Membrane recruitment of DOCK180 by binding to PtdIns(3,4,5)P_3

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DOCK180 was originally identified as one of two major proteins bound to the Crk oncogene product and became an archetype of the CDM family of proteins, including Ced-5 of *Caenorhabditis elegans* and Mbc of *Drosophila melanogaster*. Further study has suggested that DOCK180 is involved in the activation of Rac by the CrkII–p130^{Cas} complex. With the use of deletion mutants of DOCK180, we found that the C-terminal region containing a cluster of basic amino acids was required for binding to and activation of Rac. This region showed high amino-acid sequence similarity to the consensus sequence of the phosphoinositide-binding site; this led us to examine whether this basic region binds to phosphoinositides. For this purpose we used PtdIns(3,4,5)P_3-APB beads, as reported previously [Shirai, Tanaka, Terada, Sawada, Shirai, Hashimoto, Nagata, Iwamatsu, Okawa, Li et al. (1998) Biochim. Biophys. Acta **1402**, 292–302]. By using various competitors, we demonstrated the specific binding of DOCK180 to PtdIns(3,4,5)P_3. The expression of active phosphoinositide 3-kinase (PI-3K) did not enhance a DOCK180-induced increase in GTP-Rac; however, the expression of PI-3K translocated DOCK180 to the plasma membrane. Thus DOCK180 contained a phosphoinositide-binding domain, as did the other guanine nucleotide exchange factors with a Dbl homology domain, and was translocated to the plasma membrane on the activation of PI-3K.

Key words: CDM family proteins, pleckstrin homology domain, Rac.

INTRODUCTION

DOCK180 has been identified as one of two major proteins bound to an adaptor protein, Crk [1]. The activated status of the adaptor-binding proteins has been mimicked successfully by the addition of a farnesylation signal and membrane recruitment. We have shown that the membrane-targeted DOCK180 induces membrane spreading, as does activated Rac [2,3]. Further study has demonstrated that DOCK180 does indeed bind to and activate Rac in HEK-293T cells and NIH 3T3 cells [3]. Genetic studies on *Caenorhabditis elegans* and *Drosophila melanogaster* have shown that defects in their DOCK180 counterparts, Ced-5 and Mbc respectively, resulted in an abnormality of cell movement [4–7] in which Rac has an important role, probably through reorganization of the actin cytoskeleton [7,8]. Crk is involved in many signalling cascades, including those stimulated by integrins and the epidermal growth factor receptor (reviewed in [1]). DOCK180 is found in the CrkII–p130^{Cas} complex that is concentrated at focal adhesions; however, DOCK180 is not bound to epidermal growth factor receptor after ligand stimulation [9], suggesting the presence of an additional signal to determine the localization of DOCK180 after various types of stimulation.

Ras superfamily G proteins, including the Rho family, cycle between GDP-bound inactive and GTP-bound active states [10]. The GDP-bound form is converted into the active form by the guanine nucleotide exchange factor (GEF), and the GTP-bound form returns to the GDP-bound form by hydrolysis. The intrinsic GTPase activity is stimulated by GTPase-activating proteins (GAPs). The Rho-family small G proteins, which consist of the Rho, Rac and Cdc42 subfamilies, regulate the organization of the actin cytoskeleton [11]. The GEFs of Rho-family G proteins share a conserved amino-acid motif, called the Dbl homology (DH) domain [12]. The GEFs for Rho-family G proteins also share the pleckstrin homology (PH) domain, which is found in many signalling molecules and has been shown to bind to phosphoinositides [13]. Previously we reported that DOCK180 increased GTP-bound Rac and bound to nucleotide-free Rac [3]. The latter property is reminiscent of GEF, because GEF exerts catalytic activity by stabilizing the nucleotide-free form of the G proteins. However, DOCK180 does not show any significant similarity to the known GEFs for the Rho family and awaits further study *in vitro* for the demonstration of its enzymic activity.

Here we attempt to delineate a domain of DOCK180 that is essential for the activation of Rac, and we have identified a region that binds to PtdIns(3,4,5)P_3 and mediates phosphoinositide 3-kinase (PI-3K)-dependent membrane translocation of DOCK180. Thus the basic region of DOCK180 seems to function in the same way as the PH domain of DH-domain-containing GEFs for Rho-family G proteins.

