Interaction between calmodulin and microtubule-associated proteins prepared at different stages of brain development

Christophe Erneux, Heloísa Passareiro and Jacques Nunez

Institut de Recherche Interdisciplinaire (IRIBHN), Université Libre de Bruxelles, Hôpital Erasme, B-1070 Bruxelles, Belgium and *INSERM U35-CNRS, Hôpital Henri Mondor, Creteil, France*

Received 21 May 1984

Rat brain microtubules were prepared at the adult stage and from immature (i.e., 4-day-old) animals. At an early stage of development, the composition of microtubule-associated proteins is qualitatively different from that found at the adult stage [(1982) Eur. J. Biochem. 129, 465–471]. The influence of calmodulin on the time course of assembly of second cycle microtubules was compared at both stages of brain development (i.e., microtubules originating from 4-day-old and adult animals). In the presence of Ca^{2+} the inhibition of microtubule assembly was more pronounced at a young stage of brain development than at the adult stage. Cross-linking studies with ^{125}I-labeled calmodulin further established that the two major microtubule-associated proteins, MAP_2 and TAU were able to bind to calmodulin at both stages of brain development but with different intensities. The labeling with ^{125}I-labeled calmodulin was Ca^{2+}-dependent, specific, displaced by unlabeled calmodulin and trifluoperazine.

Calmodulin *Microtubule* *Rat brain development*

1. INTRODUCTION

Several lines of evidence suggest that Ca^{2+}-calmodulin regulates microtubule assembly both in the intact cell [1–4] and under in vitro conditions [1,5–8]. With purified microtubules millimolar concentrations of Ca^{2+} are required to inhibit assembly in the absence of calmodulin; in contrast, the sensitivity to Ca^{2+} is increased by two orders of magnitude in the presence of calmodulin. Since such an enhancement in Ca^{2+} sensitivity was demonstrated in preparations that contained both tubulin and MAPs, studies have been performed to identify which one of these components interacts with calmodulin.

As an interaction between calmodulin and tubulin [9] has not been confirmed [5,9], several attempts have been made to identify which one of the MAPs is involved. For instance authors in [10] have found that one of the MAPs, the TAU proteins (60–70 kDa) bind to a calmodulin–Sepharose affinity column whereas the high-molecular-mass component MAP_2 (300–350 kDa) does not. In contrast authors in [6] have reported that MAP_2 binds to calmodulin more efficiently than TAU proteins. Authors in [11], on the basis of kinetic experiments, have also proposed that Ca^{2+}-calmodulin interacts with one or more of the MAPs: since, in the absence of MAPs, tubulin polymerizes very poorly the formation of a ternary complex between Ca^{2+}, calmodulin and MAPs would prevent microtubule assembly. Very recently, it has been reported that Ca^{2+}-calmodulin binds to both MAP_2 and TAU proteins [12].

All the experiments reported above have been performed with microtubules and MAPs prepared from adult rat brain. Microtubules can be also prepared at different stages of development, i.e., when the brain is immature or during the critical

Abbreviations: MAPs, microtubule-associated proteins; MAP_2, high-molecular-mass microtubule-associated protein; TAU, another microtubule-associated protein(s); Mes, 4-morpholineethanesulfonic acid

Published by Elsevier Science Publishers B.V.
00145793/84/$3.00 © 1984 Federation of European Biochemical Societies

period of nerve cell differentiation [13]. During these periods the composition of MAPs differs markedly from that found at the adult stage [14]. For instance, the group of proteins of 60–70 kDa (TAU proteins) differ in composition, activity and peptide mapping depending on whether they are prepared at early or adult stages of development [15].

It is not known therefore whether calmodulin also regulates microtubule assembly in the immature brain, and by which mechanism(s). To answer these questions kinetic and cross-linking experiments were performed to determine whether calmodulin inhibits the assembly of microtubules prepared at early stages of brain development, and whether it interacts with one or more of the different MAPs which copolymerize with microtubules prepared from immature rat brain.

