Biochemical and Biophysical Research Communications **262,** 98–102 (1999)
Article ID bbrc.1999.1171, available online at http://www.idealibrary.com on IDEAL®

Characterization of Myosin V from PC12 Cells

Philip Wolff,* Patricia A. E. Abreu,* Enilza M. Espreafico,† M. Cristina R. Costa,‡ Roy E. Larson,‡ and Paulo Lee Ho*,[1]

*Centro de Biotecnologia, Instituto Butantan, 05503-900, São Paulo, SP, Brazil; and †Departamento de Morfologia and ‡Departamento de Bioquímica, Faculdade de Medicina de Ribeirão Preto, Universidade de São Paulo, 14049-900, Ribeirão Preto, SP, Brazil

Received July 12, 1999

PC12 cell line is a cellular model to study neurite outgrowth and neurotransmitter release mechanisms. Molecular motors may be involved in these responses and myosin V could be a candidate to mediate these effects. Overlay experiments using [^{125}I]-calmodulin showed that PC12 cells possess several calmodulin-binding proteins, some of them around 190–210 kDa. Western blots using affinity purified polyclonal antibodies raised against chicken brain myosin V revealed a component of 190 kDa, a molecular mass typical of myosin V. Furthermore, Northern blots using a myosin V probe also detected a transcript of around 12 kbp. Immunofluorescence cytochemistry demonstrated the localization of myosin V throughout the cytoplasm, in the neurites, growth cone tips, and with an intense asymmetrical perinuclear labeling. Western blot analyses of PC12 cellular extracts after FGF-2 and/or dibutyryl cAMP treatment revealed variations between myosin V and myosin II expression during neuronal differentiation. These results demonstrated the presence of myosin V in PC12 cells and also suggest a role for this motor molecule in the neuronal differentiation response in PC12 cells.

Among the most remarkable advances in cell biology over the past decade has been the discovery of a multitude of mechanochemical enzymes that are either microtubule-based (the dyneins and kinesins) or actin-based motors (the myosins) (1). The myosin family is comprised of the conventional myosin II and by the growing family of unconventional myosins (1–4). Although the conventional myosins, responsible for processes such as muscle contraction and cytokinesis have been intensively studied, comparatively less is known about the properties and biological roles of unconventional myosins. A member of the class V myosins was first characterized as a calmodulin-binding protein in rabbit and chicken brains (5–8). This was shown to be a dimeric myosin structurally organized in three domains: the N-terminal head domain containing the actin-binding and the ATP hydrolysis sites; the neck domain containing six IQ motifs which bind calmodulin and/or other light chains; the tail domain consisting of coiled-coil regions intercalated with globular regions of unknown function. The tail domain also contains a PEST sequence, known to be a motif associated with protein targets of intracellular calpain proteolysis (2). In vertebrates, several lines of evidence support the role of myosin V in vesicle or organelle transport. Myosin V colocalizes with melanosomes (9, 10, 11). The myosin V mutant mouse *dilute* has impaired pigment translocation in melanocytes resulting in diluted hair-color phenotype. Their melanosomes are aggregated near the cell nucleous although the cells have a normal dendritic morphology (9, 10, 11). Many of the *dilute* mutants also present neurological defects and null mice are born apparently normal but soon present convulsions and die within three weeks of birth. In addition, brain myosin V is found associated with synaptosomes and synaptic vesicle proteins (12, 13, 30). These data strongly suggest a role in vesicle and organelle transport in vertebrates. Moreover, inactivation of myosin V by chromophore-assisted laser inactivation showed that myosin V is also involved in the filopodial extension within growth cones (14).

Here we describe the characterization of myosin V in PC12 cells, which are a pheochromocytoma-derived cell line able to differentiate into sympathetic neuronal cells upon neurotrophic stimulus. These cells are widely used in studies of neurotoxicological and neurotransmitter release mechanisms (15–17). The identification and characterization of myosin V in PC12 cells will permit the investigation of the role of myosin V in this interesting model system.

[1] To whom all correspondence should be addressed. E-mail: pleehoib@quim.iq.usp.br.