MATERIALS AND METHODS

Expression plasmids

pCAGGS-FLAG-DOCK180, pCAGGS-His-DOCK180, pCAGGS-His-DOCK180-DOHRS (residues 1–1714), pCAGGS-His-DOCK180-del PS (residues 1–1472), pCAGGS-His-DOCK180-GS (residues 1505–1865), pCAGGS-His-DOCK180-GCB2S (residues 1505–1835), pCAGGS-His-DOCK180-GHRS (residues 1505–1714) and pCAGGS-His-DOCK180-HS (residues 1715–1865) have been described previously [9]. pCAGGS–EGFP–DOCK180-GS is derived from pCAGGS–EGFP [14] and encodes DOCK180-GS fragment (residues 1505–1865) fused to the enhanced green fluorescent

Abbreviations used: DH domain, Dbl homology domain; EGFP, enhanced green fluorescent protein; GAP, GTPase-activating protein; GEF, guanine nucleotide exchange factor; GST, glutathione S-transferase; HA, haemagglutinin; PH domain, pleckstrin homology domain; PI-3K, phosphoinositide 3-kinase, PtdSer, phosphatidylserine.

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protein (EGFP). pmycBD110 and pmycBDKN, which encode active and inactive PI-3K mutants respectively, were described previously [15,16]. Expression vectors for the wild-type and active and dominant-negative mutants of Rac1 have also been described previously [3]. pcDNA3-Tiam1 (C1199), which encodes an active form of Tiam-1, was kindly provided by John Collard (The Netherlands Cancer Institute, Amsterdam, The Netherlands) [17].

Production of glutathione S-transferase (GST)–PAK (PBD) fusion protein

Affinity purification of endogenous Rac was performed essentially as described previously [18]. A cDNA fragment covering residues 67–150 of human PAK1 was amplified by PCR, digested with *Bam*HI and *Eco*RI and subcloned into the same site of pGEX4T3 (Pharmacia Biotech, Piscataway, NJ, U.S.A.) [19]. *Escherichia coli* transformed by this plasmid was grown at 37 °C and cultured further in the presence of 0.5 mM isopropyl β-D-thiogalactoside for 2 h. Cells were harvested, resuspended in lysis buffer [50 mM Tris/HCl (pH 8)/1 % (v/v) Triton X-100/150 mM NaCl/10 % (v/v) glycerol/2 mM dithiothreitol/1 mM PMSF], sonicated and centrifuged at 30000 ***g*** at 4 °C for 20 min. The supernatant was incubated with glutathione-coupled Sepharose 4B beads (Pharmacia) for 30 min at 4 °C. The beads were washed extensively with lysis buffer. For quantification of GST–PAK (PBD) bound to the beads, a small aliquot of the beads was analysed by SDS/PAGE and staining with Coomassie Blue.

Antibodies

A rabbit antiserum against DOCK180 was developed in our laboratory [2]. Anti-His, anti-Flag (where Flag is Asp-Tyr-Lys-Asp-Asp-Asp-Asp-Lys), anti-Myc, anti-Rac and anti-haemagglutinin (anti-HA) antibodies were purchased from Qiagen (Hilden, Germany), Sigma (St Louis, MO, U.S.A.), and Santa Cruz Biotechnology (Santa Cruz, CA, U.S.A.), Upstate Biotechnology (Lake Placid, NY, U.S.A.), and Boehringer Mannheim (Mannheim, Germany).

Transfection

HEK-293T cells were transfected with expression plasmids by the calcium phosphate method. After 24 h the cells were lysed in lysis buffer [50 mM Tris/HCl (pH 7.4)/1 % (v/v) Triton X-100/10 % (v/v) glycerol/150 mM NaCl/1 mM PMSF], and cleared cell lysates were incubated for 1 h with anti-Flag, anti-His or anti-HA antibody at 4 °C. The immune complexes were precipitated for 30 min with a mixture of Protein A–Sepharose and Protein G–Sepharose at 4 °C. After being washed, the immune complexes were separated by SDS/PAGE and probed with antibodies. Antibodies were detected by an enhanced chemiluminescence system.

Observation of EGFP-fused DOCK180 in A431 cells

A431 cells were plated on a collagen-coated glass-based dish 35 mm in diameter (Asahi Techno Glass Co., Tokyo, Japan) and transfected with pCAGGS–EGFP or pCAGGS–EGFP–DOCK180-GS by using Superfect (Qiagen). Cells were imaged on a Zeiss Axiovert microscope equipped with a xenon lamp and a cooled charge-coupled device camera, which were controlled by MetaFluor software (Roper Scientific). Filters were purchased from Chroma: 86490 exciter, 86JP4bs dichroic mirror and 86528 emitter.

