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Non-adherent cell-specific expression of DOCK2, a member of the human CDM-family proteins

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Abstract

Human DOCK180, which was originally identified as a major protein bound to the Crk oncogene product, is an archetype of the CDM family of proteins, including Ced-5 of *Caenorhabditis elegans* and Mbc of *Drosophila melanogaster*. After DOCK180, at least three putative human proteins that manifest high amino acid sequence similarity to DOCK180 have been registered in the GenBank/EMBL database. We have designated one of them, KIAA0209, as DOCK2 and characterize here. DOCK2 mRNA was expressed mostly in peripheral blood cells, followed by slight expression in the spleen and thymus, whereas DOCK180 was expressed in all tissues tested except in peripheral blood cells. Immunostaining of human cadaver tissues revealed that the expression of DOCK2 was limited to the lymphocytes and macrophages of various organs. DOCK2 bound to and activated Rac1, as did DOCK180; however, DOCK2 did not bind to CrkII, which transduces signals at focal adhesions. Thus, DOCK180 and DOCK2 are regulators of Rac and function in adherent and non-adherent cells, respectively.

Keywords: DOCK180; Mbc; Ced-5; CDM-family protein

1. Introduction

CrkII belongs to the adaptor proteins consisting mostly of the SH2 and SH3 domains [1]. As a complex with its major SH2-binding protein, $p130^{Cas}$, CrkII seems to function at focal adhesions [2]. The CrkII-$p130^{Cas}$ complex regulates the cell migration [3] and activation of c-jun N-terminal kinase/stress-activated protein kinase (JNK) [4–6]. DOCK180, which was isolated as one of the two major proteins bound to the SH3 domain of an adapter protein, CrkII [7], transduces signals from the CrkII-$p130^{Cas}$ complex to both the cytoskeleton and JNK pathway by means of activation of a low molecular weight G protein, Rac [8,9]. Both the DOCK180-dependent activation of JNK and DOCK180-induced cell spreading were inhibited by a dominant-negative Rac mutant [8].

Homologs of DOCK180 have been identified and characterized in *Drosophila melanogaster* and *Caenorhabditis elegans*. Taking the acronyms of Ced-5 of *C. elegans*, DOCK180, and Mbc of *D. melanogaster*,

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these proteins are designated as the CDM-family proteins [10]. Mbc is necessary for myoblast fusion and for the migration of epithelial cells, both of which require reorganization of the cytoskeleton [11]. Recently, a genetic link between Mbc and Rac has been demonstrated, suggesting that Mbc may regulate the cytoskeleton through the activation of Rac [9]. Loss of function of Ced-5 results in the defect of engulfing dead cells and migration of distal tip cells of the nematode [10]. Thus, Ced-5 is also involved in the regulation of the cytoskeleton.

cDNA sequences of three human putative proteins, which share high sequence homology with DOCK180, have been deposited to the GenBank/EMBL databases. They are KIAA0209, isolated from the myeloblast cell line KG-1, KIAA0299 from brain, and GS034D21 from lymphoblastic cells. This finding strongly suggests that the human CDM-family proteins consist of at least four proteins; however, the role of the multiple proteins in this family is totally unknown. In this report, we characterize the KIAA0209 protein, which we renamed DOCK2 at the concession of the original depositor of the cDNA sequence [12].

2. Materials and methods

2.1. Sequence analysis

The cDNA sequences were aligned by using Clustal W [13]. The nucleotide sequences for KIAA0209, KIAA0299, and GS034D21 can be accessed through the GenBank database under GenBank Accession Numbers D86964, AB002297, and AC003077, respectively.

