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Archives of Biochemistry and Biophysics 429 (2004) 23–29
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Nuclear localization of the DOCK180/ELMO complex

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Received 19 March 2004, and in revised form 18 May 2004
Available online 2 July 2004

Abstract

DOCK180 protein plays a key role during development, cell motility, and phagocytosis. It forms a complex with another protein ELMO, and this complex acts as a guanine nucleotide exchange factor (GEF) for Rac. However, DOCK180-containing complexes have not been purified by unbiased biochemical approaches, and the nature and subcellular localization of these complexes remain unclear. Here, we show that a large fraction of endogenous DOCK180 is present as a 700 kDa nuclear complex with ELMO proteins. In addition, this nuclear DOCK180/ELMO complex has functional Rac-GEF activity. Furthermore, endogenous DOCK180 could be found in complexes with different ELMO isoforms (ELMO1, 2 or 3) in different cell lines, depending on the ELMO isoforms expressed. These studies suggest that DOCK180 may associate with different ELMO proteins to form cell-type specific complexes and may have functions in both the nucleus and the cytoplasm.

Keywords: DOCK180; ELMO; Guanine nucleotide exchange factor; Molecular complex; Nuclear localization

Mammalian DOCK180 protein and its homologues in *Drosophila* Myoblast City and *Caenorhabditis elegans* (CED-5) represent an evolutionarily conserved family of proteins involved in multiple biological processes. DOCK180 participates in actin cytoskeletal changes during cell migration and phagocytosis of apoptotic cells [1–6]. The *Drosophila* (Myoblast City) plays a role in myoblast fusion, dorsal closure, and cytoskeletal organization during embryonic development [7–9]. The *C. elegans* CED-5 is implicated in engulfment of cell corpses and in distal tip cell migration [5,10–15]. The DOCK180 family of proteins has been suggested to function as an upstream regulator of the small GTPase Rac, which mediates membrane ruffling and other cytoskeletal changes [8,10]. Although DOCK180 does not contain the conventional Dbl homology (DH) domain found in essentially all known mammalian Rac guanine nucleotide exchange factors (GEFs)[1] [16–18], DOCK180 contains a novel domain, termed Docker domain, which specifically recognizes nucleotide-free Rac and can promote loading of GTP by Rac in vitro [19]. Recent evidences suggest that in addition to Dock180, 10 additional proteins in the human genome contain a Docker domain, and that these constitute a Dock180 superfamily of proteins [20]. These different DOCK180 family members have been linked to various biological processes including cell adhesion, migration, phagocytosis of apoptotic cells, and tumor cell metastasis.

Several lines of evidence have suggested that DOCK180 forms a complex with another protein ELMO1 [5,19], which, like DOCK180, has also been identified as an evolutionarily conserved upstream

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[1] *Abbreviations used:* DTT, dithiothreitol; ELMO, engulfment and cell motility; GEF, guanine nucleotide exchange factor; IP, immunoprecipitation; PMSF, phenylmethylsulfonyl fluoride; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis.

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doi:10.1016/j.abb.2004.05.014

regulator of Rac. CED-12, the ELMO1 homologue in *C. elegans*, has been shown to function genetically at the same step as DOCK180/CED-5 in engulfment of apoptotic cells and cell migration in nematodes [5,10,13,14]. In cell culture studies, DOCK180 itself is insufficient for GTP loading of Rac, and a complex with ELMO is required [19]. Recent studies indicate that the DOCK180/ELMO complex functions as a bi-partite GEF for Rac [19,21]. At least three ELMO proteins (ELMO1, 2, and 3) have been reported in mammals, and none of them contains any obvious catalytic domains.

Although endogenous DOCK180 and ELMO1 have been shown to occur as a complex in cells [19], this complex has not been purified by unbiased biochemical approaches. Thus, the nature of the DOCK180/ELMO complex, and its localization remain unclear. Here, we have immunoisolated DOCK180-containing complexes from several mammalian cell lines. We show that DOCK180 is present in a complex with ELMO in both the nucleus and the cytoplasm, and that DOCK180 can form complexes with multiple ELMO isoforms in different cell lines.

