J. Biochem. 2010;148(5):533–538 doi:10.1093/jb/mvq116

JB THE JOURNAL OF BIOCHEMISTRY

Rapid Communication

Proline-rich domain in dynamin-2 has a low microtubule-binding activity: how is this activity controlled during mitosis in HeLa cells?

Received July 12, 2010; accepted September 20, 2010; published online September 30, 2010

Makiko Morita[1], Kozue Hamao[1], Shunsuke Izumi[2], Eiichi Okumura[3], Keita Tanaka[1], Takeo Kishimoto[3] and Hiroshi Hosoya[1,*]

[1]Department of Biological Science; [2]Department of Mathematical and Life Sciences, Graduate School of Science, Hiroshima University, Higashi-Hiroshima 739-8526; and [3]Laboratory of Cell and Developmental Biology, Graduate School of Bioscience, Tokyo Institute of Technology, Nagatsuta, Midori-ku, Yokohama 226-8501, Japan

*Hiroshi Hosoya, Department of Biological Science, Graduate School of Science, Hiroshima University, Higashi-Hiroshima 739-8526, Japan. Tel: +81 82 424 7445, Fax: +81 82 424 0734, email: hhosoya@hiroshima-u.ac.jp

The large GTPase dynamin is strongly accumulated in the constricted area including midzonal microtubules of dividing cells. The proline-rich domain (PRD) of dynamin has been considered as a microtubule-binding domain. However, it remains unclear how PRD controls dynamin–microtubule interaction in mitotic cells. Here, we found that the microtubule-binding activity of PRD is low in dynamin-2. One of the mitosis-specific kinase activities to PRD in HeLa cells was identified as cyclin B-Cdc2 kinase. The kinase phosphorylated PRD at Ser^{764} and/or Thr^{766} and reduced the microtubule-binding activity of PRD. These results suggest that phosphorylation of PRD by cyclin B-Cdc2 kinase plays an important role to control dynamin-2–microtubule interaction in mitotic HeLa cells.

Keywords: microtubules/cytoskeleton/division/protein kinases.

Abbreviations: PRD, proline-rich domain.

Dynamin is one of the large GTP-binding proteins in various eukaryotic cells (*1*). It has been reported that knockdown of dynamin causes a failure of abscission during cytokinesis in *Caenorhabditis elegans* (*2*). Recent pharmacological experiments have revealed that several inhibitors of dynamin GTPase induced cytokinesis failure in mammalian cultured cells (*3*). These data suggest that dynamin has a critical role in mediating cytokinesis.

Dynamin was initially identified as a microtubule-binding protein (*4*). Previous *in vitro* experiments revealed that the C-terminal region of dynamin-1, the proline rich domain (PRD), exhibited microtubule-binding activity (*5*). Dynamin has been shown to bind cooperatively in patches along microtubules (*6*, *7*). Furthermore, immunofluorescence analysis showed that dynamin localizes with spindle microtubules at the midzone and midbody in mammalian cultured cells (*2*, *8*). However, no co-localization between dynamin and microtubules in interphasic cells was observed (*8–10*). Consequently, conflicting evidence exists as to whether dynamin–microtubule interactions in the cell are relevant or not.

Microtubules are highly dynamic tubulin polymers involved in many diverse functions, including cell motility, vesicle and organelle transport, and spindle formation. Such activity has been attributed to the actions of cellular factors that stabilize or destabilize microtubules, such as microtubule-associated proteins (MAPs) (*11*, *12*); for example, Aizawa *et al.* (*13*) reported that the PRD is a microtubule-binding domain in MAP4. Another group reported that a mutant of τ-protein lacking the proline-rich region did not bind to microtubules (*14*), suggesting that the PRD plays a crucial role in controlling τ-microtubule interactions. On the contrary to this, several PRD-containing actin-related proteins such as vinculin and Ena/VASP have never been reported to bind to microtubules *in vitro* or *in vivo* (*15*). These results suggest that the PRD does not function as a specific microtubule-binding site in various kinds of microtubule-binding proteins.