2.2. Plasmids

cDNA of KIAA0209 is a generous gift from Dr. Nomura at the Kazusa DNA Institute [12]. An *Xho*I restriction site was introduced before the first initiation codon by PCR-mediated mutagenesis. Then the full-length cDNA was subcloned into the pCXN2 expression vector [14], with a Flag-tag at its amino-terminus, to generate pCXN2-Flag-DOCK2. Similarly, deletion mutants were constructed by subcloning of restriction fragments of DOCK2 into pCXN2. pCXN2-Flag-DOCK2-ΔN encodes amino acids from 939 to the carboxyl-terminus. pCXN2-Flag-DOCK2-ΔCS and pCXN2-Flag-DOCK2-ΔCL encode DOCK2 from the amino-terminus to amino acids 515 and 1323, respectively. Expression plasmids for CrkII, pCAGGS-CrkII, and DOCK180, pCAGGS-DOCK180 and pCXN2-Flag-DOCK180, have been reported previously [7,15]. Full-length cDNA of Vav was also subcloned into the pCXN2-Flag vector [16]. pEBG is an expression vector for glutathione S-transferase (GST) [17]. cDNAs of Rac1 and Rac2 were obtained from Dr. J.S. Gutkind and Y. Takai, respectively. Both Rac1 and Rac2 cDNAs were subcloned into pEBG to generate pEBG-Rac1 and pEBG-Rac2, respectively.

2.3. Antibodies

cDNA corresponding to the carboxyl-terminal region of DOCK2 (amino acids 1644 to 1830) was amplified by PCR and subcloned into pGEX4T (Pharmacia) to produce a GST-tagged recombinant protein. The GST-tagged DOCK2 carboxyl-terminal region was purified by glutathione Sepharose and inoculated into rabbits as described previously [15]. Rabbit polyclonal sera were first passed through a GST-loaded column and then purified on the GST-DOCK2 affinity column as described [18]. Anti-DOCK180 antibody and anti-Crk 3A8 monoclonal antibody have been described previously [7,19]. Anti-flag M5 monoclonal antibody and anti-Crk monoclonal antibody were purchased from Eastman Kodak Co. and Transduction Lab. (Lexington, KY, USA) respectively.

2.4. Cells

293T human kidney cells and NRK normal rat kidney cells were maintained in DMEM containing 10% fetal bovine serum. Jurkat human T cells, Ramos human B cells, and THP-1 human monocyte-derived cells were cultured in RPMI 1640 containing 10% fetal bovine serum.

2.5. Histochemical staining

Formalin-fixed, paraffin-embedded tissues from a human cadaver were used for analysis. Endogenous

peroxidase activity was blocked by preincubation with 1% hydrogen peroxide in phosphate-buffered saline, followed by pretreatment in a microwave oven twice for 5 min. Sections were preincubated with 1% bovine serum albumin and incubated with the anti-DOCK2 affinity-purified antibody for 1 h at 37°C, followed by detection of the antibody by a peroxidase-conjugated streptavidin-DAB readout system (DAKO Japan Co., Kyoto, Japan). Finally, the nucleus was counter-stained with Giemsa solution (Wako Chemicals, Tokyo, Japan).

2.6. Binding to CrkII

293T cells were co-transfected with pCXN2-CrkII and pCXN2-Flag-DOCK180 or pCXN2-Flag-DOCK2. Forty-eight h after transfection, cells were lysed in lysis buffer (10 mM Tris-hydrochloride, pH 7.5, 5 mM EDTA, 150 mM NaCl, 1% Triton X-100, 500 μM Na_3VO_4, 10 mM NaF, 5 μg/ml aprotinin, 1 mM phenylmethylsulfonyl fluoride). CrkII was immunoprecipitated with rabbit antiserum and protein A-Sepharose [15]. Proteins bound to the beads were separated by SDS-PAGE and analyzed by immunoblotting by the use of anti-Flag monoclonal antibody or anti-Crk monoclonal antibody, followed by detection with peroxidase-labeled anti-mouse immunoglobulin and an ECL chemiluminescence system (Amersham).

2.7. Binding to Rac1 or Rac2

Binding of DOCK180 and DOCK2 to GST-Rac1 and GST-Rac2 was analyzed as described previously [8]. Briefly, 293T cells were transfected with pEBG-Rac1 or pEBG-Rac2 and pCXN2-Flag-DOCK180 or pCXN2-Flag-DOCK2. After 48 h, cleared cell lysates were incubated with glutathione Sepharose for 30 min at 4°C. Proteins collected on glutathione Sepharose were separated by SDS-PAGE, transferred to a polyvinylidene difluoride membrane, and probed with anti-Flag monoclonal antibody.