2. MATERIALS AND METHODS

2.1. Materials

Sephacryl S-200 and low-molecular-mass calibration protein kit were from Pharmacia. Mes, GTP, EDTA and EGTA were from Sigma; SDS, acrylamide and bisacrylamide were purchased from Bio-Rad. Trifluoperazine was a gift from Smith, Kline and French. Dimethylsuberimidate was from Serva, lactoperoxidase and glucose oxidase from Calbiochem. Rat brain microtubules were purified by the temperature-dependent assembly-disassembly procedure [16]. MAPs were prepared as in [17]. Calmodulin was prepared as in [18] in which in the last step of gel filtration, Sephadex G-100, was replaced by Sephacryl S-200. Calmodulin was dialyzed against deionized water, lyophilized and stored at −70°C.

2.2. Time course of tubulin polymerisation

Tubulin polymerisation in vitro at 37°C was followed by turbidity development at 350 nm under the conditions in [13].

2.3. Cross-linking of MAPs with ^{125}I-labeled calmodulin

Iodination of calmodulin (0.2 mg) was performed at 23°C in 0.05 M phosphate buffer (pH 7.0), containing 1 μg lactoperoxidase, 34 μg glucose and 0.4 mM $CaCl_2$ (final volume 0.17 ml). $Na^{125}I$ (0.1 mCi, Amersham) in 1 μl was mixed with 10 μl KI (10 mM). A 2 μl aliquot of this solution (20 nmol I^-) was added to the incubation mixture and the reaction was initiated by addition of 5 μl glucose oxidase (1 mg/ml). After 45 min incubation, the reaction was stopped by addition of 5 μl dithiothreitol (100 mg/ml) ^{125}I-labeled calmodulin was purified on a Sephadex G-100 column, equilibrated and eluted with 0.05 M triethanolamine (pH 8.5), 0.5 mM $MgCl_2$. A single major homogeneous band that comigrated with unmodified calmodulin was detected by overloaded SDS-polyacrylamide gel electrophoresis (SDS–PAGE). The ability of ^{125}I-labeled calmodulin to stimulate a calmodulin-sensitive cyclic nucleotide phosphodiesterase from bovine brain was similar to that of the unlabeled calmodulin (not shown).

MAPs from 4-day-old and adult rat brains were dialyzed against 0.2 M triethanolamine (pH 8.5) containing 1 mM $MgCl_2$ and 0.1 mM dithiothreitol at 4°C. Cross-linking [19] in 25 μl with 10 μM ^{125}I-labeled calmodulin and 0.4–1 mg/ml of MAPs (or other proteins, see section 3) in 0.2 M triethanolamine (pH 8.5), 2 mM $MgCl_2$, 2 mM dithiothreitol and either 1 mM $CaCl_2$ or 1 mM EGTA was initiated by 2 μl of a fresh solution containing 25 mg/ml of dimethylsuberimidate in 0.2 M triethanolamine (pH 8.5).

The samples were incubated for 60 min at 23°C. The reaction was terminated by adding 5 μl of a mixture consisting of 8% SDS and 18% 2-mercaptoethanol and boiling for 2 min. The samples were subjected to SDS–PAGE as in [15]. After Coomassie blue staining, the wet gels were autoradiographed on Kodak X-AR5 X-ray films.

Protein concentration was determined as in [20] with bovine serum albumin as standard, as well as by UV absorption for purified calmodulin ($\epsilon_{M,276}$ = 3240) [21].

3. RESULTS AND DISCUSSION

Kinetic experiments were performed to establish whether Ca^{2+}-calmodulin inhibits the assembly of second cycle microtubules prepared at early stages of rat brain development. Fig.1B shows that low concentrations of either Ca^{2+} (~100 μM free Ca^{2+}) or calmodulin (10 μM), when used separately, had only trivial effects on the time course of assembly of second cycle microtubules prepared from adult rat brain. In contrast, with 4-day-old microtubules