Previously, we reported that GFP-dynamin-2-Wt (1–870) and -(1–745), with deleted an entire PRD, do not co-localize with microtubules in interphasic HeLa cells (*8*). Interestingly, GFP-dynamin-2-(1–786) deleted only the C-terminus of the PRD (C-PRD) co-localized with microtubules. These results suggest that the N-terminus of the PRD (N-PRD) and the C-PRD positively and negatively regulate the interaction of dynamin-2 with microtubules in interphasic HeLa cells, respectively (Fig. 4). However, it remains unclear how PRD controls the dynamin-2–microtubule interaction at the spindle midzone in mitotic cells.

Previous microscopic observations revealed that endogenous dynamin co-localizes with microtubules during mitosis but not at interphase in HeLa cells (*2*, *8*). This suggests that the binding ability of dynamin to microtubules is differentially regulated in interphase and during mitosis. Several *in vitro* studies indicate that brain dynamin-1 is phosphorylated by cyclin B-Cdc2 kinase or ERK2 that subsequently reduces its microtubule-binding activity (*16*, *17*). Hence, we speculated that the phosphorylation of dynamin-2

may regulate dynamin-2-microtubule binding in the cell cycle. To test this hypothesis, we initially examined whether cell extracts from interphasic and mitotic HeLa cells show an activity to phosphorylate dynamin-2. As shown in Fig. 1A, cell extracts from mitotic, but not interphasic, HeLa cells exhibited high phosphorylation activity on histone H1. The phosphorylation activity of the mitotic cell extracts to full-length of dynamin-2 was also higher than that of the interphasic cell extracts (Fig. 1B).

Previously, we showed that the N-PRD, but not the C-PRD, was required for co-localization of dynamin-2 with microtubules in HeLa cells (*8*). Then, we investigated whether dynamin-2-(714–870) containing the entire PRD (dynamin-2-PRD-Wt) was phosphorylated by HeLa cell extracts. As shown in Fig. 1C, cell extracts from mitotic, but not interphasic, HeLa cells exhibited high phosphorylation activity on dynamin-2-PRD-Wt. These data suggest that cell cycle-dependent phosphorylation of dynamin-2-PRD occurs in HeLa cells.

As shown in Fig. 1A, extracts from mitotic HeLa cells exhibited high phosphorylation activity on histone H1. This suggests that the mitotic extracts contain cyclin-dependent kinase (Cdk). To identify the kinases that phosphorylate histone H1, we conducted a phosphorylation assay with the Cdk inhibitor, roscovitine (*18*). As shown in Fig. 2A, phosphorylation of histone H1 in mitotic HeLa cell extracts was reduced in the presence of roscovitine. Next, we conducted the phosphorylation assay for dynamin-2-PRD-Wt using HeLa cell extracts in the presence of roscovitine. Phosphorylation of dynamin-2-PRD-Wt in mitotic HeLa cell extracts was also reduced in the presence of roscovitine (Fig. 2B). These results suggest that the mitosis-specific kinase activity of dynamin-2-PRD-Wt is derived mainly from cyclin B-Cdc2 kinase.

To confirm whether cyclin B-Cdc2 kinase phosphorylates dynamin-2-PRD-Wt, purified cyclin B-Cdc2 kinase was incubated with dynamin-2-PRD-Wt in the presence or absence of roscovitine. As shown