2.8. Activation of Rac1 by DOCK2

Analysis of guanine nucleotides bound to Rac1 was described previously [8]. Briefly, 293T cells were transfected with pEBG-Rac1 and pCXN2-derived expression vectors. After 3 h of labeling with $^{32}P_i$, GST-Rac1 was collected on glutathione Sepharose. The labeled guanine nucleotides on the beads were analyzed by polyethyleneimine thin-layer chromatography.

3. Results

3.1. Identification of novel human CDM-family proteins

A database search for the CDM-family proteins by the use of the DOCK180 amino acid sequence as a probe has revealed three amino acid sequences in man, DOCK2, KIAA0299, and GS034D21. We found that DocA of *Dictostylium*, also shared high sequence homology with DOCK180. DOCK180, DOCK2, Ced-5, and Mbc contain an SH3 domain at the amino-terminus. The amino acid sequences of KIAA0299, GS034D21, and DocA did not contain the amino-terminal region including SH3 (Fig. 1a). Further study is needed for determining whether these proteins truly conform only to the C-terminal region of the CDM family. Shown in Fig. 1b is the alignment of the amino acid sequences of the overlapping regions. The carboxyl-terminal regions of these proteins are extremely divergent. As a result, the Crk-binding sites of DOCK180 are not found in the other three human proteins. The phylogenetic tree shows that DOCK2 is the closest homolog to DOCK180 (Fig. 1c). KIAA0299 and GS034D21 appear to form another subfamily.

In this study, we concentrated on the characterization of DOCK2. The cDNA sequence of DOCK2 contained a 5490 bp open reading frame that encodes 1830 amino acids with a calculated molecular mass of 212 kDa. The amino acid sequence of DOCK2 showed 62.3% identity with DOCK180 when the carboxyl-terminal variable regions were omitted.

3.2. Distribution of DOCK2

The expression and distribution of DOCK2 mRNA were examined by Northern blotting analysis (Fig. 2a). A 7.4 kb transcript was expressed most abundantly in peripheral blood leukocytes, followed by thymus and spleen. Very weak expression was

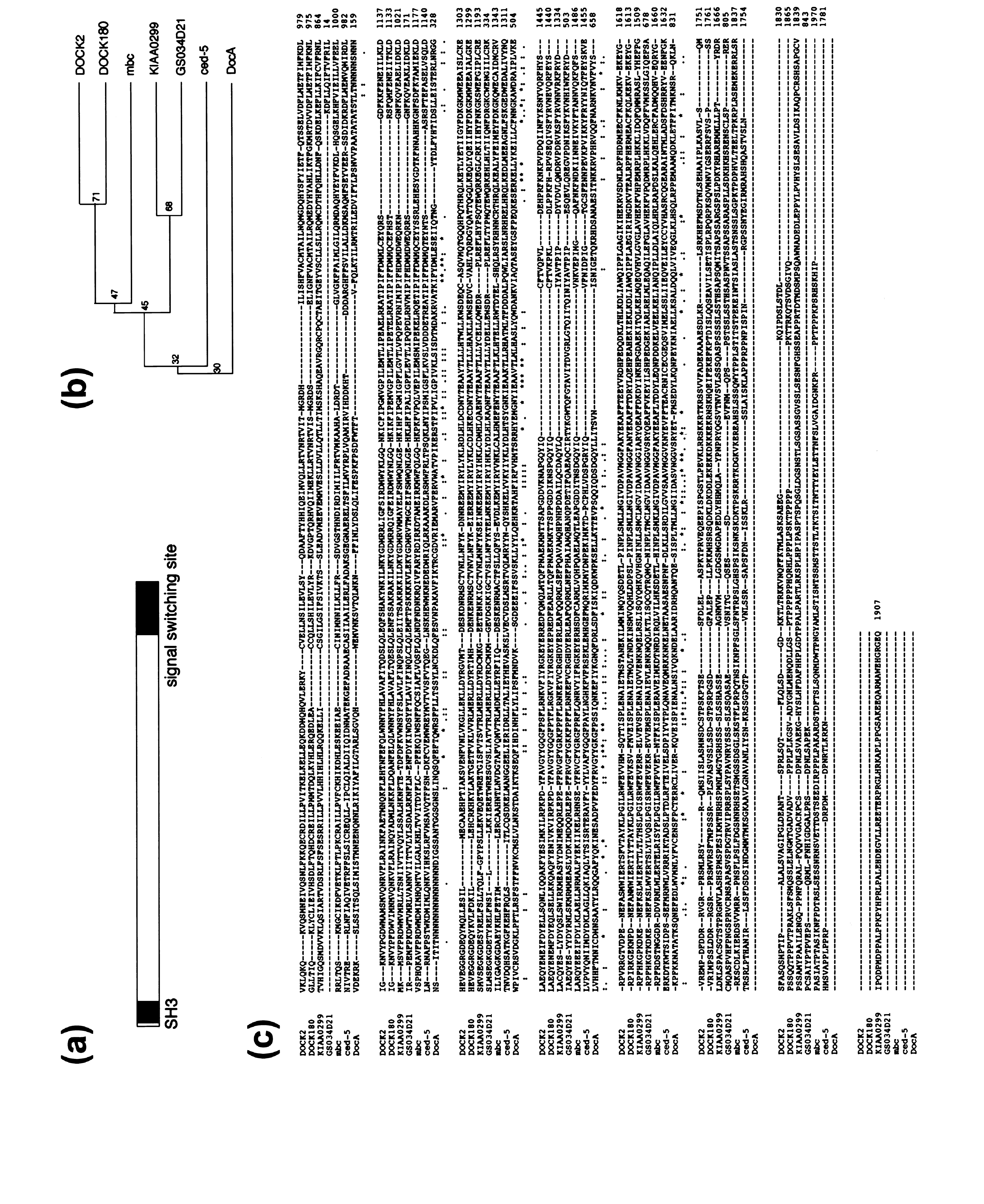
(a)
SH3
signal switching site
(b)
DOCK2
DOCK180
mbc
KIAA0299
GS034D21
ced-5
DocA
71
47
68
45
32
30
(c)
DOCK2
DOCK180
KIAA0299
GS034D21
mbc
ced-5
DocA

