Interaction of Doc2 with tctex-1, a Light Chain of Cytoplasmic Dynein

IMPLICATION IN DYNEIN-DEPENDENT VESICLE TRANSPORT*

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Fumiko Nagano§, Satoshi Orita§, Takuya Sasakiz, Akira Naito§, Gaku Sakaguchi§, Miki Maeda§, Tsuyoshi Watanabe§, Eiki Kominami§, Yasuo Uchiyama§, and Yoshimi Takai§**

From the Department of Molecular Biology and Biochemistry, the Department of Cell Biology and Anatomy I, Osaka University Medical School, Suita 565-0871, the Shionogi Institute for Medical Science, Settsu 566-0022, and the Department of Biochemistry, Juntendo University School of Medicine, Tokyo 113-0033, Japan

Doc2 has one Munc13-interacting domain at the N-terminal region and two C2-like domains interacting with Ca\(^{2+}\) and phospholipid at the C-terminal region. Doc2 consists of two isoforms, Doc2\(a\) and \(\beta\). Doc2\(\beta\) is ubiquitously expressed in neuronal cells and implicated in Ca\(^{2+}\)-dependent neurotransmitter release, whereas Doc2\(\beta\) is ubiquitously expressed and its function is unknown. We show here that both Doc2\(\alpha\) and \(\beta\) interact with rat tctex-1, a light chain of cytoplasmic dynein, in both cell-free and intact cell systems. Overexpression of the N-terminal domain of Doc2 containing the tctex-1-interacting domain induces changes in the intracellular localization of cation-independent mannose 6-phosphate receptor and its ligand, cathepsin D, which are transported from trans-Golgi network to late endosomes. Overexpression of the C-terminal fragment containing two C2-like domains shows the similar effect, but to a lesser extent, whereas overexpression of full-length Doc2 or the C-terminal fragment of rabphilin3 containing two C2-like domains does not show this effect. Because dynein is a minus-end-directed microtubule-based motor protein, these results suggest that Doc2, especially Doc2\(\alpha\), plays a role in dynein-dependent intracellular vesicle transport.

We have isolated Doc2 as a novel protein having two C2-like domains that interact with Ca\(^{2+}\) and phospholipid (1). Doc2 consists of two isoforms, Doc2\(\alpha\) and \(\beta\) (1–3). Doc2\(\alpha\) is specifically expressed in neuronal cells and localized on synaptic vesicles in nerve terminals, whereas Doc2\(\beta\) is ubiquitously expressed (1–4). Both isoforms have at least one DSR (1–39 aa) at the N-terminal region and two C2-like domains at the C-terminal region (1, 2). Overexpression of the N-terminal fragment of Doc2\(\alpha\) containing DSR or its C-terminal fragment containing the C2-like domains in PC12 cells inhibits Ca\(^{2+}\)-dependent exocytosis (5). We have recently isolated a protein interacting with Doc2\(\alpha\) and \(\beta\) and identified it to be Munc13–1 (6). Doc2\(\alpha\) and \(\beta\) interact with Munc13–1 through the region within DSR (13–37 aa). Munc13–1 is specifically expressed in neuronal cells and localized on the presynaptic plasma membrane (7, 8). Munc13–1 has two C2-like domains and one C1-like domain that interacts with diacylglycerol or phorbol ester (7, 8). The Doc2\(\alpha\)-Munc13–1 interaction is induced by the binding of diacylglycerol or phorbol ester to the C1-like domain of Munc13–1 and causes the docking of the vesicles to the plasma membrane in nerve terminals (6, 9).

In contrast to Doc2\(\alpha\), little is known about Doc2\(\beta\). To clarify the function of Doc2, particularly that of Doc2\(\beta\), it is important to isolate a Doc2-interacting protein(s) that is ubiquitously expressed. We have attempted here to isolate a Doc2-interacting protein(s) from a rat brain cDNA library by use of the yeast two-hybrid system and identified it to be tctex-1. tctex-1 interacted with both Doc2\(\alpha\) and \(\beta\) at the N-terminal region. tctex-1 was originally isolated as a candidate for involvement in the transmission ratio distortion of mouse t-haplotypes (10). tctex-1 has recently turned out to be a light chain of cytoplasmic dynein (11), a minus-end-directed microtubule-based motor protein (for reviews, see Refs. 12–15). Cytoplasmic dynein interacts with a variety of structures, such as late endosomes, lysosomes, the Golgi complex, synaptic vesicles, and ER, and is implicated in transport of these organelles and vesicle transport between these organelles (12–15). We describe here the Doc2-tctex-1-interaction and discuss the function of this interaction.