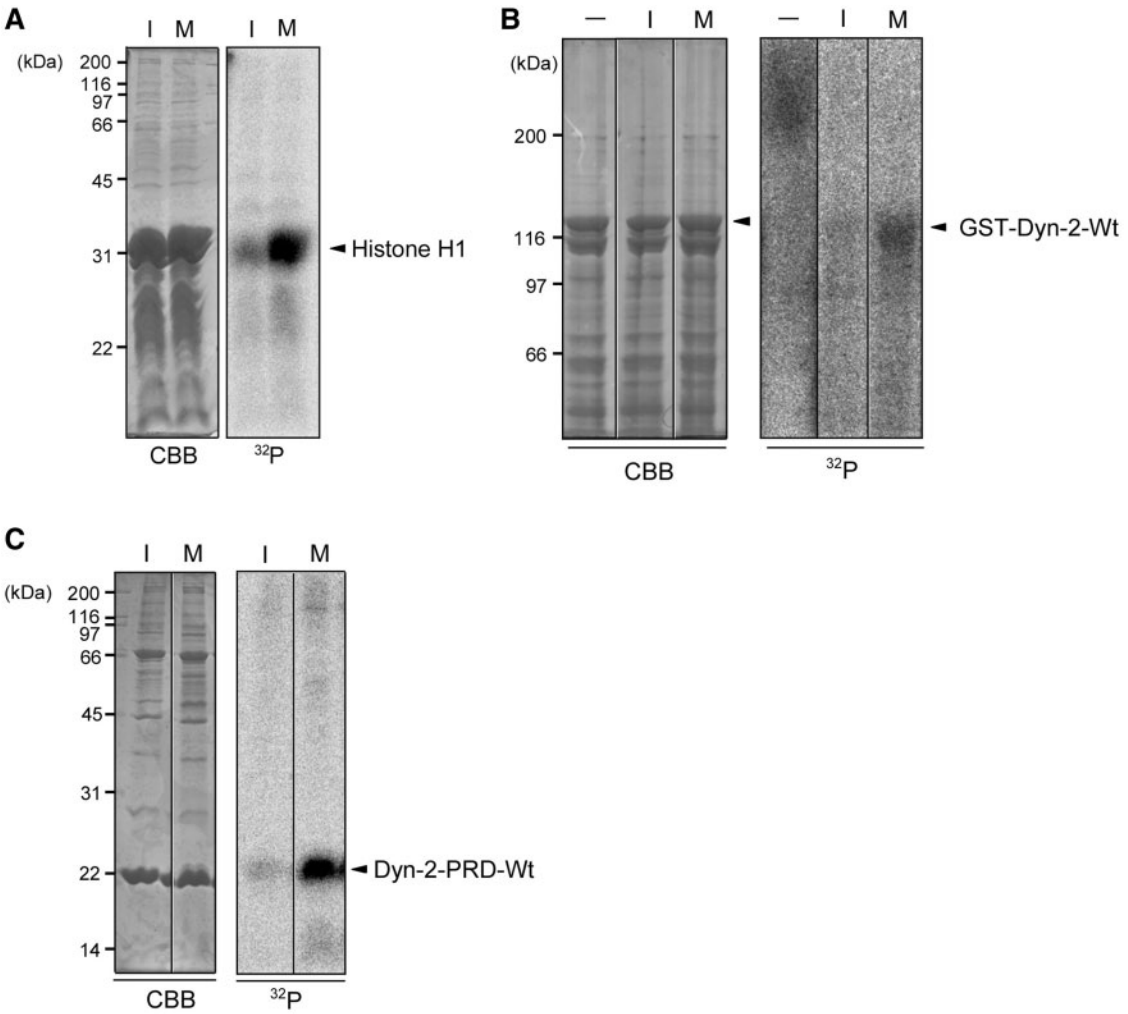

Fig. 1 PRD of dynamin-2 is phosphorylated by the extracts from mitotic HeLa cells. Phosphorylation assay of (A) histone H1, (B) GST-dynamin-2-Wt (full-length of dynamin-2) and (C) dynamin-2-PRD-Wt (C-terminus of dynamin-2) treated with (I/M) or without (-) HeLa cell extracts. I and M indicate the extracts obtained from interphase and mitotic HeLa cells, respectively. The panels show CBB staining (left) and autoradiography (right).