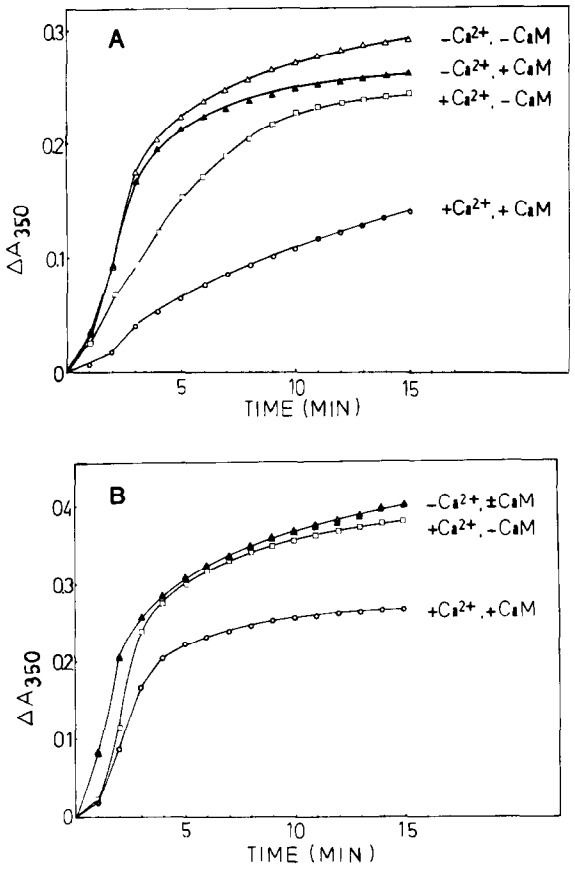

Fig.1. Ca^{2+}-calmodulin-induced inhibition of tubulin polymerisation: comparison between 4-day-old (A) and adult (B) microtubules. Microtubules (1.3 mg/ml) prepared from 4-day-old (A) and adult (B) rat brains were polymerized at 37°C as described in section 2 with the following additions: (△) none, (▲) 10 μM calmodulin, (□) 1.25 mM Ca^{2+} in (A) or 1.5 mM Ca^{2+} in (B); (○) 1.25 mM Ca^{2+} in (A) or 1.5 mM Ca^{2+} in (B) plus 10 μM calmodulin.

(fig.1A), the effects of Ca^{2+} alone seemed to be more significant than with the adult preparation. Combining Ca^{2+} and calmodulin resulted in a marked reduction in the initial rate and overall assembly and in an increase in the lag period at both stages; in addition, the effect of Ca^{2+}-calmodulin on the polymerisation of 4-day-old microtubules appears to be much more pronounced than that observed with adult microtubules. The latter conclusion was also reached at lower (1–5 μM) concentration of calmodulin (not shown).

It has been shown that the composition of the MAPs prepared at different stages of brain development is clearly different when these proteins are analyzed by SDS–PAGE. Two major groups of MAPs, MAP_2 (or HMW_2) and TAU, have been identified at an adult stage on the basis of their molecular masses on SDS gels which are ~350 kDa and ~70 kDa, respectively [22–24]. The TAU fraction is itself composed of 4 closely spaced bands which produce very similar peptide maps when treated with *Streptococcus aureus* protease [24]. In contrast, the peptide map of MAP_2 is very different from that of TAU [15].

At young stages the high-molecular-mass component, MAP_2, has a peptide map different from that of the adult one [15]. On the other hand, the young TAU entities are resolved by one-dimensional SDS–PAGE into two bands of 50 kDa ('fast' TAU) and 63 kDa ('slow' TAU) with none migrating as any of the 4 adult entities. Finally, fast and 'young' TAUs produce completely different peptide maps [15].

Cross-linking experiments revealed that both adult MAP_2 and adult TAU interact with Ca^{2+}-calmodulin (fig.2). This result is in agreement with the conclusions in [12]. The covalent complex formed between TAU and calmodulin has, as expected, an apparent molecular mass higher than that of free TAU. Since MAP_2 is a much larger protein, an increment in its molecular mass after cross-linking with calmodulin could not be calculated.

Similar cross-linking experiments performed with young MAPs led to similar conclusions: both MAP_2 and TAU were labeled by ^{125}I-labeled calmodulin (fig.2). The pattern of labeling in the young TAU region was also different from that obtained with adult TAU. Two cross-linked and labeled polypeptides were clearly separated; their molecular masses were equal to 67 kDa and 80 kDa, respectively, i.e., 17 kDa higher than the two parent young TAU proteins which have a molecular mass of 50 kDa (fast TAU) and 63 kDa (slow TAU). This indicates the formation of a 1:1 complex between fast or slow TAU and calmodulin. A 1:1 stoichiometry has also been reported using similar cross-linking techniques for the myosin kinase, whereas for the calmodulin-sensitive phosphodiesterase the ratio is 2 per mol enzyme, i.e., one for each one of the two subunits of that phosphodiesterase [25].