Experimental procedures

Cells and antibodies

HeLa S3 and CHO-K1 cells were obtained from the National Cell Culture Center. Nuclear and cytoplasmic extracts were prepared as previously described [22]. Three DOCK180 antibodies (sc-5635, sc-13163, and sc-6167) were obtained from Santa Cruz Biotechnology. Another DOCK180 antibody was kindly provided by Dr. Matsuda (Japan). The ELMO1 antibody has been described previously [19], which has weak cross-reactivity to ELMO2. The rabbit ELMO2 antibody was generated against a recombinant GST–ELMO2 fusion protein (encompassing amino acid residues 1–551 of ELMO2) expressed in *E. coli*. The specific reactivity of the ELMO2 antibody was confirmed using ELMO1, ELMO2, and ELMO3 proteins expressed in 293T cells, as well as bacterially produced recombinant proteins (data not shown). The ELMO2 antibody was purified on a protein-A column prior to use in immunoblotting.

Immunoisolation of DOCK180 complexes

DOCK180 complexes were isolated from both nuclear and cytoplasmic extracts of HeLa and CHO-K1 cells by using an immunoprecipitation protocol previously described (Lee et al. unpublished data; [23]). Briefly, 1 ml (7 mg/ml) of nuclear extract was diluted 10 fold with IP buffer (20 mM Hepes [pH 7.9], 200 mM NaCl, 10% glycerol, 0.1% NP-40, 1 mM dithiothreitol (DTT), 0.2 mM phenylmethylsulfonyl fluoride (PMSF), 2 μg/ml leupeptin, and 2 μg/ml aprotinin). The mixture was incubated with 2 μg of DOCK180 antibodies in the presence of 100 μl of protein A-beads (Amersham–Pharmacia) for at least 12 h at 4 °C. The immunoprecipitate was washed three times with the IP buffer. The complex on the beads was either used in the GEF assay or was eluted from the beads using 100 mM glycine–HCl buffer (pH2.5). The eluted complex was subjected to sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) and immunoblotting analysis. Less than 10% of the input was loaded as control for immunoblotting. For mass spectrometric analysis, the proteins were visualized by Coomassie blue staining, excised from the gel, and digested with trypsin. The peptides obtained were analyzed by matrix-assisted laser desorption ionization-time of flight mass spectrometry.

Nuclear extract fractionation

The HeLa S3 nuclear or cytoplasmic extract was directly applied to a Superose 6 column (HR16/50; Amersham) equilibrated with the column running buffer containing 20 mM Hepes (pH 7.9), 200 mM NaCl, 1 mM DTT, 0.1 mM PMSF, 5 μg/ml leupeptin, and 5% glycerol. Fractions were collected and analyzed by SDS–PAGE and immunoblotting.

In vitro GEF assay

The DOCK180 complex immobilized on the protein A beads was analyzed for Rac GEF activity as follows [19]: 1 μg of bacterially expressed and purified Rac was incubated with 50 μCi of [α-^{32}P]GTP (3000 Ci/mmol) in a loading buffer containing 40 mM Mops (pH 7.1), 1 mM EDTA, 1 mg/ml BSA, and 0.3 μM unlabeled GTP, for 20 min on ice. Magnesium chloride was then added at a final concentration of 10 mM, and the mixture was incubated on ice for an additional 10 min. [α-^{32}P]GTP-loaded Rac (50 ng) was added to the DOCK180 complex, and the mixture was diluted using an exchange buffer containing 25 mM Mops (pH 7.1), 6.25 mM magnesium chloride, 0.6 mM NaH_2PO_4, 0.5 mg/ml BSA, and 1.25 mM GDP (unlabeled), so that the final volume was 100 μl. After 15-min incubation at 30 °C, 50 μl of the exchange reaction was subjected to filter binding to nitrocellulose and scintillation counting. The presence of GEF-activity was detected by loss of radioactivity bound to Rac (as a result of the exchange reaction). [^{32}P]GTP bound to Rac when incubated with the exchange buffer alone was set at 100%. The data presented are representatives of at least three independent experiments. In a time course of this exchange assay with the DOCK180 complex, the maximal exchange on Rac was detected between 10 and 20 min. Hence, a 15-min point was chosen for the assay.