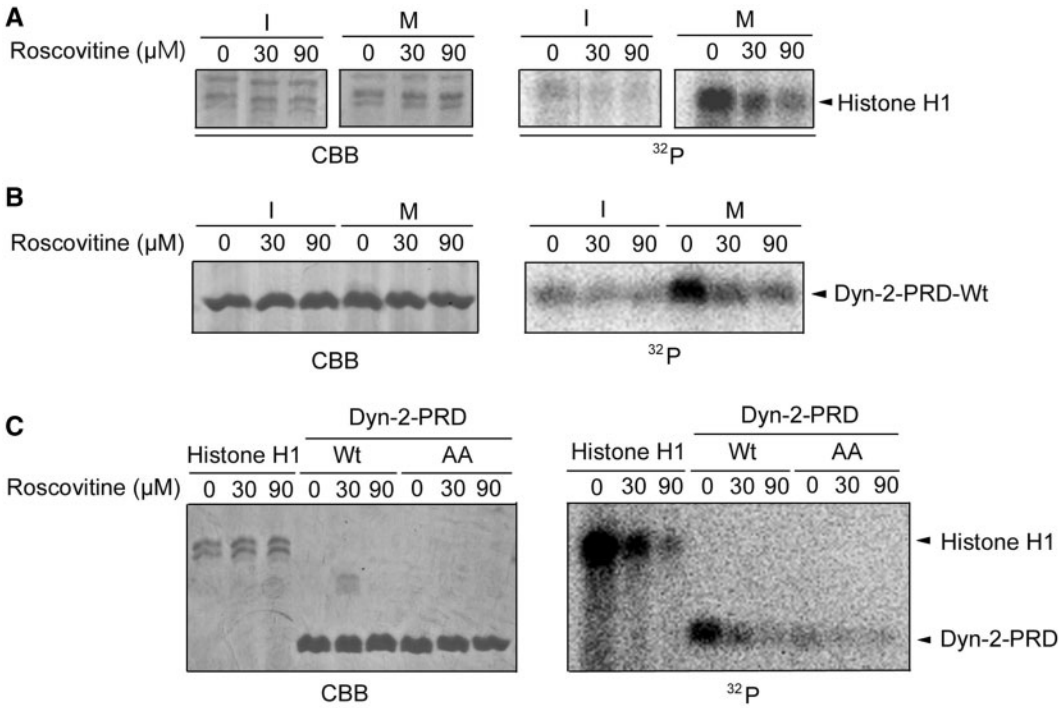

Fig. 2 Cyclin B-Cdc2 kinase phosphorylates N-terminus of dynamin-2-PRD-Wt. (A) Histone H1 and (B) dynamin-2-PRD-Wt were phosphorylated by HeLa extracts from interphase (I) or mitotic phase (M) cells pre-incubated with (30 and 90 μM, respectively) or without roscovitine for 30 min. (C) Histone H1, dynamin-2-PRD-Wt and -AA were phosphorylated by purified cyclin B-Cdc2 kinase pre-incubated with (30 and 90 μM, respectively) or without roscovitine for 30 min. Dynamin-2-PRD-AA shows the double-point mutations at Ser^{764} and Thr^{766} with Ala in dynamin-2-PRD-Wt. The panels show CBB staining (left) and autoradiography (right). Uncropped images of blots are shown in Supplementary Fig. S1.

in Fig. 2C, phosphorylation of histone H1 and dynamin-2-PRD-Wt was reduced in the presence of roscovitine, suggesting that cyclin B-Cdc2 kinase is the dynamin-2 kinase that phosphorylates dynamin-2-PRD-Wt in mitotic HeLa cells. Uncropped images of the blots are shown in Supplementary Fig. S1. Next, the phosphorylation region of dynamin-2-PRD-Wt by cyclin B-Cdc2 kinase was determined by mass spectrometry. The ESI-MS spectrum of the tryptic peptides from dynamin-2-PRD-Wt phosphorylated by cyclin B-Cdc2 kinase is shown in Supplementary Fig. S2. A pentavalent ion peak was observed at m/z 832.7. A new pentavalent ion peak was observed at m/z 850.9 (Supplementary Fig. S2B). The shift in peptide mass m/z from 832.7 to 850.9 was due to the phosphorylation. These results suggest that at least one site is phosphorylated by cyclin B-Cdc2 kinase in the N-PRD of dynamin-2. Previous reports showed that dynamin-1, an isoform of dynamin-2, was highly phosphorylated by Cdk5 at Ser^{774}, Ser^{778} and Thr^{780} (amino acid sequence: 774-SPTSSPTP-781) (*19*, *20*). We found that the sequence of SPTP in dynamin-1-(778–781) is also conserved in dynamin-2-(764–767). Next, we generated double-point mutations of Ser^{764} and Thr^{766} in dynamin-2-N-PRD with Ala (dynamin-2-PRD-AA) to determine the sites phosphorylated by cyclin B-Cdc2 kinase on dynamin-2-PRD-Wt. As shown in Fig. 2C, phosphorylation by cyclin B-Cdc2 kinase of the mutant dynamin-2-PRD-AA was greatly reduced, indicating that dynamin-2-PRD-Wt was phosphorylated by cyclin B-Cdc2 kinase at Ser^{764} or Thr^{766} during mitosis. Next, dynamin-2-PRD-Wt phosphorylated by cyclin B-Cdc2 kinase was analysed for its microtubule-binding activity. Phosphorylated dynamin-2-PRD-Wt was distinguished by an upward shift in SDS–PAGE from unphosphorylated dynamin-2-PRD-Wt. Dynamin-2-PRD-Wt cleaving GST was used, because GST-dynamin-2-PRD-Wt was only slightly bound to microtubules during ultracentrifugation and induced an insufficient phosphorylation by cyclin B-Cdc2 kinase (data not shown). In addition, the co-sedimentation assay was performed using low-speed centrifugation to avoid precipitating the dynamin-2 fragment (dynamin-2-PRD-Wt). Under this sedimentation condition, more than half of the obtained microtubules were in the precipitate (Fig. 3A). The amount of dynamin-2-PRD-Wt bound to the microtubules was quantified by imaging a SDS–PAGE gel (Fig. 3A) using ImageJ. The net amount of dynamin-2-PRD-Wt bound to the microtubules was designated as the remainder after subtracting the amount of the dynamin-2-PRD-Wt precipitated without microtubules from that precipitated with microtubules. As shown in Fig. 3B, only 15% of the unphosphorylated dynamin-2-PRD-Wt was bound to microtubules. It has been reported that almost all of MAP2 and MAP4 proteins can bind to microtubules (*21*). Compared with MAP2 and MAP4, the microtubule-binding activity of dynamin-2-PRD-Wt is weak, although it has been generally believed that PRD has a high affinity for microtubules (*13*). Moreover, cyclin B-Cdc2 kinase reduced the weak binding activity