EXPERIMENTAL PROCEDURES

Materials and Chemicals—The N-terminal fragment (1–90 aa) of human Doc2\(\alpha\) cDNA (1) was inserted into pGEX-2T vector, expressed in Escherichia coli as a GST fusion protein, and purified on a glutathione-Sepharose 4B column (Amersham Pharmacia Biotech). Mammalian expression plasmids, pEFSOS-HA and pEFSOS-Myc, and in vitro and in vivo expression plasmids, pGEM-HA and pRSET-Flag, were generated to express fusion proteins with the N-terminal HA, Myc, or Flag epitope (5, 6). The cDNA fragments encoding mouse tctex-1 (10) and human RP-3 (16) were obtained by polymerase chain reaction from mouse and human brain cDNA libraries, respectively. BHK cells were grown in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum at 37 °C in 5% CO\(_2\) (17). Anti-CIMP and anti-cathepsin D rabbit polyclonal antibodies were prepared and purified by affinity chromatography (18). Fluorescein-conjugated goat anti-rabbit IgG and Texas Red-conjugated goat anti-mouse IgG were obtained from Jackson Immunoresearch Laboratories (West Grove, PA) and Biomedia Corp. (Foster, CA), respectively.

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The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™/EBI Data Bank with accession number(s) AB010119.

** To whom correspondence should be addressed: Dept. of Molecular Biology and Biochemistry, Osaka University Medical School, 2-2 Yamada-oka, Suita 565-0871, Japan. Tel.: 81-6-879-3410; Fax: 81-6-879-3419; E-mail: yatak@molbio.med.osaka-u.ac.jp.

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1 The abbreviations used are: DSR, Doc2-specific region; aa, amino acid(s); ER, endoplasmic reticulum; GST, glutathione-S-transferase; HA, hemagglutinin; CIMP, cation-independent mannose 6-phosphate receptor; PAGE, polyacrylamide gel electrophoresis; kb, kilobase(s); X-gal, 5-bromo-4-chloro-3-indolyl \(\beta\)-galactopyranoside.

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**Doc2-Dynein Interaction**

**RESULTS AND DISCUSSION**

We first attempted to isolate a Doc2-interacting protein which is ubiquitously expressed by use of the yeast two-hybrid system with the N-terminal region (1–90 aa) of Doc2a as a bait from a rat brain cDNA library. Screening of 1 × 10^9 transformants yielded nine independent positive clones that interacted with Doc2a. The seven positive clones encoded Munc13-1 as described (6). The remaining two positive clones had the same cDNA inserts with 0.75 and 0.73 kb, respectively. Using the cDNA with 0.75 kb as a probe, we isolated the cDNA encoding the whole open reading frame from a rat brain cDNA library. The cDNA fragments encoding mouse tctex-1 (10) and human RP-3 (16) were inserted into pGAD10. The cDNA fragments encoding several Doc2a and -β deletion mutants were inserted into pBTM116 (6). After co-transformation into yeast strain L40, β-galactosidase activity was measured by liquid and filter assays (19, 20).

**Effect of Overexpression of the Doc2 Mutants on the Intracellular Localization of CIMPR and Cathepsin D in BHK Cells**—BHK cells were transfected with 3 μg of pGEM-HA encoding Doc2a and 2 μg of pPRSET-Flag encoding tctex-1, by use of a LipofectAMINE reagent (Life Technologies, Inc.). At 5 h after the transfection, the cell lysate was prepared and subjected to immunoprecipitation with 5 μg of the anti-HA monoclonal antibody bound to 20 μl of protein-A Sepharose beads (6). Comparable amounts of the pellets were subjected to SDS-PAGE, followed by immunoblot analysis with the biotinylated mouse anti-Flag antibody (Eastman Kodak, New Haven, CT).

**RESULTS AND DISCUSSION**

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The nucleotide sequence analysis of the cloned cDNA revealed that the encoded protein consisted of 113 aa and showed a sequence identity of 96% with mouse tctex-1 (GenBank™ accession number AB010119) (Fig. 1). The deduced aa sequence of this molecule showed striking sequence homology to that of human RP-3 shown). These results indicate that tctex-1 interacts with Doc2 in both cell-free and intact cell systems.

The yeast reporter strain L40 was transformed with pLexA-Doc2 and mouse tctex-1 cDNA. The tctex-1 mRNA level was examined in various rat tissues by Northern blot analysis. The tctex-1 mRNA appeared as a single band of approximately 0.8 kb and was ubiquitously expressed, although it was abundantly expressed in testis (data not shown). These results suggest that tctex-1 is a Doc2-interacting protein that is ubiquitously expressed.