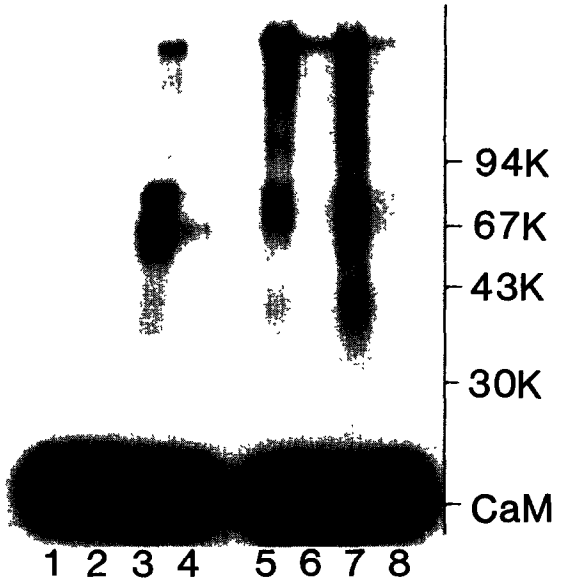

Fig.2. Cross-linking of MAPs from 4-day-old and adult rat brain microtubules to ^{125}I-labeled calmodulin. The cross-linking reaction with 10 μM ^{125}I-labeled calmodulin and 50 μg dimethylsuberimidate in the presence of either 1 mM $CaCl_2$ (lanes 1,3,5,7) or 1 mM EGTA (lanes 2,4,6,8) was followed by SDS– PAGE and autoradiography as described in section 2: (lanes 1,2) ^{125}I-labeled calmodulin in the absence of any protein; (lanes 3,4) ^{125}I-labeled calmodulin in the presence of 0.4 mg/ml MAPs from 4-day-old rat brain microtubules; ^{125}I-labeled calmodulin in the presence of MAPs of adult rat brain microtubules, respectively, at 0.8 mg/ml (lanes 5,6) and 1 mg/ml (lanes 7,8). The apparent molecular masses of MAPs, calmodulin and its cross-linked products were determined using phosphorylase *b* (94 kDa), albumin (67 kDa), ovalbumin (43 kDa), carbonic anhydrase (30 kDa), trypsin inhibitor (20 kDa) as standards in SDS–PAGE. CaM, calmodulin.

Thus although young and adult TAUs are probably different proteins, as shown both by peptide mapping [15] and after isolation of their mRNA [26], they share the property of interacting with Ca^{2+}-calmodulin.

In the region corresponding to young MAP_2 cross-linking complexes were also repeatedly observed but the labeling of this entity was always much less pronounced than that of the adult MAP_2 species. In contrast, labeling of young TAU proteins was always more pronounced than that of adult TAU.

As a criterium of specificity, the cross-linking reaction was also carried out in the presence of 1 mM EGTA to complex all the free Ca^{2+} present in the medium. Fig.2 shows that the labeling of the different young and adult MAPs by ^{125}I-labeled calmodulin was almost abolished under these conditions. Thus, the Ca^{2+}-saturated form of calmodulin is readily cross-linked with MAPs whereas the (operationally defined) Ca^{2+}-free form of this protein is not. The cross-linking reaction was inhibited by a large excess of unlabeled calmodulin (not shown).

In the presence of Ca^{2+}, trifluoperazine is known to bind to calmodulin, presumably through a hydrophobic type of interaction and thereby to inhibit the interaction between this Ca^{2+}-binding protein and several calmodulin-dependent proteins or enzymes [27]. When MAPs prepared from both 4-day-old and adult brain microtubules were incubated in the presence of ^{125}I-labeled calmodulin, Ca^{2+} and dimethylsuberimidate the labeling of the cross-linked polypeptides was partially decreased in the presence of 50 μM trifluoperazine and almost completely displaced in the presence of 100 μM of the same drug (fig.3).

Finally, the specificity of the MAPs–calmodulin cross-linking reaction was tested by incubating several other proteins (phosphorylase *b*, serum albumin, ovalbumin, carbonic anhydrase, trypsin inhibitor, α-lactalbumin) with the cross-linking reagent and calmodulin. Under the experimental conditions of fig.2, only serum albumin was slightly labeled, a result (not shown) that might be explained by the fact that this protein is hydrophobic and may therefore interact slightly with calmodulin which is also hydrophobic.