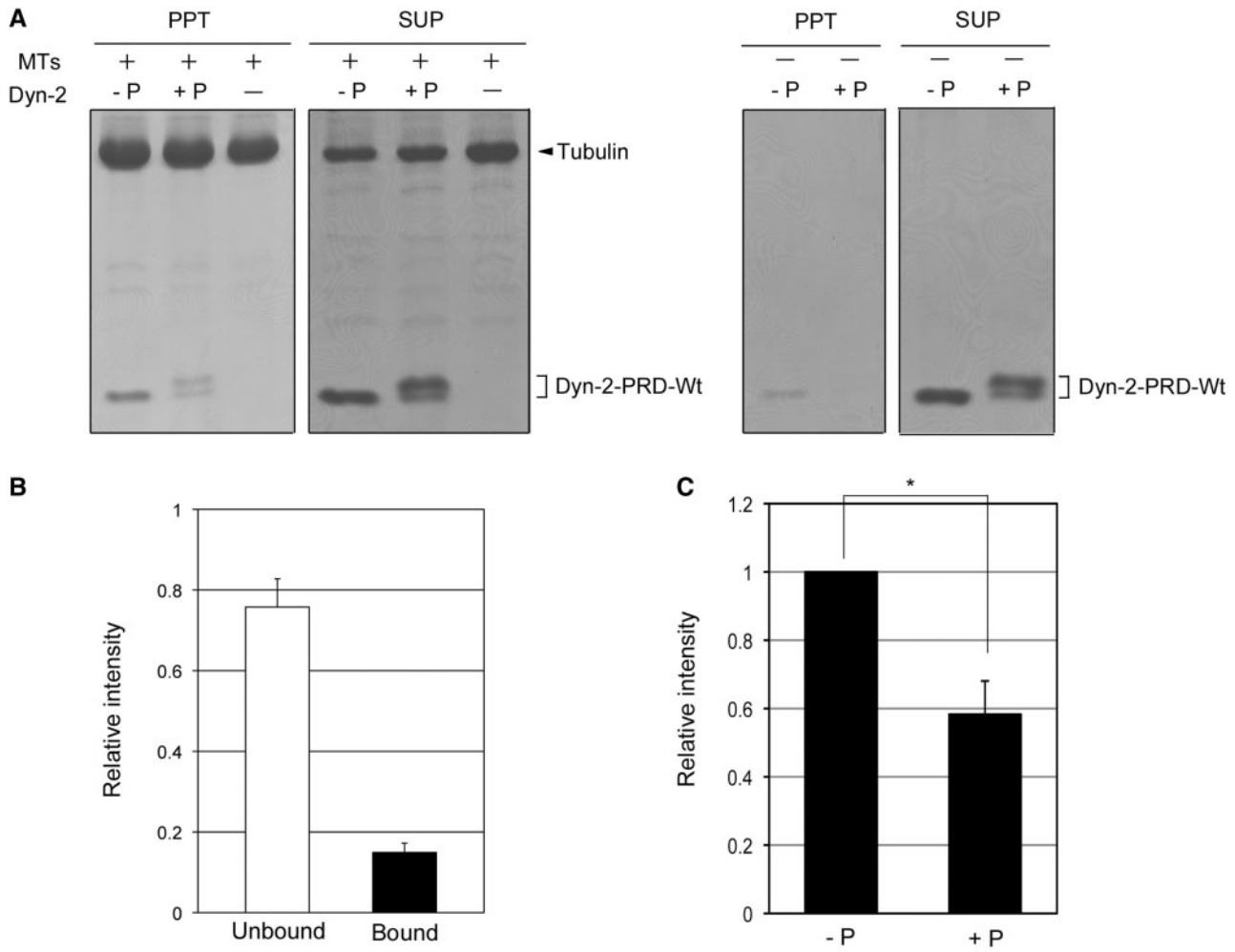

Fig. 3 Microtubule-binding activity of dynamin-2-PRD-Wt phosphorylated by cyclin B-Cdc2 kinase. (A) Mixtures of unphosphorylated (−P) or phosphorylated (+P) dynamin-2-PRD-Wt with microtubules (MTs) were centrifuged at 6,700*g* for 5 min after incubation at 30°C for 15 min under the conditions described in Supplementary 'Experimental Procedures' section. Pellets (PPT) and supernatants (SUP) were analysed by SDS–PAGE. The left and right panels show co-sedimentation assay with and without microtubules, respectively. (B) The amount of unbound unphosphorylated dynamin-2-PRD-Wt (0.76 ± 0.07) and unphosphorylated dynamin-2-PRD-Wt (0.15 ± 0.02) bound to microtubules was compared and expressed as relative intensity. (C) The amount of phosphorylated (+P, 0.583 ± 0.1) dynamin-2-PRD-Wt in the pellet was expressed as relative intensity to unphosphorylated (-P) dynamin-2-PRD-Wt in the pellet. $^*P < 0.05$ (Student's *t*-test). Experiments were repeated three times and the results are displayed as mean ± standard error.

of dynamin-2-PRD-Wt to microtubules (Fig. 3C), supporting our previous report in dynamin-1 (*16*). These results suggest that PRD is not a positive regulator to enhance the microtubule-binding activity of dynamin-2 in mitotic HeLa cells.

How then is the microtubule-binding activity of dynamin-2 controlled in the cell during mitosis? Our present results established that cyclin B-Cdc2 kinase reduced a microtubule-binding activity of dynamin-2. As it is well known that cyclin