Fig. 1. Alignment of the amino acid sequences of the CDM-family proteins. (a) The nucleotide sequences reported for each protein are demonstrated as solid lines below the schematic representation of the structure of the CDM-family proteins. Alignment (b) and dendrogram (c) of the CDM-family proteins were drawn by the use of Clustal W. Amino acid sequences overlapping in all seven CDM-family proteins were used for the analysis. Asterisks indicate amino acids conserved absolutely. Double stars demonstrate the conserved amino acid substitutions. The single stars are semi-conserved amino acid substitutions.
←

detected in the small intestine and colon. This distribution contrasts clearly with that of DOCK180, which was expressed in all tissues except peripheral blood leukocytes. For the detection of DOCK2 protein, we raised antisera by immunizing rabbits with the bacterially expressed DOCK2 carboxyl-terminal region. Among various cell lines tested, expression of DOCK2 protein was limited to those derived from T cells, B cells, and monocytes. Representative data are shown in Fig. 2b. DOCK2 was detected in Jurkat T cells, Ramos B cells, and THP-1 monocytes, but not in 293T kidney cells. Again, we found that expression of DOCK180 was complementary to that of DOCK2. These results were also confirmed by Northern blotting analysis (data not shown).

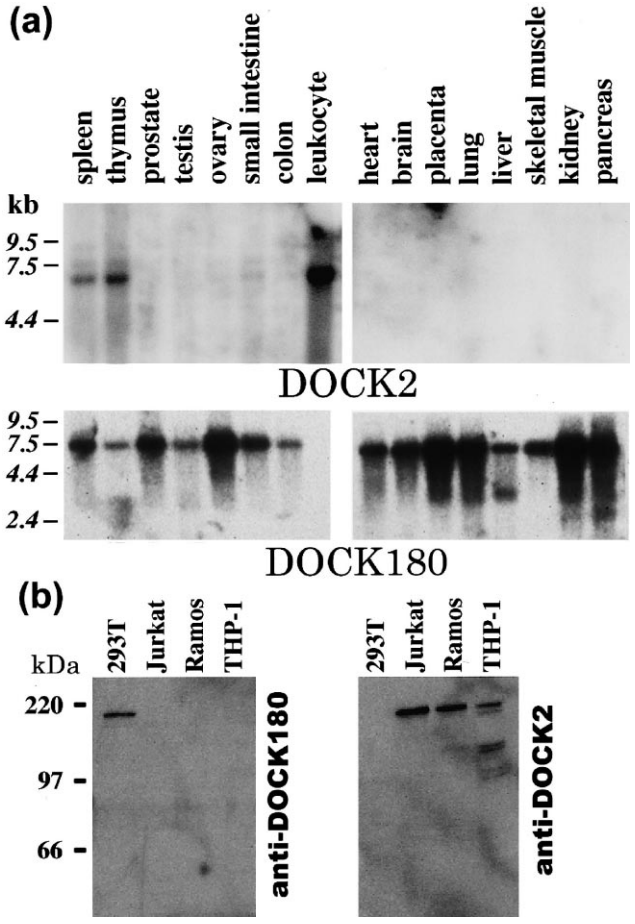

Fig. 2. Distribution of DOCK2 in tissues and cell lines. (a) Nylon filters blotted with human mRNAs of various tissues were hybridized with [^{32}P]-dCTP-labeled cDNA probes of DOCK2 and DOCK180. (b) 293T, Jurkat, Ramos, and THP-1 cells are derived from human kidney cells, T cells, B cells, and monocytes, respectively. Total cell lysates were separated by SDS-polyacrylamide gel electrophoresis and analyzed by immunoblotting with antisera against DOCK180 or DOCK2, as indicated on the right.

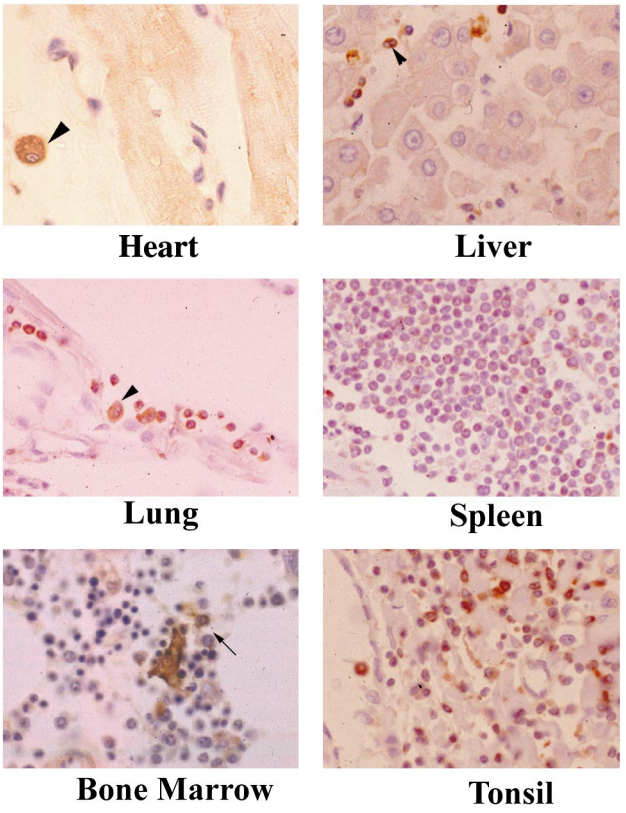

Fig. 3. Immunohistochemical analysis of DOCK2. Formalin-fixed, paraffin-embedded tissues from human cadavers were used for analysis. Specimens were incubated with anti-DOCK2 serum, followed by detection by a peroxidase-conjugated streptavidin-DAB readout system. The nuclei of the cells were counter-stained with Giemsa. Arrows and arrowheads indicate lymphocytes and macrophages, respectively.