The minimum region of Doc2a required for the interaction with tctex-1 was 1–32 aa of Doc2a (Fig. 2A, a). Doc2β also interacted with tctex-1 (Fig. 2A, b). The tctex-1-interacting region of Doc2a showed striking sequence homology to that of Doc2β (85% identity). Although RP-3 showed sequence homology to tctex-1, RP-3 did not interact with Doc2a or -β (Fig. 2A, b). These results suggest that the Doc2-tctex-1 interaction is specific.

The Doc2-tetex-1 interaction was confirmed by the binding of in vitro translated, [35S]methionine-labeled tetex-1 to the GST-tagged N-terminal region of Doc2a (1–90 aa) (Fig. 2B). In vitro translated RP-3 did not bind to the GST-tagged N-terminal region of Doc2a. The Doc2-tetex-1 interaction was furthermore confirmed by co-immunoprecipitation from cultured HeLa cells of Flag-tagged full-length tetex-1 and HA-tagged full-length Doc2a (Fig. 2C). Flag-tagged full-length RP-3 was not co-immunoprecipitated with HA-tagged full-length Doc2a (data not shown). These results indicate that tetex-1 interacts with Doc2 in both cell-free and intact cell systems.

It was shown that dynin is involved in the vesicle transport from early to late endosomes (24). CIMPR and its ligand, cathepsin D, are transported from trans-Golgi network to early endosomes and then from early to late endosomes in BHK cells (25). We finally examined whether the Doc2-tetex-1 interaction is involved in this dynin-dependent vesicle transport. When the intracellular localization of CIMPR in BHK cells was analyzed by immunocytochemistry using the anti-
CIMPR antibody, CIMPR was distributed in a compact juxtanuclear region corresponding to late endosomes (Fig. 3 A).

Overexpression of the N-terminal fragment of Doc2a containing the tctex-1-binding region induced the dispersion of CIMPR. The severity of this change varied somewhat among transfected cells, usually as a result of variation in expression levels of Doc2a. Overexpression of the C-terminal fragment containing C2-like domains showed the similar effect, but to a lesser extent. In contrast, overexpression of full-length Doc2a did not affect the intracellular localization of CIMPR (data not shown). To determine the specificity of the effect of the Doc2 mutants, we used the similar mutants of rabphilin3, which is a downstream target of the Rab3 small G protein subfamily implicated in Ca2+ -dependent exocytosis and has two C2-like domains (26), and full-length RP-3, which does not interact with Doc2 in both cell-free and intact cell systems. Overexpression of the N-terminal fragment of rabphilin3 containing Rab3-binding domain, its C-terminal fragment containing C2-like domains (data not shown) (27), or full-length RP-3 (Fig. 3 B) showed no effect. As to the effect of overexpression of the Doc2 mutants on the localization of cathepsin D, large dot-like structures were scattered throughout the cytoplasm in the untransfected cells (Fig. 3 B). This staining indicates that mature cathepsin D is localized in lysosomes. Overexpression of the N-terminal fragment of Doc2a caused the disappearance of large dot-like structures. Overexpression of its C-terminal fragment showed the similar effect, but to a lesser extent. In contrast, overexpression of full-length Doc2a, the N-terminal fragment of rabphilin3, its C-terminal fragment (data not shown), or full-length RP-3 (Fig. 3 B) did not affect the intracellular localization of cathepsin D. These results suggest that Doc2 is involved at least in the vesicle transport from early to late endosomes in cooperation with dynein in BHK cells.

Cytoplasmic dynein consists of two heavy chains, three in-
termediate chains, four light intermediate chains, and three light chains (11–15). Of these subunits, the heavy chains have ATPase activity and interact with microtubules (12–15). The intermediate chains and dynactin are suggested to interact with cargos (13–15). The function of the light chains has been unknown, but our present results indicate that one of the light chains with a molecular mass of 14 kDa interacts with Doc2. The intracellular localization of Doc2β is unknown in nonneuronal cells, but our previous result, that Doc2α is localized on synaptic vesicles in neuronal cells (1), suggests that Doc2β is associated with some specific vesicles in nonneuronal cells. Taken together, Doc2α may link dynactin to its cargos through interaction with tctex-1. Doc2 probably associates with cargos through its C-terminal region because overexpression of the C-terminal fragment of Doc2α shows a similar effect of overexpression of its N-terminal fragment containing the tctex-1-interacting domain.