B-Cdc2 kinase is active in metaphase but not after anaphase, the kinase might largely contribute no localization of dynamin-2 with microtubules in metaphase. Then, the inactivation of cyclin B-Cdc2 kinase after anaphase might induce microtubule-binding of dynamin-2 at the spindle midzone. Previous reports have revealed that the microtubule-binding activity of MAP4-PRD is enhanced by the assembly-promoting (AP) sequence region nearby the PRD (*13*, *22*). Recently, we found that GFP-dynamin-2-(1–786), but not Wt (1–870) or -(1–745), co-localized with microtubules in interphasic HeLa cells, suggesting that the C-PRD inhibits the binding of dynamin-2 to microtubules in the cell (*8*) (Fig. 4). These results demonstrate that specific region(s) nearby or in PRD play important roles to control microtubule-binding activity of PRD in the cell. Thus, one possible explanation is that, if the inhibitory activity of the C-PRD is cancelled by, for example, phosphorylation, microtubule-binding activity of the N-PRD might be enhanced in telophase. As shown in Fig. 2B, the phosphorylation of dynamin-2-PRD-Wt by mitotic HeLa extracts was not completely suppressed with roscovitine. Based on this data, we preliminarily identified a mitosis-specific kinase other than cyclin B-Cdc2 kinase that phosphorylated dynamin-2-PRD-Wt. As the predicted sites for phosphorylation by this second kinase were present on both the N-PRD and C-PRD (data not shown), we are now attempting to elucidate how the phosphorylation by this second kinase controls the

