transduction, the culture medium was replaced with 1 ml of growth medium containing 10% calf serum. The cells were then trypsinized and reseeded at 5.5×10^6 cells/75 cm² flask (Corning Inc., Corning, NY). The final PCR products containing a Kozac sequence (CACC) followed by a sequence encoding the HA epitope at the 5′-end of the DOK1 coding sequence were cloned into the pLentib6/V5-D-TOPO expression vector (Invitrogen). The complete sequence was verified by DNA sequencing.

**Generation of Virus Stocks—**293FT cells (Invitrogen) were prepared for generation of virus stocks of each individual pLenti construct. Cells were plated at 5×10^5/75 cm² flask (Corning Inc., Corning, NY) the day before transfection in the growth medium (DMEM-H with 10% FBS with streptomyacin at 100 ng/ml and penicillin at 100 units/ml). On the day of transfection, the culture medium was replaced with 5 ml of Opti-MEM I (Invitrogen) without antibiotics or serum. DNA-Lipofectamine complexes for each transfection were prepared and added along with total 8 ml of Opti-MEM I medium according to the manufacturer’s protocol (Invitrogen). The next day the medium containing the DNA-Lipofectamine complex was removed and replaced with 12 ml of growth medium. The virus-containing supernatants were harvested at 48-h post-transfection, filtered through a 0.2-μm filter, and stored as 1-ml aliquots at −80 °C.

**Incorporation of pSMCs into 3-D Constructs—**pSMCs were prepared from porcine aortas as described previously (18). The peptides were synthesized by the Protein Chemistry Core Facility at the University of North Carolina at Chapel Hill. Purity and sequence confirmation were determined by mass spectrometry.

**Cell Culture—**pSMCs were prepared from porcine aortas as described previously (19). The cells were maintained in DMEM-H with 10% fetal bovine serum (HyClone, Logan, UT) and streptomycin (100 μg/ml), and penicillin (100 units/ml). The smooth muscle cells that were used in these experiments were used between passages 4–16.

**Expression of pSMCs in 3-D Constructs —**pSMCs were prepared from porcine aortas as described previously (18). The cells were maintained in DMEM-H with 10% fetal bovine serum (HyClone, Logan, UT) and streptomycin (100 μg/ml), and penicillin (100 units/ml). The smooth muscle cells that were used in these experiments were used between passages 4–16.

**Generation of pLenti Expression Vectors—**The full-length human DOK1 cDNA was generated by reverse transcription-PCR from mRNA that had been derived from human fibroblasts (GM10 Coriell Institute, Camden, NJ). The full-length DOK1 sequence was PCR-amplified from pcDNA-DOK1 WT as a template. The primers used to generate the first fragment were the forward primer from above plus 5′-CCCTGTTCCGCGGAGCAGGACAAGG-3′ plus the reverse primer from above. The two fragments were designed to overlap across the region of the mutation (bold letters). They were annealed and subsequently extended by Taq polymerase (Clontech) to generate a full-length DOK1 sequence containing the alanine substitutions. The final PCR products containing a Kozac sequence (CACC) followed by a sequence encoding the HA epitope at the 5′-end of the DOK1 coding sequence were cloned into the pLentib6/V5-D-TOPO expression vector (Invitrogen). The complete sequence was verified by DNA sequencing.

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**RESULTS**

**DOK1 Is Associated with Phosphorylated β3 Subunit in pSMCs**—Consistent with our previous finding (5), tyrosine phosphorylation of the β3 subunit was detected at high level in the basal state in confluent cultures, and IGF-1 stimulation decreased the level of phosphorylated β3 (89.8 ± 5% reduction, mean ± S.E., n = 3, p < 0.01). In contrast, in subconfluent cultures, there was a low level of β3 phosphorylation basally, and IGF-1 stimulated an increase in β3 phosphorylation (4.22 ± 0.13-fold increase compared with basal level, mean ± S.E., n = 3, p < 0.01) (Fig. 1). When DOK1 and β3 association was evaluated, the amount of β3 that associated with DOK1 correlated with levels of β3 phosphorylation. IGF-1 decreased the amount of DOK1 associated with β3 to 14 ± 5% of the basal level in high density cultures and stimulated a 3.06 ± 1.06-fold increase in subconfluent cultures (mean ± S.E., n = 3, p < 0.01 in both cases). These results suggested the association between DOK1 and the β3 subunit was phosphorylation-dependent, and they are consistent with previous studies showing a direct association between DOK1 and the β3 subunit (17). In contrast, we could not detect an association between β3 and IRS-2 or between β3 and Grb-2-associated binder 2 (Gab2).