3.3. Immunohistochemical study of DOCK2

Because the mRNA used for the Northern analysis was extracted from organs, the faint bands detected in the small intestine and colon may not necessarily reflect the expression of DOCK2 in the parenchymal cells of these organs. Thus, we examined the expression of DOCK2 immunohistochemically (Fig. 3). In the heart, liver, and lung, myocytes, hepatocytes, and lung epithelial cells did not express DOCK2; however, macrophages in the interstitium or alveolus were detected by the anti-DOCK2 antibody. Lymphocytes in the lymph nodes showed strong reactivity with anti-DOCK2. Most of the hematopoietic cells also expressed DOCK2, although the level of expression varied from a cell to cell. In the tonsil, again, cells positive for DOCK2 were limited to macrophages and lymphocytes.

3.4. Inability to bind to CrkII

We recently reported that DOCK180 is involved in signaling from focal contacts by means of the CrkII-p130^{Cas} complex [20]. Because both the CrkII and p130^{Cas} proteins are expressed rather ubiquitously [2,15,21], DOCK2 may function similarly to DOCK180 in non-adherent cells. Therefore, we examined whether DOCK2 binds to CrkII (Fig. 4). 293T cells expressing CrkII with either DOCK180 or DOCK2 were lysed and immunoprecipitated with anti-CRK rabbit serum. DOCK180 co-precipi-

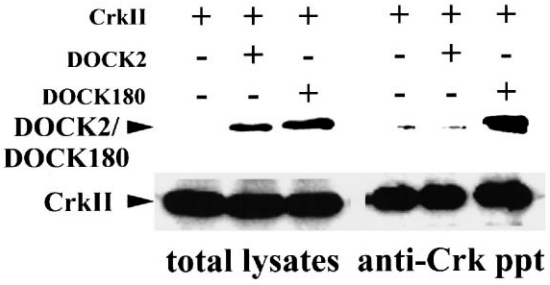

Fig. 4. Binding of DOCK180, but not DOCK2, to CrkII. 293T cells were co-transfected with pCXN2-CrkII (indicated as CrkII) and pCXN2-Flag-DOCK2 (DOCK2) or pCXN2-Flag-DOCK180 (DOCK180). Cells were lysed and immunoprecipitated with anti-Crk rabbit serum. Total cell lysates and immunoprecipitates were separated by SDS-PAGE and analyzed by immunoblotting with anti-Flag monoclonal antibody (top) or anti-Crk monoclonal antibody (bottom).