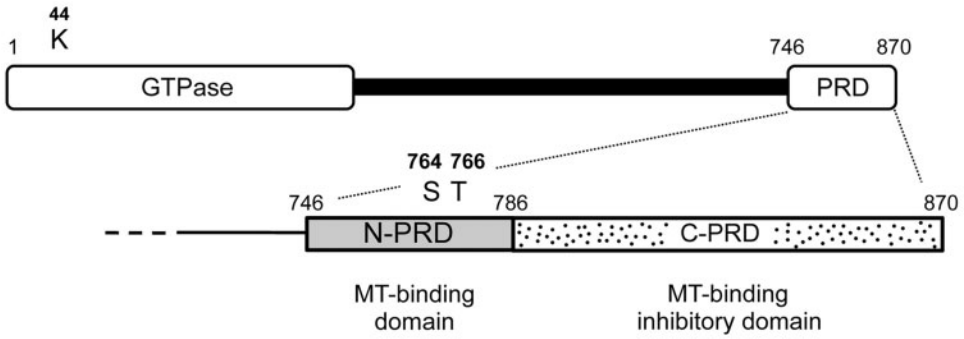

Fig. 4 Schematic representation of dynamin-2-PRD. GTPase, GTP-binding domain; PRD, proline rich domain; N-PRD, N-terminal half of PRD (residues 746–786); C-PRD, C-terminal half of PRD (residues 787–870). A mutant for GTP-binding domain substituted Ala for Lys^{44} in dynamin-2-(1–786) does not co-localize with microtubules in the cell. Phosphorylation sites by cyclin B-Cdc2 kinase are shown at Ser^{764} and/or Thr^{766} in N-PRD. N-PRD is a microtubule (MT)-binding domain and C-PRD is a MT-binding inhibitory domain.

roles of the C- to N-PRD. Our previous data revealed that the GTP-binding domain of dynamin-2 controls microtubule-binding of dynamin-2, although it is not a microtubule-binding domain in the cell (*8*). Thus, the second possibility is that, although the GTP-binding domain locates an N-terminus of dynamin-2, it might be possible to locate close to the N-PRD in a three-dimensional dynamin-2 molecule and cooperatively regulates the microtubule-binding activity of dynamin-2 via the PRD. Further experiments are required to examine how the GTP-binding domain regulates the microtubule-binding activity of the PRD in dynamin-2.

Supplementary Data

Supplementary Data are available at *JB* Online.

Conflict of interest
None declared.