Analysis of GTP-bound Rac

Cells were washed in ice-cold PBS containing 5 mM $MgCl_2$, lysed in lysis buffer containing 5 mM $MgCl_2$, then centrifuged for 10 min at 15000 ***g*** at 4 °C. The supernatant was incubated for 30 min with the GST–PAK (PBD) protein pre-bound to glutathione–Sepharose beads at 4 °C. The beads were washed, separated by SDS/PAGE and analysed by immunoblotting with anti-Rac1 antibody.

Binding of DOCK180 to Rac

HEK-293T cells were transfected with pEBG-RhoA, pEBG-Rac1 or pEBG-Cdc42Hs. After 48 h the cells were lysed in EDTA-lysis buffer [25 mM Tris/HCl (pH 7.5)/150 mM NaCl/5 mM EDTA/10 mM NaF/500 μM Na_3VO_4/1 μg/ml aprotinin/1 mM PMSF]. The cleared cell lysates were incubated for 30 min with glutathione–Sepharose at 4 °C. Proteins collected on glutathione–Sepharose were washed three times with lysis buffer, separated by SDS/PAGE and transferred to a PVDF membrane. The filter was blocked with TBS-T containing 2 % (v/v) non-fat milk and probed with anti-DOCK180 antibody or anti-GST antibody, followed by horseradish-peroxidase-conjugated secondary antibody and chemiluminescence reagents (Amersham). Similarly, pEB-HA-RacV12 and pEB-HA-RacN17 were co-transfected with pCA-FLAG-DOCK180 and lysed in the lysis buffer described above. Cleared cell lysates were incubated for 2 h with anti-DOCK180 antibody and Protein A–Sepharose at 4 °C. Immune complexes were washed several times, separated by SDS/PAGE and analysed by immunoblotting with anti-HA antibody.

Membrane fractionation

HEK-293T cells were transfected with pCAGGS-Flag-DOCK180 and pCAGGS, pmycBD110 or pmycBDKN. After 24 h, cells were scraped and suspended in buffer A [20 mM Tris/HCl (pH 7.5)/150 mM NaCl/50 mM NaF/1 mM Na_3VO_4/1 mM PMSF]. After rapid freezing in liquid nitrogen and thawing in a water bath, cells were centrifuged at 18500 ***g*** for 10 min. The supernatant was removed and used as a cytosolic fraction. The pellet was washed once with buffer A, lysed in buffer B [20 mM Tris/HCl (pH 7.5)/150 mM NaCl/50 mM NaF/1 mM Na_3VO_4/1 % (v/v) Triton X-100/1 mM PMSF] and cleared by centrifugation at 10000 ***g*** for 10 min for preparation of the membrane fraction. One-fifth volume of buffer A was used for preparation of the membrane fraction. Both the cytosolic and membrane fractions were separated by SDS/PAGE and analysed by immunoblotting with anti-DOCK antibody.

Phospholipids

Preparation of synthetic PtdIns(3,4,5)P_3 [distearoyl PtdIns(3,4,5)P_3] covalently bound to the beads and synthetic PtdIns(3,4)P_2 has been described previously [20]. Phosphatidylserine (PtdSer) was purchased from Avanti (Alabaster, AL, U.S.A.). D-*myo*-Ins(1,3,4,5)P_4 and PtdIns(4,5)P_2 were purchased from Dojin (Kumamoto, Japan) and Boehringer Mannheim (Indianapolis, IN, U.S.A.) respectively. PtdIns(4,5)P_2, PtdIns(3,4)P_2 and PtdIns(3,4,5)P_3 were mixed with a 10-fold excess of PtdSer in chloroform and dried, then and dissolved in DMSO at a concentration of 2 mg/ml. The lipid solutions were added to buffer C [20 mM Tris/HCl (pH 7.0)/150 mM NaCl/1 mM EDTA/1 mM EGTA] and mixed immediately to form micelles.

PtdIns(3,4,5)P_3-binding assay

The synthesis of a PtdIns(3,4,5)P_3 analogue, PtdIns(3,4,5)P_3-APB, and the preparation of PtdIns(3,4,5)P_3-APB-carrying beads were reported previously [20]. COS7 cells were transfected with pCAGGS-Flag-DOCK180 by the DEAE-dextran method. After 2 days, cells were harvested, homogenized in buffer D [20 mM Tris/HCl (pH 7.0)/150 mM NaCl/1 mM EDTA/1 mM EGTA/1 mM PMSF] and cleared by centrifugation at 10000 ***g*** for 10 min. The supernatant was further centrifuged at 100000 ***g*** for 1 h and then incubated with PtdIns(3,4,5)P_3-APB beads at 4 °C for 4 h. In some experiments the cell lysates were incubated with phospholipid micelles or Ins(1,3,4,5)P_4 before the addition of PtdIns(3,4,5)P_3-APB beads. Proteins bound to the beads were subjected to immunoblotting with anti-DOCK180 polyclonal antibody.