0006-291X/99 $30.00

MATERIALS AND METHODS

Materials. Dibutyryladenosine 3′:5′ cyclic monophosphate (dbcAMP), and poly-d-lysine were purchased from Sigma (St. Louis, MO). FGF-2 was from human placenta (18). The immunoconjugates, the nitrocellulose membranes and the random primer oligolabeling kit were from Gibco-BRL (Gaithersburg, MD). [^{125}I]-Protein A, [^{125}I]-calmodulin, [α-^{32}P]-dCTP and GeneScreen hybridization transfer membranes were purchased from Du Pont NEN (Boston, MA). The ECL Western blotting analysis system was purchased from Amersham Pharmacia Biotech (Uppsala, Sweden).

Cell culture and immunofluorescence. PC12 cells were a generous gift of Dr. A. Howllet (Saint Louis University, St. Louis, MO). Cells were maintained as previously described (19) and they were plated on a poly-d-lysine (10 μg/ml) treated glass coverslips (1.0 $\times$ 1.0 cm) at 4.0 $\times$ 10^4 cells/well in a 24 wells plate. The cells were grown in the presence of 1 mM dbcAMP and/or 5 ng/ml FGF-2 for 4 days, fixed by 3.7% formaldehyde in phosphate buffered saline (PBS) for 5 min, washed with PBS and permeabilized with 0.2% Triton X-100 in PBS. The cells were incubated with affinity purified rabbit anti-chicken myosin V generated against the globular tail (8) at 3.2 μg/ml for 90 min and fluoresceine conjugate at 1:200 dilution for 60 min. The immunoreaction was visualized by a Nikon Fluophot microscope.

Overlay and Western blotting. PC12 cells grown in the presence or absence of dbcAMP and/or FGF-2 were extracted on ice with 25 mM Tris-HCl pH 8.5, 0.2% SDS, 10 mM EDTA, 10 mM EGTA, 2 mM 2-mercaptoethanol, 1 mM PMSF and 10 U/ml of aprotinin. The cellular DNA was mechanically broken by the passage of the cellular extract through a syringe for 10 times, followed by boiling for 10 min. The extracts were clarified by centrifugation at maximum speed in a microcentrifuge and the supernatant recovered and kept at −80°C until use. SDS–PAGE was performed in 6–8% acrylamide-bisacrylamide (29:1) gels and the samples (100 μg/lane) were electrotransferred to nitrocellulose membranes by a semi-dry system (Amersham Pharmacia Biotech). For the overlay experiments, the membranes were incubated with [^{125}I]-calmodulin (1 $\times$ 10^6 cpm/ml) for 60 min, washed and exposed to X-ray films. For the Western blot experiments, the membranes were incubated with anti-myosin V (8) or anti-myosin II (11) for 60–120 min and visualized by [^{125}I]-Protein A (1 $\times$ 10^6 cpm/ml) and exposure to X-ray films or by chemiluminescence after previous incubation with peroxidase-conjugated anti-rabbit Ig G. Densitometric analyses were done using the Sharp JX-325 scanner and the ImageMaster software considering the volume of the bands (Amersham Pharmacia Biotech, Uppsala, Sweden).

RNA extraction and Northern hybridization. Total RNA from PC12 cells was purified according to Chirgwin *et al.*, 1979 (20). RNA samples (20 μg) were fractionated in 1% agarose-formaldehyde gels and transferred to nylon membranes (21). After baking (120 min, 80°C), membranes were hybridized to [^{32}P]-labeled myosin V probe obtained by random primer extension (22). The probe used corresponds to the C-terminal globular domain of myosin V (8).

RESULTS AND DISCUSSION

Myosin V was first characterized as a calmodulin-binding protein (5–8). Using [^{125}I]-calmodulin overlay assay, we detected several calmodulin-binding proteins in the PC12 cellular extracts (Fig. 1A). Two strong bands were detected and probably correspond to the α- and β-subunits of Ca^{2+}/calmodulin-dependent protein kinase II (6, 23). Other bands were also detected at about 65–70 kDa, 90–98 kDa and 140–220 kDa. Some of these components may be myosin I and other unconventional myosins (24, 25). To better define the presence of myosin V, a Western blot was performed, using monospecific polyclonal antibodies affinity purified against the tail domain of myosin V (8). A 190 kDa band typical of myosin V was detected (Fig. 1A). Myosin V is not the major calmodulin-binding protein in PC12 cells. Furthermore, a Northern blot of total RNA from PC12 cells showed a transcript of 12 kbp. This size corresponds to the highest transcript found in the mouse brain (26). Together, these results demonstrated the expression of myosin V at both protein and RNA levels in PC12 cells.