Immunofluorescence

Cell fixation and blocking followed a published protocol [24]. Cells were incubated with a DOCK180 antibody (Santa Cruz Biotechnology) in blocking buffer (5% milk in PBS with 0.2% Triton X-100) at 4 °C overnight, and then stained with Alexa Fluor 568-conjugated secondary antibody (Molecular Probes). DNA was stained using the TOTO-3 dye (Molecular Probes). Confocal images were obtained with a Zeiss LSM 410 (Carl Zeiss, Germany). As a negative control, cells were incubated with only the secondary antibody, which had negligible fluorescence under these conditions (data not shown).

Results

Purification of DOCK180/ELMO complex in nuclear extract of HeLa cells

In our studies to identify components of nuclear chromatin remodeling and helicase complexes from HeLa nuclear extract by immunoisolation [23,25–28], we identified DOCK180 as a component by mass spectrometry (data not shown). Subsequent analyses showed that DOCK180 does not associate with these nuclear proteins, and is likely a contaminant isolated due to antibody cross-reactivity. However, since DOCK180 has not been reported to be present in nucleus, and DOCK180 complex has not been purified by unbiased biochemical approach, we decided to immunopurify the nuclear and cytosolic DOCK180 complex for further characterization.

We isolated the endogenous DOCK180/ELMO complex from human HeLa nuclear extracts by direct immunoprecipitation with a DOCK180-specific antibody. Three major polypeptides (180, 72, and 70 kDa) were immunoisolated by a DOCK180 antibody, but not by a preimmune serum or by protein A beads alone (Fig. 1A). The same three polypeptides were immunoisolated by two additional antibodies against other regions of DOCK180 (H4 and C19) (Fig. 1B), suggesting that these three polypeptides are not contaminants isolated due to antibody cross-reactivity, but are components of one complex. The 180 kDa polypeptide was identified as DOCK180 by both mass spectrometry and immunoblotting analysis (Fig. 1C). Two other polypeptides were identified by mass spectrometry as ELMO2 and ELMO3, both of which are homologues of ELMO1, but encoded by distinct genes. The identity of ELMO2 was further confirmed by immunoblotting analysis (Fig. 1C). Since specific antibodies against ELMO3 are not yet available, we could not identify this by immunoblotting.

Interestingly, the complex of ELMO with DOCK180 is highly stable, as it resists the extraction conditions used, as well as washing with 1 M salt (data not shown). In contrast, although nucleotide-free Rac, $p130^{Cas}$ and CrkII have been shown to interact with DOCK180 [1–5], we failed to detect these proteins as part of the DOCK180 complex (data not shown), implying that the interactions between these polypeptides and DOCK180 are not stable enough to withstand the isolation procedure used here.

Superose 6 gel-filtration fractionation revealed that DOCK180 in HeLa nuclear extract fractionates as a single peak (Fig. 1D), corresponding to a complex with molecular weight close to 700 kDa. The same analysis also showed that ELMO2 similarly fractionates as a single peak, and that this peak overlaps with that of DOCK180, consistent with the notion that these proteins are components of a single complex. The 700 kDa molecular weight of this complex is roughly twice the sum of the molecular weights of DOCK180, ELMO2, and ELMO3, perhaps suggesting the presence of two copies of Dock180 and ELMO.

DOCK180/ELMO complex localizes in both the nucleus and the cytoplasm

Immunoblotting analysis revealed that DOCK180 is present in both nuclear and cytoplasmic extracts (Fig. 2A). A known nuclear protein, BRCA2, was detected only in the nuclear extract. Immunoprecipitation with a DOCK180 antibody from the cytoplasmic extract obtained the same three polypeptides as those from the nuclear extract (Fig. 2B). However, the amount of DOCK180 complex isolated from the cytoplasmic extract is reproducibly lower than that obtained from the nuclear extract, consistent with the lower amount of DOCK180 detected by immunoblotting in the cytoplasmic extract (Fig. 2A).

We performed indirect immunofluorescence to localize DOCK180 in HeLa cells. DOCK180 was detected in both the nucleus and cytoplasm (Fig. 2C). The intensity of immunofluorescence in the cytoplasm is also lower than that in the nucleus, consistent with the biochemical data above that DOCK180 is more abundant in the nucleus.