**Inhibition of DOK1-β3 Association Blocks SHP-2 Association with β3**—To test the hypothesis that DOK1 may mediate SHP-2 association with the β3 subunit, we incubated subconfluent SMCs with a synthetic peptide that contained the sequence. This resulted in a 90% decrease in the amount of SHP-2 that associated with β3, and this decrease was significant (p < 0.01) (Fig. 2). This result suggests that DOK1-β3 association is required for SHP-2 association with β3.

**Statistical Analysis**—Student’s t test was used to compare the differences between control and treatment groups or control cells and cells expressing mutant proteins. p ≤ 0.05 was considered statistically significant.

**Cell Migration Assay**—pSMCs were seeded in 6-well dishes and grown to confluency. A razor blade was used to scrape an area of cells, leaving a denuded area and a sharp visible wound line. The wounded monolayers were then incubated with 0.2% fetal bovine serum-containing medium for 14 h then reincubated with IGF-I (100 ng/ml) for indicated times. The cell lysates were immunoprecipitated (IP) with anti-β3 or anti-DOK1 antibodies. The membranes were immunoblotted with anti-phosphotyrosine antibody (pTyr, first panel) or anti-β3 antibody (third panel). The total amount of β3 and DOK1 that had been immunoprecipitated was detected by reprobing the membrane with either anti-β3 or anti-DOK1 antibody. Results are the mean ± S.E. of three separate experiments. **, p < 0.01.

**Cell Proliferation Assay**—Assessment of SMC proliferation was performed as described previously (20). Cells were incubated in the presence or absence of the DOK1-β3, DOK1-SHP2 peptide (10 µg/ml) with or without 100 ng/ml IGF-I for 48 h at 37 °C. The cells were then fixed and stained (Diff Quick, Dade Behring, Newark, DE), and the number of cells migrating into the wound area was counted. The results are the mean ± S.E. of eight determinations in each of the three separate experiments.

**Statistical Analysis**—Student’s t test was used to compare the differences between control and treatment groups or control cells and cells expressing mutant proteins. p ≤ 0.05 was considered statistically significant.
bind phosphorylated β3 and prevent the binding of DOK1 to β3. In control cultures, IGF-I induced DOK1 binding to β3 after 5 min (Fig. 2A, first panel) and at the same time point, there was a corresponding increase of SHP-2 association with β3 (Fig. 2A, third panel). Exposure to the peptide abolished the IGF-I-induced increase in DOK1 binding to β3, and it markedly inhibited SHP-2 association with β3. However, there was no significant impairment of IGF-I-induced β3 phosphorylation. Quantitative analysis of the tyrosine phosphorylation of β3 showed that IGF-I induced a 4.36 ± 0.84-fold increase in control cultures and a 4.08 ± 1.59-fold increase in β3 phosphorylation in the presence of the blocking peptide (mean ± S.E., n = 3, p = 0.86). These results suggested that DOK1 might be mediating SHP-2 association with β3. To confirm this hypothesis, we generated SMCs expressing a DOK1 mutant that had arginines 207 and 208 substituted with alamines (DOK1-AA). The expression of a mutant containing these substitutions blocked phosphotyrosine-mediated binding of DOK1 and altered the function of DOK1 in NIH-3T3 cells (15). Fig. 2B shows that DOK1WT and DOK1-AA were expressed in SMCs at similar levels. In cells expressing wild type DOK1, IGF-I increased the binding of DOK1 to β3, and this was associated with a corresponding increase in the association of SHP-2 with β3. However, in cells expressing the DOK1-AA mutant, the ability of IGF-I to stimulate an increase in DOK1 binding to β3 was abolished, and the association between β3 and SHP-2 was decreased (Fig. 2C). These results provided in vivo evidence that DOK1 binds to β3 via its PTB domain and this binding mediates the recruitment of SHP-2 to the β3 subunit.
**Figure 5. Inhibition of SHP-2/β3 association decreased IGF-I dependent phosphorylation of Akt and p44/p42MAPK.** A, non-transfected pSMCs were serum-starved overnight then incubated with IGF-I for the indicated times in the absence or presence of the DOK1-β3 or DOK1-SHP2 blocking peptides. 30 µl of cell lysate was used to detect phosphorylation of Akt and p44/p42MAPK by immunoblotting (IB) with anti-phospho-Akt and anti-phospho-p44/p42MAPK. The protein levels were shown by probing the membrane with anti-Akt or anti-p44/p42MAPK antibodies. Densitometric analysis of phosphorylation of Akt (B) and p44/p42MAPK (C) were derived from at three independent experiments. Results are the mean ± S.E.