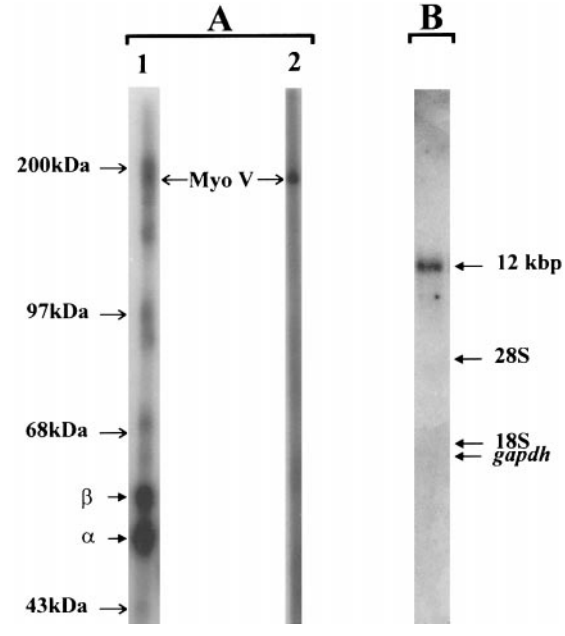

FIG. 1. Characterization of PC12 myosin V. (A) Proteins extracted from PC12 cells were resolved in a 8% SDS–PAGE, blotted on a nitrocellulose membrane, and stained with Ponceau S. The lane was cut in two. One side was used for [^{125}I]-calmodulin overlay (1) and the other side was used for Western blot using monospecific anti-myosin V and revealed by [^{125}I]-Protein A (2). α and β indicate the subunits of the Ca^{2+}/calmodulin-dependent protein kinase. Myosin V is also indicated (Myo V); (B) Northern blot using the myosin V cDNA coding for the globular-tail domain as a probe. The positions of the gadph, 28S and 18S rRNAs are indicated.

PC12 cells originate from a cell line that differentiates into sympathetic neuronal cells upon stimulus by neurotrophic factors. For this reason, we were interested to see how the myosin V distributes in this cell line under different neurotrophic stimuli. Figure 2 shows the immunofluorescence of PC12 cells treated with FGF-2 and/or dbcAMP probed with anti-myosin V antibodies. Under all conditions, myosin V had an asymetric perinuclear distribution. During differentiation, myosin V was also found in the neurites and growth cones. Under the double treatment, where the neurotrophic action of FGF-2 is potentiated by dbcAMP (19), we could also see some patches of myosin V along the neurites. These overall immunofluorescence patterns