RESULTS

Detection of GTP-Rac in the cells

We have shown previously that DOCK180 promotes the guanine nucleotide exchange of Rac1 by measuring the uptake of ^{32}P-labelled guanine nucleotides by recombinant Rac1. To gain further insight into the physiological role of DOCK180 with Rac, we set up an assay method for endogenous GTP-Rac1 based essentially on a method for detecting GTP-Rap1 described previously [21]. In brief, cells were lysed and incubated with a GST–PAK (PBD) fusion protein encoding the Cdc42/Rac-binding region of human PAK1 (residues 67–150). GTP-Rac bound to GST–PAK (PBD) was quantified by immunoblotting with anti-Rac antibody. First we confirmed that the GST–PAK (PBD) fusion protein bound only to the active form of Rac1 by using a GTPase-deficient mutant of Rac1, Rac1V12 (Figure 1A).

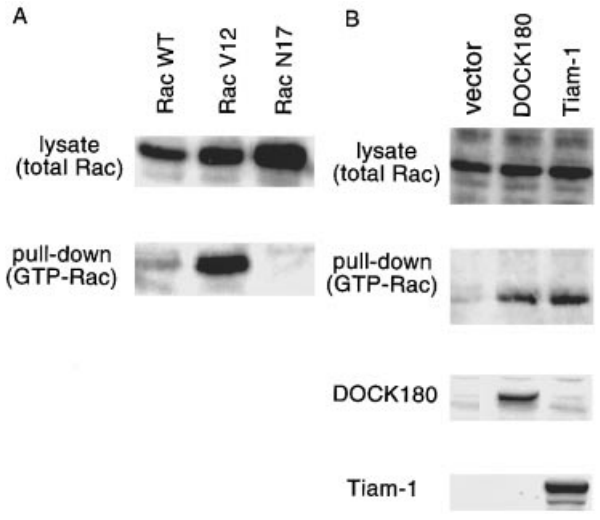

Figure 1 Detection of GTP-bound Rac by use of GST–PAK (PBD)

(**A**) HEK-293T cells were transfected with expression vectors encoding the wild-type (WT), constitutively active mutant (V12) and dominant-negative mutant (N17) of HA-tagged Rac1 proteins as indicated. Cells were lysed and incubated with GST–PAK (PBD) pre-bound to glutathione–Sepharose beads. Total cell lysate (lysate) and proteins bound to the beads (pull-down) were separated by SDS/PAGE and analysed by immunoblotting with anti-HA antibody. (**B**) HEK-293T cells were transfected with expression vectors encoding Flag-tagged DOCK180 or HA-tagged Tiam-1(C1199) as indicated. Proteins bound to the GST–PAK (PBD) were analysed as in (**A**). Total cell lysates were also analysed by immunoblotting with anti-Flag or anti-HA antibody for the detection of DOCK180 and Tiam-1.

With this method we found that DOCK180 activated endogenous Rac1 as efficiently as did the activated form of Tiam-1, a DH-domain-containing GEF for Rac1, when these proteins were expressed in HEK-293T cells (Figure 1B).

Requirement of the C-terminal basic region of DOCK180 for the binding to and activation of Rac

The C-terminal regions of the CDM family of proteins are extremely divergent [4,7]. We therefore examined the effect of the deletion of the C-terminus of DOCK180. As shown in Figure 2, the wild-type DOCK180 and DOHRS mutant, which lacked the Crk SH3-binding domain, bound to Rac1; however, the del PS mutant, in which the C-terminus was further deleted to residue 1472, failed to do so. Neither the wild-type nor any of the mutants of DOCK180 bound to Rho or Cdc42H. As shown in Figure 2(C), the wild-type and DOHRS mutant, but not the del PS mutant, increased the amount of GTP-Rac in the transfected cells, indicating that residues 1472–1714 were required for the binding to and activation of Rac. However, Rac was not activated by any of the DOCK180 mutants consisting only of the C-terminal region, namely GS, HS and GHRS (Figure 2C, right panel). We also attempted to delete the N-terminal region of DOCK180; however, most of the N-terminal deletion mutants that we tested failed to activate Rac, preventing further domain analysis. In the region deleted in del PS, but not in DOHRS, we noticed a cluster of basic amino acids (Figure 2D) that aligned with the consensus sequence of the PtdIns(4,5)P_2-binding site [22]. The DH domain containing GEFs for the Rho-family G protein always possesses the PH domain, which associates with phosphoinositides [13,23]. We therefore speculated that this basic region might serve as the PH domain in DOCK180.