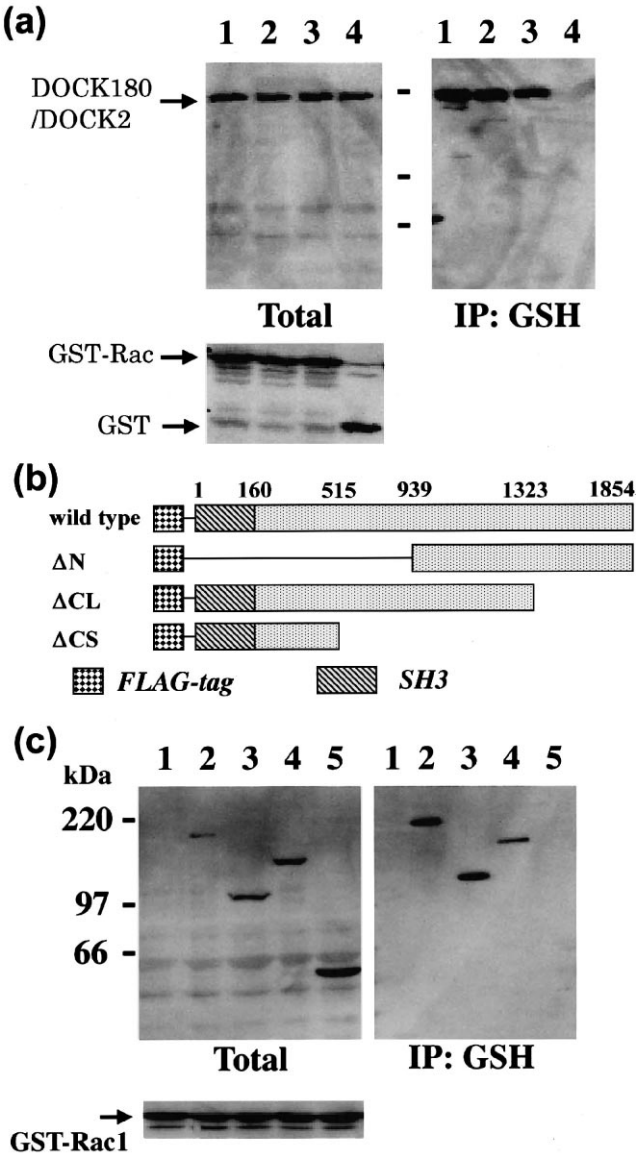

Fig. 5. Binding of DOCK2 to Rac1 and Rac2. (a) 293T cells transfected with the expression vectors were lysed, and GST-tagged Rac proteins were collected on glutathione Sepharose. Total cell lysates (left panel) and proteins bound to the beads (right panel) were analyzed by immunoblotting with anti-Flag monoclonal antibody (upper panels) or anti-GST polyclonal antibody (lower panel). Transfected plasmids are: lanes 1, pCXN2-Flag-DOCK1 and pEBG-Rac1; lanes 2, pCXN2-Flag-DOCK2 and pEBG-Rac1; lanes 3, pCXN-Flag-DOCK2 and pEBG-Rac2; lanes 4, pCXN-Flag-DOCK2 and pEBG. (b) Schematic representation of DOCK2 mutants; wild-type, pCXN-Flag-DOCK2; ΔN, pCXN-Flag-DOCK2-ΔN; ΔCL, pCXN-Flag-DOCK2-ΔCL; ΔCS, pCXN-Flag-DOCK2-ΔCS. (c) 293T cells were transfected with pEBG-Rac1 and the vectors denoted at the top and analyzed as in (a).

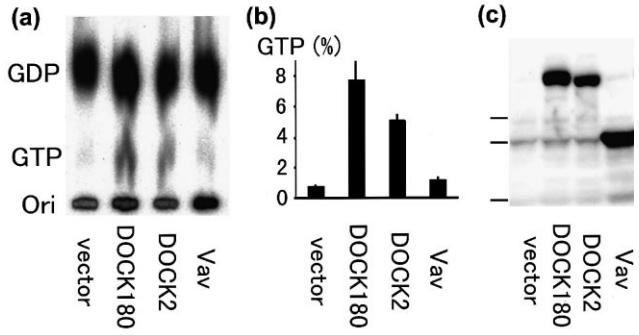

Fig. 6. Activation of Rac1 by DOCK2. (a) 293T cells were transfected with pGEX-Rac1 and expression vectors for the proteins indicated at the bottom. Cells were labeled with $^{32}P_i$, and the guanine nucleotides bound to Rac were separated by TLC as described in the text. (b) The ratio of GTP versus total guanine nucleotides were calculated from two independent experiments. Errors are shown as bars. (c) In a parallel experiment, cell lysates were analyzed by immunoblotting with anti-Flag antibody. Bars at the left indicate the positions of molecular size markers; 116, 99, and 66 kDa from the top.