Cytoplasmic dynein is implicated in a broad range of cellular functions in addition to the vesicle transport from early to late endosomes (12–15): chromosome segregation, spindle formation, nuclear migration, the distribution of organelles (Golgi endosomes (12–15): chromosome segregation, spindle formation, nuclear migration, the distribution of organelles (Golgi complex, late endosomes, and lysosomes), the retrograde organelle transport in axon, and the transport of intermediate compartment from ER to Golgi complex. Doc2 may be involved in these dynex-dependent functions. Further study is necessary to establish the physiological role of the Doc2-tctex-1 system.

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REFERENCES
1. Orita, S., Sasaki, T., Naito, A., Komuro, R., Ohtsuka, T., Maeda, M., Suzuki, H., Igarashi, H., and Takai, Y. (1995) Biochem. Biophys. Res. Commun. 209, 439–448
2. Sakaguchi, G., Orita, S., Maeda, M., Igarashi, H., and Takai, Y. (1995) Biochem. Biophys. Res. Commun. 217, 1053–1061
3. Verhage, M., de Vries, K. J., Rashol, H., Burkh, J. P. H., Gispen, W. H., and Sudhof, T. C. (1997) Neuron 18, 453–461
4. Naito, A., Orita, S., Wanaka, A., Sasaki, T., Sakaguchi, G., Maeda, M., Igarashi, H., Toyama, M., and Takai, Y. (1997) Mol. Brain Res. 44, 198–204
5. Orita, S., Sasaki, T., Komuro, R., Sakaguchi, G., Maeda, M., Igarashi, H., and Takai, Y. (1996) J. Biol. Chem. 271, 7257–7260
6. Naito, A., Naito, A., Sakaguchi, G., Maeda, M., Igarashi, H., Sasaki, T., and Takai, Y. (1997) J. Biol. Chem. 272, 16081–16084
7. Brose, N., Hofmann, K., Hata, Y., and Sudhof, T. C. (1995) J. Biol. Chem. 270, 25273–25280
8. Betz, A., Ashery, U., Rickmann, M., Augustin, I., Neher, E., Sudhof, T. C., Retig, J., and Brose, N. (1998) Neuron 21, 123–136
9. Mechida, S., Orita, S., Sakaguchi, T., Sasaki, T., and Takai, Y. (1998) Proc. Natl. Acad. Sci. U. S. A. 95, 11418–11422
10. Lader, E., Ha, H. S., O'Neill, M., Artzt, K., and Bennett, D. (1989) Cell 58, 969–979
11. King, S. M., Dillman, J. F., III, Benashski, S. E., Lye, R. J., Patel-King, R. S., and Pfeffer, K. K. (1996) J. Biol. Chem. 271, 32281–32287
12. Holzbaur, E. L. F., and Vallee, R. B. (1994) Annu. Rev. Cell Biol. 10, 339–372
13. Vallee, R. B., and Sheetz, M. P. (1996) Science 271, 1539–1544
14. Hirokawa, N., Noda, Y., and Okada, Y. (1998) Curr. Opin. Cell Biol. 10, 60–73
15. Hirokawa, N. (1998) Science 279, 519–526
16. Roux, A. F., Rommens, J., McDowell, C., Anson-Cartwright, L., Bell, S., Schappert, K., Fishman, G. A., and Musarella, M. (1994) Hum. Mol. Genet. 3, 257–263
17. Bastos, R., Lin, A., Enarson, M., and Burke, B. (1996) J. Cell Biol. 134, 1141–1156
18. Muno, D., Ishidoh, K., Ueno, T., and Kominami, E. (1993) Arch. Biochem. Biophys. 306, 103–110
19. Miller, J. H. (1972) Experiments in Molecular Genetics, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY
20. Vojtek, A. B., Hollenberg, S. M., and Cooper, J. A. (1993) Cell 74, 205–214
21. Laemmli, U. K. (1970) Nature 227, 680–685
22. Towbin, H., Staehelin, T., and Gordon, J. (1979) Proc. Natl. Acad. Sci. U. S. A. 76, 4350–4354
23. Bradford, M. M. (1976) Anal. Biochem. 72, 248–254
24. Aniento, F., Emans, N., Griffiths, G., and Gruenberg, J. (1993) J. Cell Biol. 123, 1373–1387
25. Press, B., Peng, Y., Hoflack, B., and Wandinger-Ness, A. (1998) J. Cell Biol. 140, 1075–1089
26. Shirataki, H., Kaibuchi, K., Sakoda, T., Kishida, S., Yamaguchi, T., Wada, K., Miyazaki, M., and Takai, Y. (1993) Mol. Cell. Biol. 13, 2061–2068
27. Yamaguchi, T., Shirataki, H., Kishida, S., Miyazaki, M., Nishikawa, K., Wada, K., Numata, S., Kaibuchi, K., and Takai, Y. (1993) J. Biol. Chem. 268, 27164–27170