Binding of DOCK180 to PtdIns(3,4,5)P_3

We examined the binding of DOCK180 to PtdIns(3,4,5)P_3 by using beads covalently bound to PtdIns(3,4,5)P_3-APB. COS7 cells expressing DOCK180 were lysed and applied to PtdIns(3,4,5)P_3-APB beads in the absence or presence of PtdIns(3,4,5)P_3 in the micelles of PtdSer. DOCK180 retained on the beads was detected by immunoblotting. As shown in Figure 3(A), DOCK180 bound to PtdIns(3,4,5)P_3-APB beads and this binding was inhibited by free PtdIns(3,4,5)P_3. However, none of PtdIns(4,5)P_2, PtdIns(3,4)P_2 or Ins(1,3,4,5)P_4 inhibited the binding of DOCK180 to PtdIns(3,4,5)P_3-APB beads. To delineate the PtdIns(3,4,5)P_3-binding site, we used DOCK180 mutants consisting of the C-terminal region of DOCK180 (Figure 2). All mutants except del HS bound to PtdIns(3,4,5)P_3-APB beads, indicating that residues 1505–1714 of DOCK180, including the basic region, were required for binding to PtdIns(3,4,5)P_3 (Figure 3).

Membrane recruitment of DOCK180 by expression of the active PI-3K

On the basis of these observations, we reasoned that accumulation of PtdIns(3,4,5)P_3 by PI-3K recruited DOCK180 to the plasma membrane and enhanced DOCK180-dependent Rac activation. To demonstrate this we used constitutively active mutants of PI-3K, mycBD110, which has a structure similar to that of p110* reported by Hu et al. [24], and mycBDKN, which is a kinase-negative counterpart of mycBD110. First we measured the level of GTP-Rac in HEK-293T cells expressing mycBD110 or mycBDKN, with or without DOCK180 or the membrane-targeted DOCK180, DOCK-F. Against our expectation, mem-

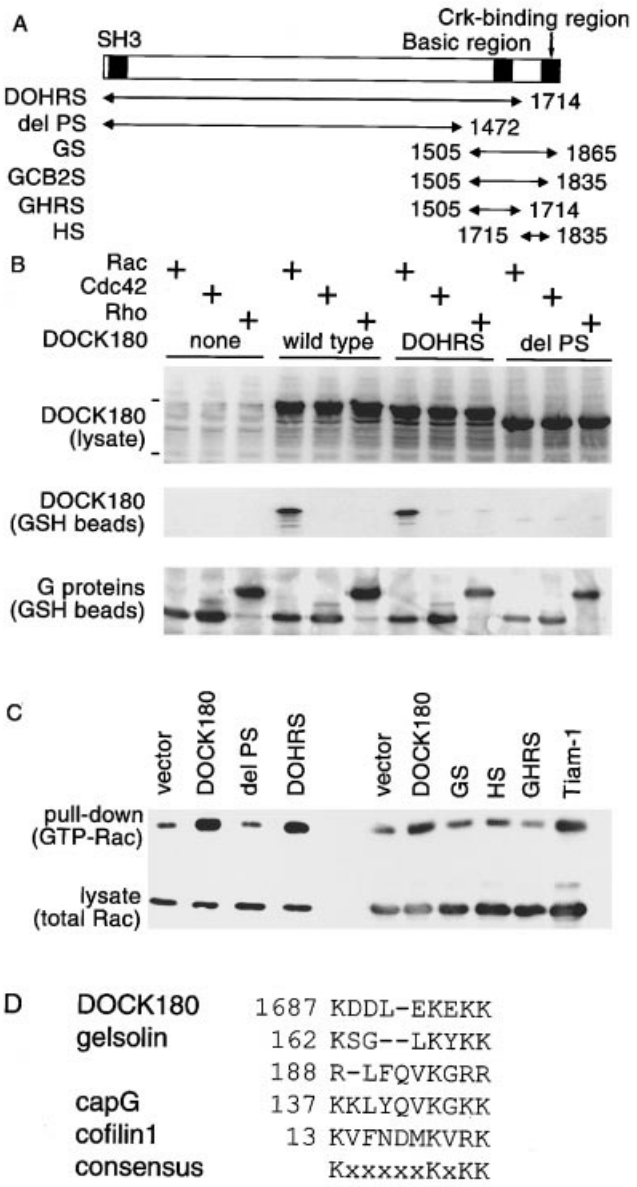

Figure 2 Requirement of the basic region of DOCK180 for binding to and activation of Rac