**Disruption of DOK1 and SHP-2 Association Inhibits SHP-2 Binding to β3**—Because we showed that DOK1 is required for SHP-2 binding to β3 and DOK1 contains the YXXL motifs that are potential binding sites for SHP-2, we hypothesized that DOK1 would bind to SHP-2 through this domain and that this interaction was required for SHP-2 binding to β3. Therefore we determined whether 1) DOK1 bound SHP-2 through its YXXL motifs and 2) whether disrupting the binding altered the interaction between β3 and SHP-2. Fig. 3A illustrates the regions of DOK1 that contain the PH domain at the N terminus followed by the PTB domain. Multiple tyrosine residues, including Tyr203 and Tyr337, that are located within YXXL motifs are also shown. In the basal state, there is low level of SHP-2 association with DOK1, and the level is significantly enhanced after IGF-I stimulation for 5 min (2.73 ± 0.28-fold increase compared with basal state, p < 0.05, Fig. 3, B and C). Pretreatment of cultures with the synthetic peptide that contains a DOK-1 SH2 recognition sequence (i.e. Y307WDL) abolished the association between SHP-2 and DOK1 (Fig. 3D, first panel). In contrast to the disrupting peptide used in Fig. 2A this peptide did not block DOK1 and β3 subunit association (Fig. 3D, second panel). β3 and SHP-2 association was also inhibited following peptide exposure (Fig. 3D, fourth panel). These results further support the conclusion that SHP-2 binding to β3 is mediated by DOK1.

**Inhibiting SHP-2-β3 Association Impairs SHP-2 Recruitment to SHPS-1**—Our previous studies have shown that inhibiting of SHP-2 and β3 association by inhibiting β3 phosphorylation leads to impaired SHP-2 transfer to SHPS-1 upon IGF-I stimulation (5). Therefore we analyzed SHP-2 recruitment to SHPS-1 in the presence of either the DOK1-β3 or the DOK1-SHP2 blocking peptide. In control cultures, IGF-I induced SHP-2 association with SHPS-1 after 5 min. However, this association was abolished following exposure to either the DOK1-β3 or the DOK1-SHP-2 blocking peptide (Fig. 4, A and B). Compared with SMCs expressing DOK1-WT, in which IGF-I induces a significant increase in SHP-2 binding to SHPS-1, expression of the DOK1-AA mutant also inhibited the increase in the amount of SHP-2 that is transferred to SHPS-1 after IGF-I stimulation (Fig. 4, C and D). These results suggested that the degree of DOK1-AA expression is sufficient to exert a dominant negative effect on SHP-2 transfer to SHPS-1 following IGF-I stimulation.