In conclusion, our data show that:

(i) The Ca^{2+}-calmodulin complex inhibits the assembly of microtubules prepared at young stages of brain development. At this stage in the rat brain, the neurons of the cortex are already formed but not differentiated, whereas in the cerebellum neuronal proliferation only begins after birth. It is interesting in these respects that calmodulin antibodies decorate the microtubules of the spindle in the dividing cells [28].

(ii) Both at a young and at an adult stage the two main groups of MAPs, MAP_2 and TAU, form

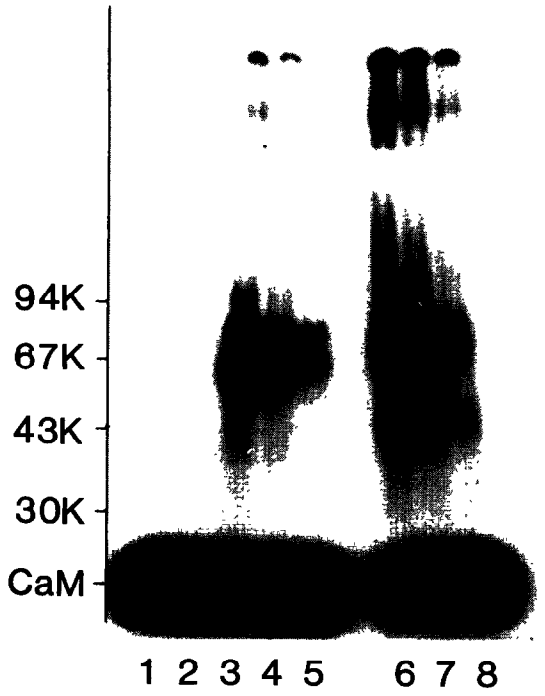

Fig.3. Effect of trifluoperazine on the cross-linking efficacy of MAPs to ^{125}I-labeled calmodulin. The cross-linking reaction with 10 μM ^{125}I-labeled calmodulin and 50 μg dimethylsuberimidate (except lane 1) was allowed to proceed in the presence of 1 mM $CaCl_2$ with the following: (lanes 1,2) ^{125}I-labeled calmodulin in the absence of any protein (lane 1: no cross-linker); (lanes 3–5) ^{125}I-labeled calmodulin in the presence of 0.4 mg/ml MAPs from 4-day-old rat brain microtubules; (lanes 6–8) ^{125}I-labeled calmodulin in the presence of 0.7 mg/ml MAPs from adult rat brain microtubules with the addition of 0 mM (lanes 3,6), 50 μM (lanes 4,7) and 100 μM (lanes 5,8) trifluoperazine. CaM, calmodulin.

cross-linked derivatives with Ca^{2+}-calmodulin. Based on experiments with young TAU we propose a 1:1 stoichiometry. This result provides direct proof of the proposal made in [11] that the inhibition of microtubule assembly by Ca^{2+}-calmodulin results from the formation of a ternary complex between this protein and MAPs. It also implies that the Ca^{2+}–calmodulin–MAPs complex is inactive in promoting tubulin assembly. In other words, calmodulin and tubulin seem to compete for the binding to MAPs. This is consistent with the observation that the concentration of calmodulin required to inhibit microtubule assembly is approximately equimolar with that of tubulin.

(iii) In contrast to the conclusions in [10] but in agreement with the recent data in [12], not only adult TAU but also adult MAP_2 interact with calmodulin.

(iv) The labeling of adult MAP_2 by ^{125}I-labeled calmodulin always seems stronger than that of adult TAU. The situation appears to be reversed at early stages of brain development since the cross-linking reaction is systematically less efficient with young MAP_2 than with young TAU. It is not known whether the stronger inhibition of microtubule assembly by Ca^{2+}-calmodulin observed at young stages of development (fig.1) is related to these differences in cross-linking efficiency.

Irrespective of the answer to this question, it remains that most of the young and adult MAPs seem to be able to interact with Ca^{2+}-calmodulin. This suggests a fundamental function of MAPs as target proteins for Ca^{2+}-calmodulin. Although the various young and adult MAPs are probably different proteins with different cellular functions, it is nevertheless conceivable that they might all share a similar domain responsible for the interaction with calmodulin.