(**A**) Schematic representation of DOCK180 and its mutants. The numbers of the first and last amino acid residues are indicated. (**B**) HEK-293T cells were transfected with expression vectors of the His-tagged DOCK180 proteins and GST-tagged G proteins as indicated. After 24 h, cells were lysed and incubated with glutathione–Sepharose beads. Total cell lysates (top) and proteins bound to the glutathione beads (middle and bottom) were separated by SDS/PAGE and analysed by immunoblotting with anti-His or anti-GST antibody. (**C**) HEK-293T cells were transfected with DOCK180 vectors as indicated. After 24 h, cells were lysed and incubated with GST–PAK (PBD) pre-bound to glutathione–Sepharose. Total cell lysates and proteins bound to the beads were separated by SDS/PAGE and analysed by immunoblotting with anti-Rac1 antibody. (**D**) PtdIns(4,5)P_2-binding sequences of gelsolin, capG and coffilin1 [22] are aligned with the basic region of DOCK180.

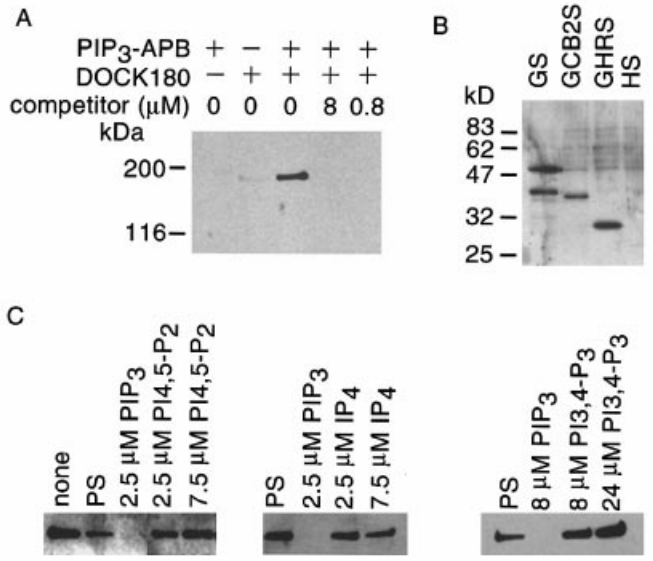

Figure 3 Specific binding of DOCK180 to PtdIns(3,4,5)P_3

(**A**) COS7 cells were transfected with pCAGGS (lane 1) or pCAGGS-Flag-DOCK180 (lanes 2–5). After 2 days, cells were lysed and cleared by centrifugation. PtdIns(3,4,5)P_3 was added to 8 and 0.8 μM as a competitor, as indicated. Lysates were incubated with control beads or PtdIns(3,4,5)P_3-APB [(PIP$_3$-APB)]-bound beads. Proteins bound to the beads were separated by SDS/PAGE and analysed by immunoblotting with anti-DOCK180 antibody. (**B**) COS7 cells were transfected with His-tagged DOCK180 mutants, GS, GCB2S, GHRS and HS, the structures of which are shown in Figure 2(A). After 2 days, cells were analysed as in (**A**). (**C**) The cytosolic fraction of HEK-293T cells transfected with pCAGGS-Flag-DOCK180 was incubated with micelles containing PtdSer (PS) or other phosphoinositides as indicated, 1 h before the addition of PtdIns(3,4,5)P_3-APB beads. Bound DOCK180 was detected as in (**A**). Abbreviations: PIP$_3$, PtdIns(3,4,5)P_3; PI4,5-P$_2$, PtdIns(4,5)P_2; IP$_4$, Ins(1,3,4,5)P_4. The positions of molecular mass markers are indicated (in kDa) at the left of panels (**A**) and (**B**).

brane targeting of DOCK180 did not enhance Rac activation. Moreover, neither mycBD110 nor mycBDKN affected the DOCK180-induced accumulation of GTP-Rac (Figure 4A). We also observed that an inhibitor of PI-3K, wortmannin, did not affect the increase in GTP-Rac by the expression of either wild-type DOCK180 or the DOHRS mutant (Figure 4B).