**IGF-I-mediated Phosphorylation of Akt and p44/p42MAPK Is Decreased in the Presence of DOK1-β3 and DOK1-SHP2 Blocking Peptide or in Cells Expressing the DOK1-AA Mutant**—Impaired recruitment of SHP-2 to SHPS-1 or expression of a SHP-2 mutant with attenuated phosphatase activity have been linked to deficient MAPK and phosphatidylinositol 3-kinase activation in response to growth factor stimulation including insulin and IGF-I (21–23). We therefore analyzed IGF-I-induced phosphorylation of Akt and p44/p42MAPK in control cultures and in SMCs that had been exposed to either the DOK1-β3 blocking peptide or the DOK1-SHP2 blocking peptide prior to IGF-I addition. Fig. 5 shows that IGF-I-induced phosphorylation of Akt and p44/p42MAPK after 5 and 10 min in control cultures. In the presence of the DOK1-β3 blocking peptide, however, the IGF-I-induced responses were significantly decreased. Similarly, prior exposure to the DOK1-SHP2 blocking peptide also significantly decreased IGF-I-dependent phosphorylation of both Akt and p44/p42MAPK. To confirm this result the phosphorylation of Akt and p44/p42MAPK in response to IGF-I was compared between SMCs expressing DOK1-WT and the DOK1-AA mutant. IGF-I-dependent Akt and MAPK phosphorylation were significantly impaired in cells expressing the DOK1-AA mutant (Fig. 6). Because blocking the association of DOK1 and β3...
has an effect on the ability of IGF-I to stimulate Akt and p44/p42 MAPK phosphorylation that is similar to blocking SHP-2 and DOK1 association, these results indicate that IGF-I-dependent activation of both phosphatidylinositol 3-kinase and MAPK pathways requires association of SHP-2-β3 association and that DOK1 and β3 association alone is not sufficient.

**IGF-I-dependent Cell Migration and Proliferation Are Impaired in the Presence of DOK1-β3 or the DOK1-SHP2 Blocking Peptide and in Cells Expressing the DOK1-AA Mutant**—We have previously shown that activation of the phosphatidylinositol 3-kinase and MAPK pathways are responsible for IGF-I-mediated cell migration and proliferation in cultured SMCs (24). To evaluate the consequences of impaired DOK1-β3 association and hence inhibition of SHP-2 transfer to β3 and subsequently to SHPS-1, we analyzed IGF-I-dependent cell migration and proliferation responses in the presence of the DOK1-β3 blocking peptide and in SMCs expressing the DOK1-AA mutant. The effects of DOK1-SHP-2 blocking peptide were also analyzed. Fig. 7A shows that IGF-I induced a 2.28 ± 0.30-fold increase in cell migration and a 2.21 ± 0.12-fold increase in proliferation in control cultures. In the presence of the peptide that blocks the DOK1-β3 association, the cell migration response to IGF-I was significantly decreased 1.21 ± 0.14-fold increase (p < 0.01 compared with control cultures). The increase in cell proliferation was also decreased (e.g. Fig. 7. 1.42 ± 0.24-fold, *, p < 0.05 compared with control). Prior exposure of SMCs to the DOK1-SHP-2 blocking peptide also significantly decreased IGF-I-dependent cell migration and proliferation responses (e.g. a 1.46 ± 0.11 increase in migration and a 1.30 ± 0.22-fold increase in cell proliferation, p < 0.05 in both cases compared with control). Compared with SMCs expressing DOK1WT in which IGF-I induced 1.95 ± 0.19 and 2.12 ± 0.36-fold increases in cell migration and proliferation, respectively, cells expressing the DOK1-AA mutant showed significant impairment of the IGF-I-stimulated increase in cell migration (Fig. 7, 1.24 ± 0.18-fold, **, p < 0.01 compared with DOK1WT) and in cell proliferation (Fig. 7B, 1.05 ± 0.07-fold, *, p < 0.05). As an additional control, IGF-I was shown to induce 2.34 ± 0.21-fold increase in migration and 2.27 ± 0.17-fold increase in proliferation in non-transfected wild type pSMCs. These responses are not significantly different compared with the responses of SMCs expressing wild type DOK1 (Fig. 7B, p = 0.375 in migration, p = 0.465 in proliferation). In addition, there was no significant difference in the basal growth rate of wild type SMCs as compared with SMCs expressing DOK1-WT (data not shown).

**DISCUSSION**

In other cell types, activation of SHP-2 catalytic activity has been shown to correlate with the capacity of IGF-I to stimulate phosphatidylinositol 3-(21) or MAP kinase (25). However the role of transfer of SHP-2 to β3 or other integrins in mediating their activation was not examined. We have previously shown that SHP-2 associates with the β3 subunit of the αVβ3 integrin in response to high culture density or following stimulation of low density cultures with IGF-I. This association is dependent upon β3 phosphorylation, and it is required for SHP-2 localization to the plasma membrane as well as its transfer to SHPS-1 and for IGF-I to stimulate mitogenesis (5).