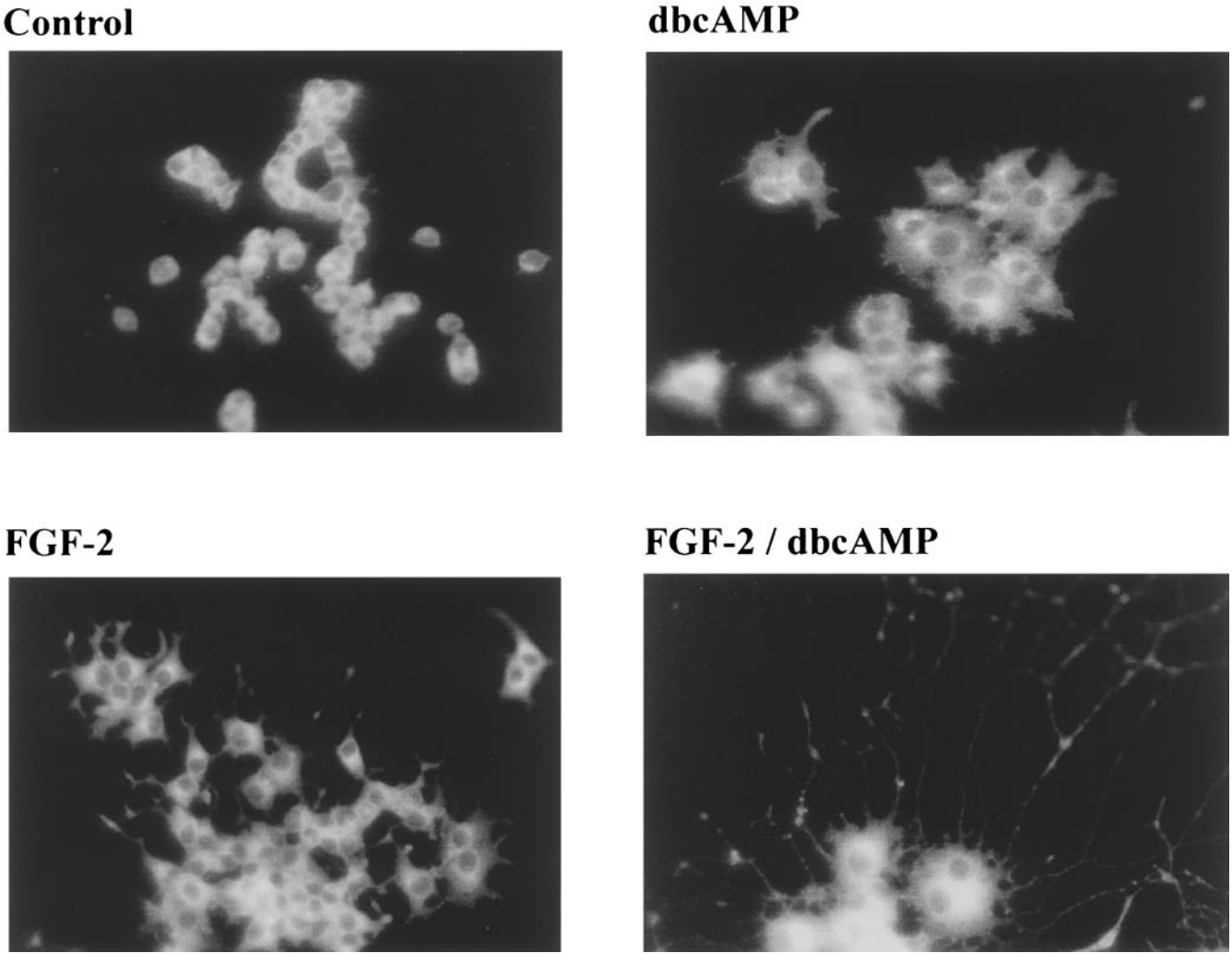

FIG. 2. Immunolocalization of myosin V in PC12 cells. The cells were treated for 5 days with FGF-2 (5 ng/ml) and/or dbcAMP (1 mM), then collected and processed as described in Materials and Methods.

are very similar to those observed in primary neuronal cells from rat hippocampus (8).

Western blots were also performed using cellular extracts of cells treated with FGF-2 and/or dbcAMP over different periods. Although equal amounts of protein from each sample were loaded on the gels, the apparent amount of myosin V varied depending on the type and time of treatments (Fig. 3A). In order to compare the expression of myosin V in these experiments with another protein, the same blot was reprobed with anti-myosin II. Densitometric analysis of the appropriate bands showed that myosin II expression also varied considerably, but in a more consistent way, increasing several fold over control levels under all treatments (Fig. 3B, C). Myosin V expression, on the other hand, increased 2–3 fold under certain conditions, but also seemed to be suppressed in some other conditions. These results suggest that myosin V and myosin II are differentially expressed depending on the time and type of treatments (dbcAMP and/or FGF-2) and may reflect the specialized roles of these molecular motors during the differentiation process elicited by these factors. It is interesting to note that cAMP pathway was shown to be involved in the up regulation of Myosin V in B16 melanoma cells treated with α-melanocyte-stimulating hormone for 48 h (11). There is also a significant increase in Myosin V in cells treated with dbcAMP alone or with dbcAMP and FGF-2 for 48 h. Changes in calpain, a calcium-activated neutral protease, have been reported in the neuronal differentiation of PC12 cells (27) and myosin V was shown to be a calpain substrate *in vitro*, generating a stable 65 kDa head fragment and a 80 kDa tail fragment (7, 28). However, neither of these fragments were observed in the experiments presented here, suggesting that myosin V is not a major *in vivo* substrate for calpain during the neuronal differentiation induced by FGF-2 and/or dbcAMP.