References

1. Praefcke, G.J. and McMahon, H.T. (2004) The dynamin superfamily: universal membrane tubulation and fission molecules? *Nat. Rev. Mol. Cell Biol.* **5**, 133–147
2. Thompson, H.M., Skop, A.R., Euteneuer, U., Meyer, B.J., and McNiven, M.A. (2002) The large GTPase dynamin associates with the spindle midzone and is required for cytokinesis. *Curr. Biol.* **12**, 2111–2117
3. Joshi, S., Perera, S., Gilbert, J., Smith, C.M., Mariana, A., Gordon, C.P., Sakoff, J.A., McCluskey, A., Robinson, P.J., Braithwaite, A.W., and Chircop Nee Fabbro, M. (2010) The dynamin inhibitors MiTMAB and OcTMAB induce cytokinesis failure and inhibit cell proliferation in human cancer cells. *Mol. Cancer Ther.* **9**, OF1–OF12
4. Shpetner, H.S. and Vallee, R.B. (1989) Identification of dynamin, a novel mechanochemical enzyme that mediates interactions between microtubules. *Cell* **59**, 421–432
5. Herskovits, J.S., Shpetner, H.S., Burgess, C.C., and Vallee, R.B. (1993) Microtubules and Src homology 3 domains stimulate the dynamin GTPase via its C-terminal domain. *Proc. Natl Acad. Sci. USA* **90**, 11468–11472
6. Maeda, K., Nakata, T., Noda, Y., Sato-Yoshitake, R., and Hirokawa, N. (1992) Interaction of dynamin with microtubules: its structure and GTPase activity investigated by using highly purified dynamin. *Mol. Biol. Cell* **3**, 1181–1194
7. Shpetner, H.S. and Vallee, R. (1992) Dynamin is a GTPase stimulated to high levels of activity by microtubules. *Nature* **355**, 733–735
8. Hamao, K., Morita, M., and Hosoya, H. (2009) New function of the proline rich domain in dynamin-2 to negatively regulate its interaction with microtubules in mammalian cells. *Exp. Cell Res.* **315**, 1336–1345
9. Scaife, R. and Margolis, R.L. (1990) Biochemical and immunochemical analysis of rat brain dynamin interaction with microtubules and organelles *in vivo* and *in vitro*. *J. Cell Biol* **111**, 3023–3033
10. Cao, H., Garcia, F., and McNiven, M.A. (1998) Differential distribution of dynamin isoforms in mammalian cells. *Mol. Biol. Cell* **9**, 2595–2609
11. Mandelkow, E. and Mandelkow, E.M. (1995) Microtubules and microtubule-associated proteins. *Curr. Opin. Cell Biol.* **7**, 72–81
12. Andersen, S.S. (2000) Spindle assembly and the art of regulating microtubule dynamics by MAPs and Stathmin/Op18. *Trends Cell Biol.* **10**, 261–267
13. Aizawa, H., Emori, Y., Mori, A., Murofushi, H., Sakai, H., and Suzuki, K. (1991) Functional analyses of the domain structure of microtubule-associated protein-4 (MAP-U). *J. Biol. Chem.* **266**, 9841–9846
14. Kanai, Y., Chen, J., and Hirokawa, N. (1992) Microtubule bundling by tau proteins *in vivo*: analysis of functional domains. *EMBO J.* **11**, 3953–3961
15. Krause, M., Dent, E.W., Bear, J.E., Loureiro, J.J., and Gertler, F.B. (2003) Ena/VASP proteins: regulators of the actin cytoskeleton and cell migration. *Annu. Rev. Cell Dev. Biol.* **19**, 541–564
16. Hosoya, H., Komatsu, S., Shimizu, T., Inagaki, M., Ikegami, M., and Yazaki, K. (1994) Phosphorylation of dynamin by cdc2 kinase. *Biochem. Biophys. Res. Commun.* **202**, 1127–1133
17. Earnest, S., Khokhlatchev, A., Albanesi, J.P., and Barylko, B. (1996) Phosphorylation of dynamin by ERK2 inhibits the dynamin-microtubule interaction. *FEBS Lett.* **396**, 62–66
18. De Azevedo, W.F., Leclerc, S., Meijer, L., Havlicek, L., Strnad, M., and Kim, S.H. (1997) Inhibition of cyclin-dependent kinases by purine analogues: crystal structure of human cdk2 complexed with roscovitine. *Eur. J. Biochem.* **243**, 518–526
19. Tomizawa, K., Sunada, S., Lu, Y.F., Oda, Y., Kinuta, M., Ohshima, T., Saito, T., Wei, F.Y., Matsushita, M.,

Li, S.T., Tsutsui, K., Hisanaga, S., Mikoshiba, K., Takei, K., and Matsui, H. (2003) Cophosphorylation of amphiphysin I and dynamin I by Cdk5 regulates clathrin-mediated endocytosis of synaptic vesicles. *J. Cell Biol* **163**, 813–824

20. Graham, M.E., Anggono, V., Bache, N., Larsen, M.R., Craft, G.E., and Robinson, P.J. (2007) The *in vivo* phosphorylation sites of rat brain dynamin I. *J. Biol. Chem.* **282**, 14695–14707
21. Kawachi, A., Ichihara, K., Hisanaga, S., Iida, J., Toyota, H., Hotani, H., and Itoh, T.J. (2003) Different protofilament-dependence of the microtubule binding between MAP2 and MAP4. *Biochem. Biophys. Res. Commun.* **305**, 72–78
22. Katsuki, M., Tokuraku, K., Murofushi, H., and Kotani, S. (1999) Functional analysis of microtubule-binding domain of bovine MAP4. *Cell Struct. Funct.* **24**, 337–344