The tyrosines that are phosphorylated in the β3 subunit are contained within NXXY motifs and cannot bind directly to SHP-2, because SHP-2 does not contain a PTB domain. Our current study shows that DOK1 mediates the association of SHP-2 with β3. This requires two distinct binding sites within DOK1, its PTB domain that binds to phosphorylated β3 and the Y337WDL SH2 recognition sequence that binds to the SH2 domain of SHP-2. Our studies also demonstrate that inhibition of SHP-2 association with β3 either by blocking DOK1 binding to β3 or by blocking DOK1 binding to SHP-2 was associated with impaired IGF-I-induced activation of the phosphatidylinositol 3-kinase and MAPK pathways and, hence, failure to stimulate increases in cell migration and proliferation. These findings support the conclusion that DOK1-mediated SHP-2 recruitment to β3 is an important step in IGF-I receptor-linked signaling in pSMC.

Our results suggest that phosphorylation of β3 is a key event in DOK1/SHP-2 transfer. β3 phosphorylation can be accomplished by growing the cultures to high density or by stimulation with IGF-I. The addition of αVβ3 ligands to subconfluent cultures or the increased availability of these ligands in confluent cultures also stimulates β3 phosphorylation (5). Our current findings therefore emphasize the role of cooperativity between integrin ligand occupancy and IGF-IR activation in modulating cellular responses to IGF-I and the necessity for activation of both signaling pathways to obtain an optimal IGF-I response.

It has been reported that IRS family proteins including IRS-1 and Gab1 can associate with integrin β subunits. For example, in rat fibroblasts that overexpress insulin receptors, IRS-1 can associate with the αVβ3 integrin in response to insulin stimulation (10). Similarly, Chinese hamster ovary cells that have been transfected with β1A subunit show co-immunoprecipitation between β1A and IRS-1, whereas expression of β1C in these cells induces binding of Gab1 and SHP-2 to the β1C subunit (26). These associations correlate with the
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enhanced extracellular matrix protein-mediated cell attachment in response to growth factor stimulation (10, 26), and in the case of the αvβ3 integrin with an enhanced mitogenic response to insulin (10). Our studies show that in primary SMC cultures where there is no exogenous expression of either αvβ3 or IGF-IR, DOK1 but not IRS-1, IRS-2 or Gab2 associates with the β3 subunit. Most importantly, we provided functional evidence of a role for DOK1 by demonstrating that a blockade of DOK1-mediated SHP-2 binding to β3 leads to a disruption in both MAP kinase activation and the mitogenic response to IGF-I.

Both in vitro and in vivo evidence have been provided for DOK1 binding to the cytoplasmic tail of β3 integrin (17). A GST-PTB domain fusion protein of DOK1 was shown to bind to the recombinant integrin β3 tail in vitro, and intact DOK1 was shown to co-immunoprecipitate with the αIIbβ3 integrin in Chinese hamster ovary cells that were stably expressing αIIbβ3 (17). This interaction was dependent upon the integrity of the NPXY motif within the β3 cytoplasmic tail, because the mutation of the tyrosine residue in the NPXY motif blocked DOK1 and β3 association (17). Furthermore, when the two arginines within the PTB domain of DOK1 were mutated to alanines, the interaction between DOK1 and the phosphotyrosine moiety was disrupted (15). Those results demonstrated that DOK1 and β3 association is a direct interaction that occurs via the PTB domain of DOK1 and the NPXY motif of the β3 subunit. Our results are consistent with these observations, because the exposure of SMC to a peptide encompassing part of the PTB domain of DOK1 containing the Arg207, Arg208 sequence or overexpression of the DOK1-R207A, R208A mutant disrupted association between DOK1 and β3. Our finding of lack of association between IRS-1, IRS-2, and β3 was also supported by the studies of Calderwood et al. (17) in which they screened a large series of recombinant PTB domains for their ability to bind to β3. They were able to identify DOK1 and Numb, a negative regulator of Notch signaling but not IRS-1 or IRS-2.