DOCK180/ELMO complex isolated from the nucleus contains GEF activity

DOCK180 has previously been shown to function as a two-part guanine nucleotide exchange factor for Rac [19]. To test whether the nuclear localized DOCK180/ELMO complex is functional in activating Rac, we tested the activity of the complex purified from cells in an in vitro GEF assay. We found that the DOCK180 complexes from either HeLa nuclear or cytoplasmic extracts display robust GEF activity toward Rac (Fig. 3). Since the DOCK180 complex isolated from HeLa cells lacks ELMO1 but contains ELMO2 and

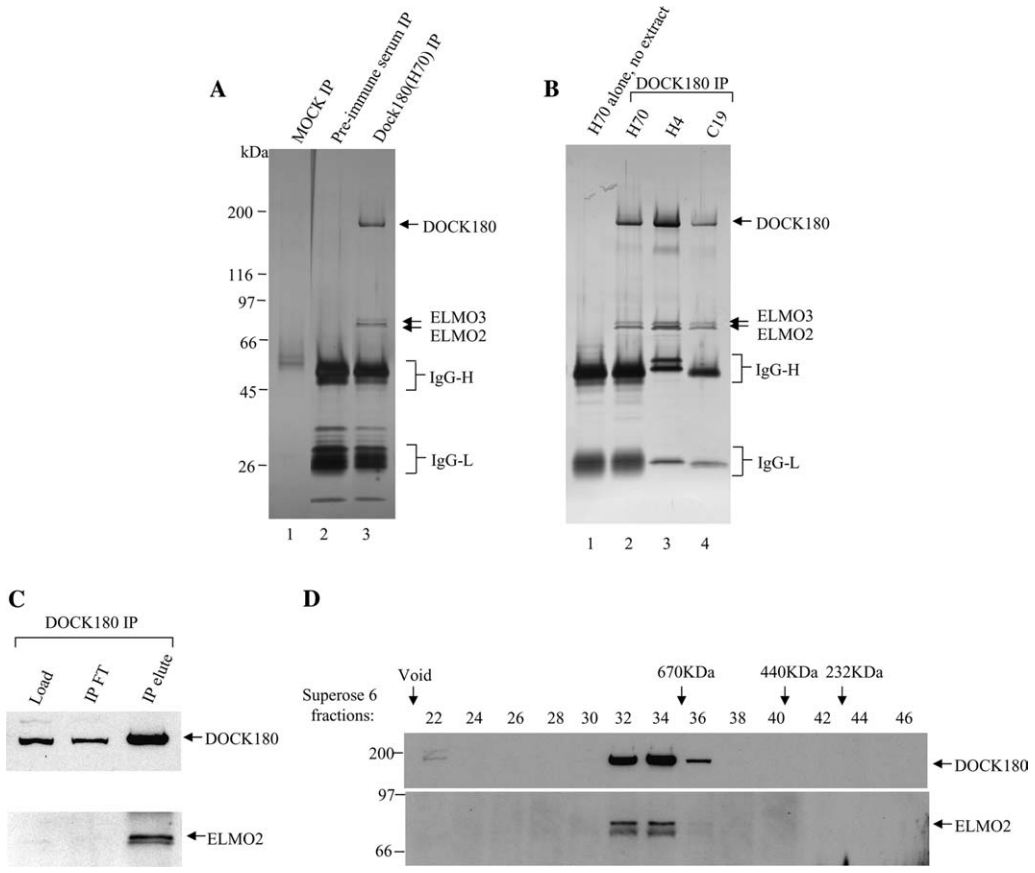

Fig. 1. Purification of the DOCK180/ELMO complex from HeLa cell nuclear extracts. (A) Silver-stained SDS–PAGE gel (8–16%) with the DOCK180/ELMO complex isolated by immunoprecipitation with DOCK180 polyclonal antibody from HeLa cell nuclear extracts. Polypeptides identified by mass spectrometry are indicated. The presence of the heavy and light chains of the immunoglobulins (IgG-H and IgG-L) is also indicated. Lane 1: mock immunoprecipitate using HeLa nuclear (NE) without DOCK180 antibody; lane 2: preimmune serum IP; and lane 3: DOCK180 complex isolated by immunoprecipitation with DOCK180 antibody. (B) Silver-stained SDS–PAGE gel (8–16%) with the DOCK180/ELMO complex immunoisolated by three different DOCK180 antibodies (H70, H4, and C19) from HeLa cell nuclear extract. The polypeptides derived from a mock immunoprecipitation using antibody alone without the nuclear extract are also shown (lane 1). (C) Immunoblotting analysis of the load (Load), IP, flow-through (FT), and IP eluted fraction (elute) after immunoprecipitation with DOCK180 antibody. The immunoreactivity for each antibody is indicated. (D) Immunoblotting of different fractions from the Superose 6 fractionation of HeLa nuclear extract reveals that ELMO2 co-fractionates with DOCK180. The immunoreactivity to DOCK180 and ELMO2 is indicated. The arrows at the top denote the elution positions of calibration proteins of known molecular weights.