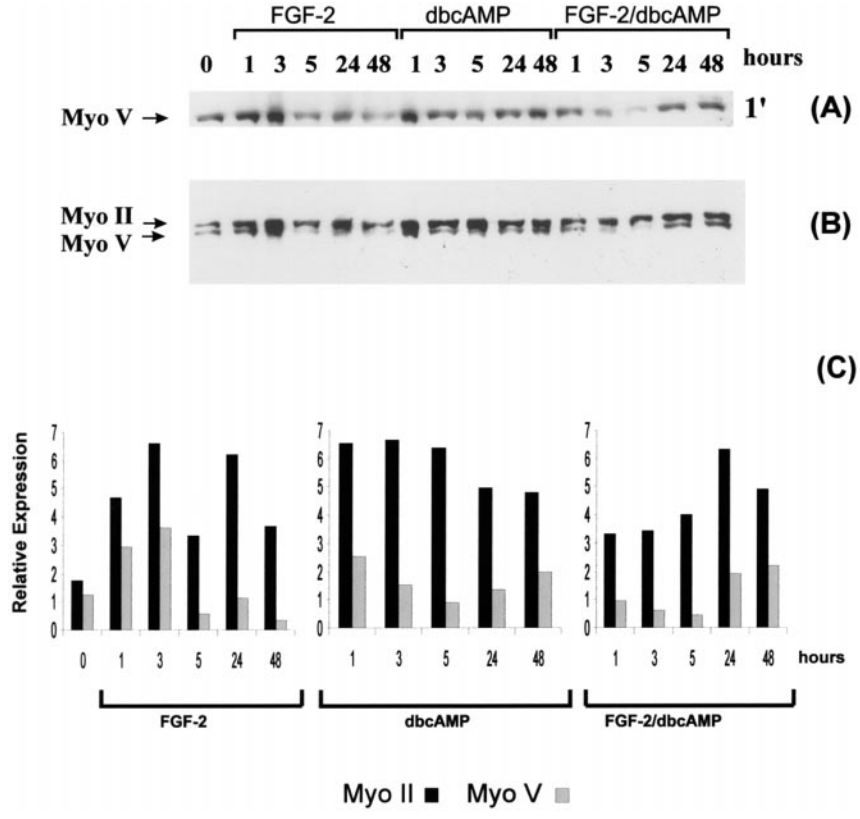

FIG. 3. Western blot of PC12 cellular extracts after different times of exposure to FGF-2 and/or dbcAMP. The PC12 cells were treated with FGF-2 and/or dbcAMP for different times (0, 1, 3, 5, 24 and 48 h) as indicated. The cellular extract derived from each treatment was fractionated on a 6% SDS–PAGE, blotted to a nitrocellulose membrane and probed with anti-myosin V polyclonal antibodies (A) or both anti-myosin V and anti-myosin II polyclonal antibodies (B) after stripping the membrane used in (A). The blots were revealed by ECL (Amersham Pharmacia Biotech). Densitometric analysis of immunoreactive myosin V and myosin II are shown in (C).

In conclusion, we have described here the identification and characterization of myosin V in PC12 cells. Our results indicate that myosin V and myosin II are differentially expressed during the differentiation process induced by dbcAMP and/or FGF-2. Myosin V may be a multifunctional protein. It seems that myosin V may play a role in organelle transport and in neuronal growth cone motility (14, 29). Recently, myosin V was shown to be associated with synaptosomes, synaptic vesicles and synaptobrevin via Ca^{2+}-dependent interactions (12, 13). Thus, the PC12 cell line may be a useful system to study the role of myosin V in these processes, being an established cell line with neuronal properties that are easily manipulated in culture.

ACKNOWLEDGMENTS

We are grateful to Dr. Alexandra A. C. Nascimento for providing us the anti-myosin II antiserum. This work was supported by grants from the Fundação de Amparao à Pesquisa do Estado de São Paulo, Grant 93/3552-9 (to R.E.L., P.L.H. and E.M.E.), Programa de Apoio ao Desenvolvimento Científico e Tecnológico (PADCT), 62.0099/95.0 (to R.E.L. and E.M.E.), and Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq), 522791/95-6 (to R.E.L. and E.M.E.). M.C.R.C. was a Predoctoral Fellow supported by Coordenação de Aperfeiçoamento de Pessoal de Niível Superior (CAPES).