ACKNOWLEDGEMENTS

This work was realized under contract of the Ministère de la Politique Scientifique (Actions Concertées, Belgium). C.E. is Chargé de Recherches au Fonds National de la Recherche Scientifique. We wish to thank Dr J.E. Dumont for criticism and Mrs D. Leemans for typing the manuscript.

REFERENCES

[1] Marcum, J.M., Dedman, J.R., Brinkley, B.R. and Means, A.R. (1978) Proc. Natl. Acad. Sci. USA 75, 3771–3775.
[2] Anderson, B.A., Osborm, M. and Weber, K. (1978) Cytobiologie 17, 354–364.
[3] Welsh, M.J., Dedman, J.R., Brinkley, B.R. and Means, A.R. (1978) Proc. Natl. Acad. Sci. USA 75, 1867–1871.
[4] Keith, C., Dipaola, M., Maxfield, F.R. and Shelanski, M.L. (1983) J. Cell Biol. 97, 1918–1924.

[5] Schliwa, M., Euteneuer, U., Bulinski, J.C. and Izant, J.G. (1981) Proc. Natl. Acad. Sci. USA 78, 1037–1041.
[6] Rebhun, L.I., Jemiolo, D., Keller, T., Burgess, W. and Kretsinger, R. (1980) in: Microtubules and Microtubule inhibitors (De Brabauder, M. and De Mey, J. eds) pp. 243–252. Elsevier, Amsterdam, New York.
[7] Jemiolo, D.K., Burgess, W.H., Rebhun, L.I. and Kretsinger, R.H. (1980) J. Cell Biol. 87, 248a (abstract).
[8] Berkowitz, J.A. and Wolff, J. (1981) J. Biol. Chem. 256, 11216–11223.
[9] Kumagai, H., Nishida, E., Ishiguro, K. and Murofushi, H. (1980) J. Biochem. (Tokyo) 87, 667–670.
[10] Sobue, K., Fujita, M., Muramoto, K. and Kakiuchi, S. (1981) FEBS Lett. 132, 137–140.
[11] Lee, Y.C. and Wolff, J. (1982) J. Biol. Chem. 257, 6306–6310.
[12] Lee, Y.C. and Wolff, J. (1984) J. Biol. Chem. 259, 1226–1230.
[13] Francon, J., Fellous, A., Lennon, A.M. and Nunez, J. (1978) Eur. J. Biochem. 85, 43–53.
[14] Mareck, A., Fellous, A., Francon, J. and Nunez, J. (1980) Nature 284, 353–355.
[15] Francon, J., Lennon, A.M., Fellous, A., Mareck, A., Pierre, M. and Nunez, J. (1982) Eur. J. Biochem. 129, 465–471.
[16] Shelanski, M.L., Gaskin, F. and Cantor, R.C. (1973) Proc. Natl. Acad. Sci. USA 70, 765–768.
[17] Fellous, A., Francon, J., Lennon, A.M. and Nunez, J. (1977) Eur. J. Biochem. 78, 167–174.
[18] Yasawa, M., Sakuma, M. and Yagi, K. (1980) J. Biochem. 87, 1313–1320.
[19] Adelstein, A.S. and Klee, C.B. (1981) J. Biol. Chem. 256, 7501–7509.
[20] Bradford, M.M. (1976) Anal. Biochem. 72, 248–254.
[21] Watterson, D.M., Harrelson, W.G., Keller, P.M., Sharief, F. and Vanaman, T.C. (1976) J. Biol. Chem. 251, 4501–4513.
[22] Dentler, W.L., Granett, S. and Rosenbaum, J.L. (1975) J. Cell Biol. 65, 237–241.
[23] Murphy, D.B., Johnson, K. and Borisy, G.G. (1977) Biochemistry 16, 2598–2605.
[24] Cleveland, D.W., Hwo, S.Y. and Kirschner, M.W. (1977) J. Mol. Biol. 116, 227–247.
[25] Manalan, A.S. and Klee, C.B. (1983) Chem. Scr. 21, 137–142.
[26] Ginzburg, I., Scherson, T., Giveon, D., Behar, L. and Littauer, U.Z. (1982) Proc. Natl. Acad. Sci. USA 79, 4892–4896.
[27] Weiss, B. (1983) Methods Enzymol. 102, 171–184.
[28] Welsh, M.J., Dedman, J.R., Brinkley, B.R. and Means, A.R. (1979) J. Cell Biol. 81, 624–634.