ELMO3 (see below), these data suggest that ELMO2 and ELMO3 can participate in promoting the Rac-GEF activity of DOCK180.

DOCK180 associates with distinct ELMO proteins in different cell lines

Although ELMO1 and ELMO2 have been reported to interact with DOCK180 [5,19,20], we detected ELMO2 and ELMO3, but not ELMO1, in the DOCK180/ELMO complex isolated from HeLa cells (Figs. 1A–C). Subsequent analyses showed that ELMO1 is not detectably expressed in HeLa cells (Fig. 4A). It has been reported that DOCK180 co-immunoprecipitates with ELMO1 in the LR73 cell line (a Chinese hamster ovary cell variant) [19]. Consistent with this, immunoprecipitation of DOCK180 from CHO K1 cells identified a complex containing ELMO1 and ELMO2, but not ELMO3 (Figs. 4B and C). This suggested that the DOCK180 complexes in CHO K1 and LR73 cells are different from that of HeLa cells, which contains ELMO2 and ELMO3, but not ELMO1. Thus, DOCK180 may associate with different ELMO homologues in different cell lines.

Discussion

DOCK180 and ELMO proteins function as key upstream regulators of Rac during development and cell migration in several higher organisms. However, the molecular nature of the endogenous DOCK180/ELMO complex and the subcellular localization of these complexes have not been addressed. In this study, we used an unbiased biochemical approach to immunoisolate endogenous DOCK180 complexes from three different