tated with CRKII as reported previously; however, DOCK2 did not. Thus, DOCK2 cannot bind to CrkII even when both proteins are expressed in excess. In accordance with this finding, we could not observe co-immunoprecipitation of Crk and DOCK2 in Jurkat cells (data not shown).

3.5. Binding of DOCK2 to Rac1 and Rac2

Recently, we have shown that DOCK180 binds to and activates Rac1 [8]. Thus, we tested whether DOCK2 also binds to Rac1 and Rac2 in 293T cells. 293T cells were transfected with expression vectors for GST, GST-Rac1 or GST-Rac2 and Flag-DOCK1 and Flag-DOCK2. GST and GST-tagged proteins were precipitated with glutathione beads from cell lysate and proteins bound to the beads were analyzed by immunoblotting using anti-Flag antibody. As shown in Fig. 5a, DOCK2 bound to both GST-Rac1 and GST-Rac2, but not to GST. For the determination of the region necessary for the binding to Rac, we used deletion mutants of DOCK2 shown in Fig. 5b. Since both ΔN and ΔCL mutants bound to GST-Rac1, the Rac1-binding domain was assigned to amino acids 939 to 1323. However, it should be pointed out that the ΔCL mutant (amino acids 1–1323) bound to Rac1 significantly less than did wild-type DOCK2, suggesting that more of the carboxyl-terminal region appears to be required for optimal binding.