Next we examined the subcellular localization of DOCK180 in the presence of the PI-3K mutant. As shown in Figure 4(C), expression of the active PI-3K, mycBD110, enhanced the association of DOCK180 with the plasma membrane. In contrast, expression of the kinase-negative mutant of PI-3K, mycBDKN, did not increase the amount of DOCK180 in the plasma membrane fraction. These results demonstrated that PI-3K did not increase the level of GTP-Rac by DOCK180 but recruited DOCK180 to the membrane compartment, probably through the accumulation of PtdIns(3,4,5)P_3.

It has been shown that the PH domain of ARNO, a GEF for ADP-ribosylation factor, is sufficient to recruit green fluorescent protein to the plasma membrane on activation of PI-3K [25]. Similarly to this study, we confirmed that the C-terminal region of DOCK180 was sufficient to recruit DOCK180 to the plasma membrane by the use of the DOCK180 GS mutant fused to EGFP (EGFP–DOCK-GS). We observed the accumulation of EGFP–DOCK-GS in the nucleus in addition to the diffuse localization in the cytoplasm of A431 cells (Figure 4D). The nuclear localization of EGFP–DOCK-GS suggests that the basic region of DOCK180 might also function as a nuclear targeting signal. In the presence of mycBD110, we observed the accumulation of EGFP–DOCK-GS at the periphery of the plasma membrane. We did not observe a similar accumulation when the authentic EGFP was co-expressed with mycBD110 (results not shown). We therefore concluded that the C-terminus of DOCK180 was sufficient to recruit EGFP to the plasma membrane.