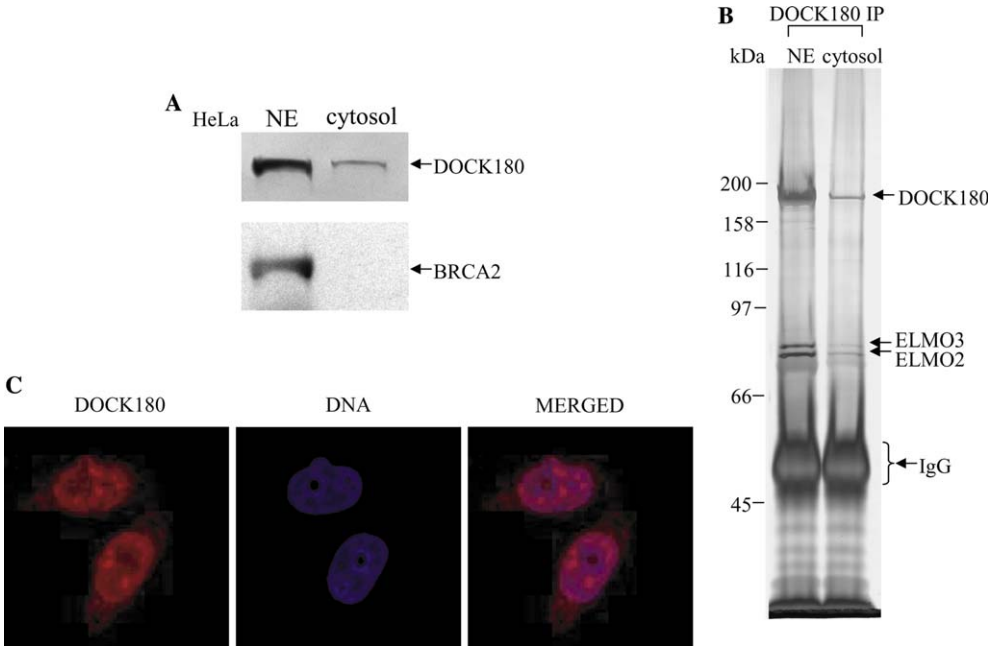

Fig. 2. DOCK180 complex can be found in both nuclear and cytoplasmic extracts from HeLa cells. (A) Immunoblotting analysis of nuclear and cytoplasmic extracts for Dock180. Immunoblotting of BRCA2, a known nuclear protein, is also shown as control. The same amounts of nuclear and cytoplasmic extracts were loaded in the two lanes. (B) DOCK180 complexes purified from HeLa nuclear and cytoplasmic extracts were separated by SDS–PAGE and visualized by silver-staining. Lane 1: DOCK180/ELMO complex from HeLa cell nuclear extract. Lane 2: DOCK180/ELMO complex from HeLa cell cytoplasmic extract. (C) DOCK180 localizes to both the nucleus and cytoplasm in HeLa cells as analyzed by indirect immunofluorescence. A rabbit polyclonal antibody against DOCK180 (DOCK180 H-70: red) was used, while the DNA was visualized by TOTO-3 staining (Blue).

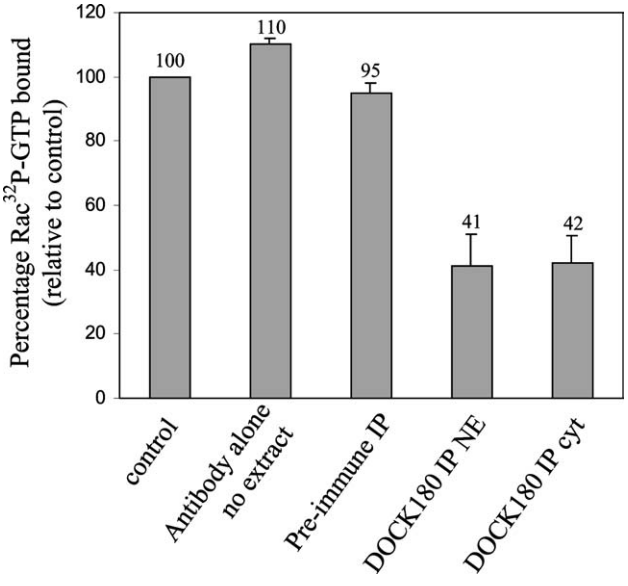

Fig. 3. DOCK180 complex purified from HeLa cell nuclear extract has a Rac guanine nucleotide exchange factor (GEF) activity. DOCK180 complexes purified from either the nuclear or cytoplasmic extracts were tested for GEF activity using as substrate [α-^{32}P]GTP-loaded Rac. The presence of GEF activity was detected by loss of radioactivity bound to Rac as a result of the exchange reaction (see Experimental procedures). [^{32}P]GTP bound to Rac when incubated with exchange buffer alone was set at 100%.

mammalian cell lines. Interestingly, in addition to their presence in the cytoplasm, we observe a significant fraction of both DOCK180 and ELMO proteins in the nucleus. This was seen in HeLa cells as well as two different CHO cell lines. Since the DOCK180/ELMO complex in the nuclear extract possessed robust Rac-GEF activity, an interesting possibility is that the DOCK180 may regulate Rac activation in the nucleus. Although we did not detect Rac in a complex with DOCK180/ELMO, this might be either because only nucleotide-free Rac associates with DOCK180, or Rac could not form a stable complex with DOCK180 under the conditions of purification; moreover, if only a small fraction of Rac were to localize to the nucleus, this might have been difficult to detect. While smaller proteins could move readily between the nucleus and the cytoplasm, the existence of a DOCK180 complex ($\sim$700 kDa) in the nucleus suggests that DOCK180/ELMO may be actively transferred to the nucleus. The biological significance of the nuclear localization of DOCK180/ELMO complex remains to be determined, but it is noteworthy that other proteins in the Rac signaling pathway, such as the p21 activated kinase (PAK) have been detected in the nucleus previously ([29]). Thus, it is tempting to speculate that the DOCK180 complex may have a nuclear function. It would be of