3.6. Activation of Rac1 by DOCK2

We examined whether DOCK2 promotes the guanine nucleotide exchange reaction of Rac1 by expressing GST-Rac1 with DOCK2 (Fig. 6a and b). DOCK2 increased the GTP/GDP ratio on Rac1, as did DOCK180. Vav, which is known as a guanine nucleotide exchange protein for Rac1 [22], increased the GTP/GDP ratio only slightly in our assay system. The levels of protein expression among DOCK180, DOCK2, and Vav were comparable (Fig. 6c). Thus, at least in 293T cells, DOCK2 facilitates the guanine nucleotide exchange reaction more efficiently than does Vav. Vav may require an additional signal, such as tyrosine phosphorylation [22], for its optimal enzymatic activity.

4. Discussion

The CDM-family proteins are evolutionarily conserved from nematodes to man (Fig. 1). Moreover, the presence of at least four human proteins belonging to this family demonstrates that the CDM-family proteins have also diverged into several human proteins during evolution. We have focused on the similarities and the differences between DOCK180 and DOCK2, which are most closely related to each other among the four human CDM-family proteins.

The expression of DOCK180 and DOCK2 is strictly exclusive from one the other, as far as we have examined. DOCK2 is expressed only in non-adherent cells such as T cells, B cells, and macrophages in the peripheral blood and tissues, whereas DOCK180 is expressed only in adherent cells. To examine whether this differential expression depends on the state of the cells, we treated THP-1 monocyte-derived cells with phorbol ester, which induces differentiation of THP-1 cells into macrophage-like adherent cells [23]. We observed that phorbol ester cannot induce DOCK180 nor down-regulate DOCK2 in THP-1 cells after differentiation (data not shown). Thus, the expression of DOCK2 appears to be related to the cell lineage rather than to the adhesion signals.

We have positioned DOCK180 downstream of the p130^{Cas}-CrkII complex, which is activated by integrin stimulation [20]. Because both p130^{Cas} and CrkII are expressed ubiquitously [2,15], we speculated that DOCK2 may substitute for the role of DOCK180 in these cells. However, we never detected binding of CrkII to DOCK2; thus, there was no involvement of DOCK2 in CrkII signaling. Neither of the other putative human DOCK180-like proteins, KIAA0299 and GS034D21, contains the Crk-binding sequence at its carboxyl-terminus; therefore, each of the DOCK180-like proteins may be positioned in different signaling cascades.

DOCK2 binds to and activates Rac1 in a manner similar to DOCK180 [8]. However, the effects of these two proteins on the morphology of the cells are astonishingly different. DOCK180 per se does not induce a morphologic change in adherent cells; however, with the membrane localization signals or with the co-expression of p130^{Cas} and CrkII, DOCK180 induces spreading of the cells [7,20]. In contrast, the expression of DOCK2 induced rounding and detachment of NRK cells from the dishes (data not shown). A similar phenomenon was observed when we used NIH 3T3 cells (data not shown). Thus, apparently the activation of Rac1 by DOCK180 or by DOCK2 does not result in the same outcome for the cell morphology. The difference in the intracellular localization of DOCK180 and DOCK2 might account for this discrepancy.

Rac proteins play pivotal roles in the reorganization of the cell architecture and also in gene regulation in most cells [24]. However, Rac is also implicated in a function specific to macrophages and granulocytes. In these cells, Rac1 is involved in NADPH oxidation by means of binding to p67-phox [25–27]. Mutations in p67-phox that abrogate the binding to Rac cause chronic granulomatous disease [28]. The coincidence of the expression of DOCK2 in phagocytic cells may suggest the involvement of DOCK2 in phagocytosis. It is noteworthy that DOCK180 can complement only one of the two defects in *ced*-5 mutant nematodes [10]; DOCK180 expression restores the distal tip cell migration of the *ced*-5 mutant, but not the engulfment of the apoptotic body. This observation suggests the involvement of other DOCK180-like proteins, possibly DOCK2, in phagocytosis in man. It is not yet known whether DOCK2 plays any role in phagocytosis and NADPH oxidation.

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