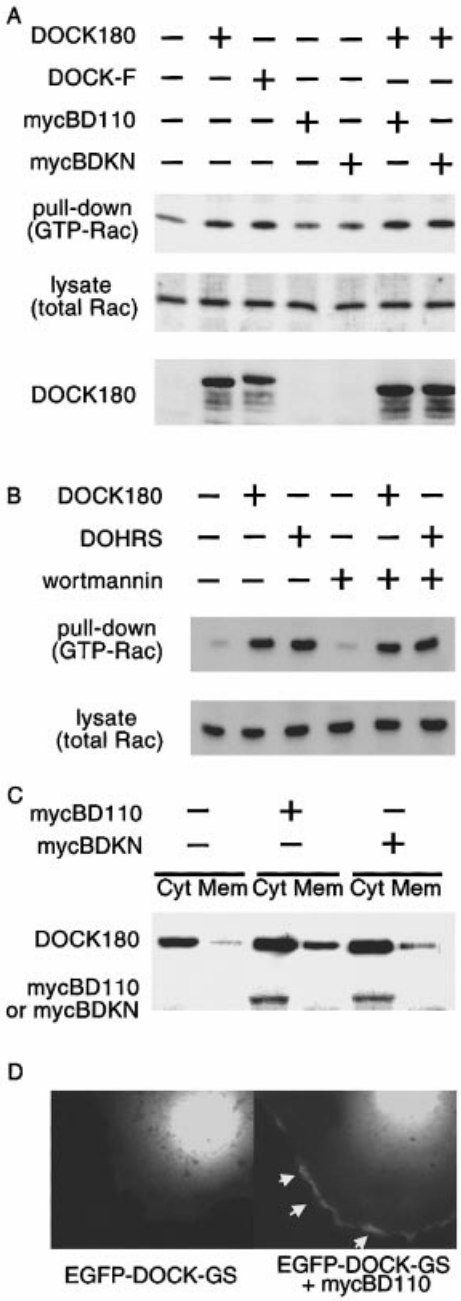

Figure 4 Effect of PtdIns(3,4,5)P_3 on DOCK180

(**A**) HEK-293T cells were transfected with expression vectors of DOCK180, mycBD110 and mycBDKN as indicated. mycBD110 and mycBDKN are constitutively active and kinase-negative PI-3K mutants respectively. After 24 h, cells were lysed and incubated with GST–PAK (PBD). GST–PAK (PBD) was collected with glutathione–agarose, separated by SDS/PAGE and analysed by immunoblotting with anti-Rac1 antibody. Total cell lysates were also analysed by immunoblotting. (**B**) HEK-293T cells were transfected with DOCK180 or DOHRS mutant as indicated. After 24 h, cells were treated for 30 min with wortmannin, lysed in lysis buffer, cleared by centrifugation and incubated with GST–PAK (PBD). GST–PAK (PBD) was collected with glutathione–agarose, separated by SDS/PAGE and analysed by immunoblotting with anti-HA antibody. Total cell lysates were also analysed by immunoblotting. (**C**) HEK-293T cells were transfected with expression vectors of Flag-DOCK180 and mycBD110 or mycBDKN as indicated. Cells were fractionated into a soluble cytosolic fraction (Cyt) and a crude membrane fraction (Mem). Aliquots of these fractions were analysed by immunoblotting with anti-Flag antibody and anti-Myc antibody. (**D**) A431 cells were transfected with pCAGGS–EGFP–DOCK-GS with or without pmycBD110. After 24 h, cells were observed with a Zeiss Axiovert fluorescence microscope equipped with a xenon lamp and a cooled charge-coupled device camera. Arrows indicate the accumulation of EGFP–DOCK-GS at the periphery of the A431 cell.

DISCUSSION

All DH-domain-containing proteins possess associated C-terminal PH domains [13,23]. The high affinity of the PH domain for phosphoinositides enables the DH-domain-containing proteins to locate to the membrane compartments on activation of PI-3K [23]. This recruitment to the plasma membrane seems to be crucial in inducing morphological change by these DH-domain-containing GEFs such as Tiam-1 and Lfc [26–28]. Similarly to these DH-domain-containing GEFs, only the membrane-targeted DOCK180 induces the spreading of NIH 3T3 cells [2]. Because in the present study we could not detect any difference in the levels of increase in GTP-Rac by the membrane-targeted DOCK180 and the wild-type DOCK180, the induction of a morphological change by DOCK180 seems to require an additional signal that regulates the localization of DOCK180. The PI-3K-dependent membrane recruitment of DOCK180 strongly suggests that PtdIns(3,4,5)P_3 has such a role in the signalling to DOCK180.

In Lfc, the PH domain is not necessary for the induction of DNA synthesis in Swiss 3T3 cells; however, Sos and Vav require an intact PH domain for the activation of Rac [29,30]. Moreover, determination of the structure of the DH-domain-containing GEFs has raised the possibility that the PH domain regulates the interaction of the DH domain with G proteins and thus enhances the guanine nucleotide exchange activity of the DH domain [31–33]. We found that the basic region of DOCK180 was required for the activation of Rac. Therefore, again, the basic region of DOCK180 seems to have a role similar to that of the PH domain of the DH-domain-containing GEFs.

Previously, we searched for PtdIns(3,4,5)P_3-binding proteins by using PtdIns(3,4,5)P_3-APB beads. Proteins identified by this method include Akt, GAP1m and Tec, all of which contain the PH domain [20]. In that study we showed that DOCK180 also bound specifically to PtdIns(3,4,5)P_3-APB beads under a condition very similar to that used in a previous study [20]. Thus DOCK180 seems to bind to PtdIns(3,4,5)P_3 as efficiently as do Akt, GAP1m and Tec. Alignment of the C-terminal basic region of DOCK180 to the PtdIns(4,5)P_2-binding domains of gelsolin, capG and cofilin1 suggests that ionic interaction of the basic amino acids with phosphate residues of PtdIns(3,4,5)P_3 and PtdIns(4,5)P_2 is important for binding (Figure 2D). However, it is also apparent that this ionic interaction is not sufficient for the binding of DOCK180 to PtdIns(3,4,5)P_3 because Ins(1,3,4,5)P_4 cannot compete with PtdIns(3,4,5)P_3 in binding to DOCK180 (Figure 3C). Therefore regions other than this basic domain must participate in the recognition of PtdIns(3,4,5)P_3.

The PTB domain, originally identified as a phosphotyrosine-binding domain, has been shown to have a structural resemblance to the PH domain [34]. In concord with this finding, the PTB domain of Shc has been shown to bind to PtdIns(3,4,5)P_3 and PtdIns(4,5)P_2 [35]. Similarly, despite a lack of any detectable amino-acid sequence similarity between the C-terminal region of DOCK180 and the PH domain, it is possible that the structure of the basic region of DOCK180 resembles the PH domain.

In conclusion, we have shown that the basic region of DOCK180 functions in the same way as the PH domain of the DH-domain-containing GEFs Tiam-1, Sos and Lfc, in that the basic region is bound to PtdIns(3,4,5)P_3 and is required for Rac activation.

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