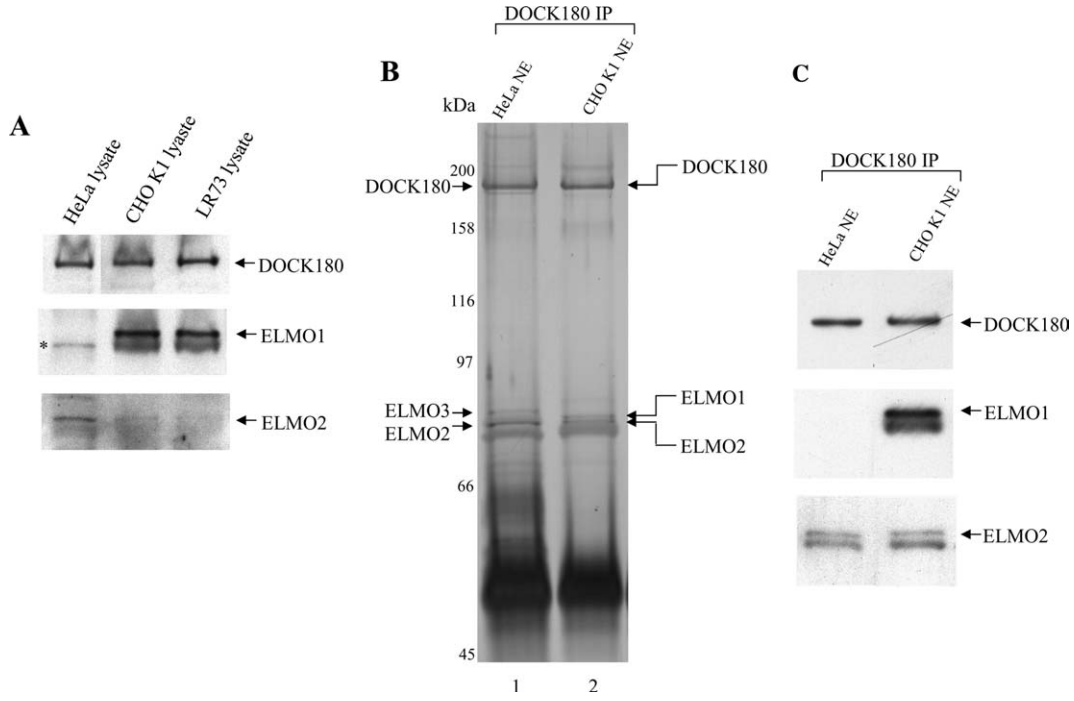

Fig. 4. DOCK180 forms different ELMO-containing complexes in different cell lines. (A) Immunoblotting of DOCK180 complexes from HeLa, LR73, and CHO-K1 cells. The band indicated by asterisk is likely a nonspecific protein, which cross-reacts with ELMO1. (B) Silver-stained SDS–PAGE gel with the DOCK180 complex purified from HeLa and CHO-K1 nuclear extracts. Lane 1: DOCK180/ELMO complex isolated from HeLa cell nuclear extract; lane 2: DOCK180/ELMO complex isolated from CHO-K1 nuclear extracts. Notably, DOCK180 complexes from these two cell lines contain different ELMO homologues. (C) Immunoblotting of DOCK180 complexes from HeLa and CHO K1 cells indicates the presence of different ELMO homologues.

interest to determine whether other members of the DOCK180 family also move to the nucleus.

While the complex from HeLa cells contains DOCK180, ELMO2, and ELMO3, the complex from CHO cells contains DOCK180, ELMO1, and ELMO2. These results suggest that DOCK180 can be part of distinct complexes in different cell types by associating with different members of the ELMO family. Also, these studies provide the first evidence that more than one ELMO protein may be part of the complex with DOCK180, with possible functional implications for Rac activation via this complex.

In summary, our studies here make the novel observations that a significant fraction of the DOCK180/ELMO complex exists in the nucleus, DOCK180 and ELMO exist in a complex larger than a simple 1:1 stoichiometry, and that DOCK180 may form complexes with distinct ELMO proteins in different cell types. These observations may have important implications toward our understanding of signaling via these proteins during multiple biological processes.

Acknowledgments

We thank Dr. Matsuda for providing anti-DOCK180 antibody. We thank National Cell Culture Center for providing cultured cells. This work was supported by grants from Ellison Medical Foundation and Rett syndrome Research Foundation (W.W.) and by grants from the NIGMS (to K.S.R.).

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