The significance of disruption of the interaction between DOK1 and β3 is that SHP-2 binding to β3 is inhibited. We have previously shown that SHP-2 and β3 association is a prerequisite for recruitment of SHP-2 to SHPS-1 and for the appropriate timing of SHP-2 transfer to IGF-IR (5). SHP-2 binding to β3 therefore plays an important role in regulating its subsequent transfer to SHPS-1, which is necessary for IGF-I to stimulate cell migration and proliferation (5, 6). The current study illustrates that SHP-2 transfer to β3 is mediated by the binding of its SH2 domain to DOK1 and that it also requires binding of DOK1 to tyrosine phosphorylated β3. Inhibition of either of these events resulted in failure to transfer SHP-2 to β3 and to SHPS-1. In addition, the fact that a blockade of DOK1-SHP-2 association, which did not alter DOK1 association with β3, elicited effects on SHP-2 transfer that were comparable with blocking DOK1-β3 association indicated that DOK1 functions as a linker protein that mediates SHP-2 transfer to β3 and thereby facilitates its subsequent transfer to phosphorylated SHPS-1. We have shown previously that SHP-2 transfer to SHPS-1 is required for IGF-I stimulated increases in MAP kinase activation as well as stimulation of SMC migration and proliferation (24). Our current results confirm those earlier studies by showing that blocking DOK1-mediated transfer of SHP-2 to β3, which resulted in impaired SHP-2 transfer to SHPS-1, also resulted in an impaired cell migration and proliferation responses to IGF-I.

Previous studies have provided support for the conclusion that DOK1 is a positive regulator of cell growth and migration. Hosooka et al. (27) reported that expression of a functional dominant negative DOK1 mutant suppressed cell migration and Ras activation and thereby cellular proliferation in mouse melanoma cells. In addition, overexpression of wild type DOK1 in Chinese hamster ovary cells enhanced insulin-stimulated migration (28). However, other studies have suggested that DOK1 is a negative regulator for mitogenic signaling (5, 14, 27).
16). For example, overexpression of wild type DOK1 inhibited PDGF-induced MAPK activation (16) as well as Ras and Erk activation induced by gliial cell-derived neurotrophic factor in human neuroectodermal tumor cell lines (14). Recruitment of Ras-GAP, a negative regulator of Ras activation, to DOK1 following DOK1 phosphorylation has been proposed to be responsible for decreasing mitogenic signaling (14). In our current study, we did not characterize the association of Ras-GAP with DOK1. It is possible that disruption of DOK1-SHP-2 binding may lead to sustained DOK1 phosphorylation and enhanced binding of Ras-GAP, thereby subsequently inhibiting IGF-IR-linked downstream signaling. Nevertheless, our results indicate that DOK1 has a direct role in mediating SHP-2 and β3 association and inhibition of this interaction resulted in impaired IGF-IR-mediated stimulation of MAP kinase activation and cell proliferation.

The association between SHP-2 and DOK1 is a novel finding. This association appears to be mediated via the YXXL/I motifs because pre-incubating cells with the DOK1 blocking peptide abolished SHP-2 association with DOK1. The SH2 domain of SHP-2 has been shown to bind phosphorylated tyrosines that are contained in the YXXL/I motifs (8). Therefore these results suggest that IGF-I induced phosphorylation of DOK1, leads to enhanced SHP-2 recruitment. This is consistent with the concept that DOK1 can function as a scaffolding protein for signaling molecule assembly. In addition to Ras-GAP, the adapter protein Nck (29), the non-receptor tyrosine kinase Csk (30), and the X-linked lymphoproliferative syndrome gene product SH2D1A (30) have all been shown to be associated with tyrosine-phosphorylated DOK1. These associations are required for DOK1 to facilitate downstream signaling. For example, Nck binding to Tyr^{361} of DOK1 is required for RET tyrosine kinase-mediated JNK phosphorylation in neuroectodermal cell line (14), whereas association of SH2D1A with phosphorylated DOK1 at Tyr^{1482} has been shown to be important in the normal effective host response to Epstein-Barr virus infection (30). Here we demonstrate that by mediating SHP-2 recruitment, tyrosine phosphorylation of DOK1 plays a positive role for IGF-IR downstream signaling.

In summary, our studies provide evidence that in SMCs, DOK1 associates with SHP-2 in response to IGF-I and that the DOK1-SHP-2 complex associates with the tyrosine-phosphorylated β3 subunit. Therefore DOK1 acts as a linker protein for SHP-2 association with the β3 and thereby positively regulates IGF-IR-linked downstream signaling events and IGF-I-stimulated cell migration and proliferation.

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