REFERENCES

1. Titus, M. A. (1997) *Trends in Cell Biology* **7,** 119–123.
2. Larson, R. E. (1996) *Braz. J. Med. Biol. Res.* **29,** 309–318.
3. Moosecker, M. S., and Cheney, R. E. (1995) *Ann. Rev. Cell Dev. Biol.* **11,** 633–675.
4. Mermall, V., Post, P. L., and Moosecker, M. S. (1998) *Science* **279,** 527–533.
5. Larson, R. E., Pitta, D. E., and Ferro, J. A. (1988) *Braz. J. Med. Biol. Res.* **21,** 213–217.
6. Larson, R. E., Espindola, F. S., and Espreafico, E. M. (1990) *J. Neurochem.* **54,** 1288–1294.
7. Espindola, F. S., Espreafico, E. M., Coelho, M. V., Martins, A. R., Costa, F. R. C., Moosecker, M. S., and Larson, R. E. (1992) *J. Cell Biol.* **118,** 359–368.
8. Espreafico, E. M., Cheney, R. E., Matteoli, M., Nascimento, A. A. C., De Camilli, P. V., Larson, R. E., and Moosecker, M. S. (1992) *J. Cell Biol.* **119,** 1541–1557.
9. Wei, Q., Wu, X., and Hammer, J. A., III (1997) *J. Mus. Res. Cell Motil.* **18,** 517–527.
10. Wu, X., Bowers, B., Wei, Q., Kocker, B., and Hammer, J. A., III (1997) *J. Cell Sci.* **110,** 847–859.

11. Nascimento, A. A. C., Amaral, R. G., Bizario, J. C. S., Larson, R. E., and Espreafico, E. M. (1997) *Mol. Biol. Cell* **8,** 1971–1988.
12. Mani, F., Espreafico, E. M., and Larson, R. E. (1994) *Braz. J. Med. Biol. Res.* **27,** 2639–2643.
13. Prekeris, R., and Terrian, D. M. (1997) *J. Cell. Biol.* **137,** 1589–1601.
14. Wang, F. S., Wolenski, J. S., Cheney, R. E., Moosecker, M. S., and Jay, D. G. (1996) *Science* **273,** 660–663.
15. Greene, L. A., and Tischler, A. S. (1982) *in* Advances in Cellular Neurobiology (Fedoroff, S., and Hertz, L., Eds.), Vol. 3, Academic Press, NY.
16. Guroff, G. (1995) *in* Cell Culture in the Neuroscience (Bottenstein, J. E., and Sato, G., Eds.), Plenum Publishing Corporation, NY.
17. Shafer, T. J., and Atchison, W. D. (1991) *NeuroToxicol.* **12,** 473–492.
18. Wolff, P., Tanaka, A. M., Chenker, E., Cabrera-Crespo, J., Raw, I., and Ho, P. L. (1996) *Biochimie* **78,** 190–194.
19. Ho, P. L., and Raw, I. (1992) *J. Cell. Physiol.* **150,** 647–656.
20. Chirgwin, J., Aeyble, A., McDonald, R., and Rutter, W. (1979) *Biochemistry* **8,** 5294–5299.
21. Thomas, P. S. (1980) *Proc. Natl. Acad. Sci. USA* **77,** 5201–5205.
22. Feinberg, A. P., and Vogelstein, B. (1984) *Anal. Biochem.* **137,** 266–267.
23. Hanson, P. I., and Schulman, H. (1992) *Annu. Rev. Biochem.* **61,** 559–601.
24. Wagner, M. C., Barylko, B., and Albanesi, J. P. (1992) *J. Cell Biol.* **119,** 163–170.
25. Sherr, E. H., Joyce, M. P., and Greene, L. A. (1993) *J. Cell Biol.* **120,** 1405–1416.
26. Mercer, J. A., Seperak, P. K., Strobel, M. C., Copeland, N. G., and Jenkins, N. A. (1991) *Nature* **349,** 709–713.
27. Saido, T. C., Sorimachi, H., and Suzuki, K. (1994) *FASEB J.* **8,** 814–822.
28. Nascimento, A. C., Cheney, R. E., Tauhata, S. B. F., Larson, R. E., and Moosecker, M. S. (1996) *J. Biol. Chem.* **271,** 17561–17569.
29. Lin, C. H., Espreafico, E. M., Moosecker, M. S., and Forscher, P. (1996) *Neuron* **16,** 769–782.
30. Costa, M. C. R., Mani, F., Santoro, W., Jr., Espreafico, E. M., and Larson, R. E. (1999) *J. Biol. Chem.* **274,** 15811–15819.