Binding of microtubule protein to DNA and chromatin: possibility of simultaneous linkage of microtubule to nucleic acid and assembly of the microtubule structure

Alfredo Villasante, Victor G. Corces*, Rafael Manso-Martínez and Jesús Avila

Centro de Biología Molecular (C.S.I.C.-U.A.M.), Universidad Autónoma de Madrid, Facultad de Ciencias, Canto Blanco, Madrid-34, Spain

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ABSTRACT

Microtubule protein binds to DNA through microtubule associated polypeptides (MAPs). Among MAPs there is one high molecular weight polypeptide (MAP$_2$) which interacts with DNA fundamentally through certain polynucleotide sequences. This interaction is not affected by the presence of histones and other chromosomal proteins. DNA can associate to assembled microtubules and when a determinate DNA/protein ratio is reached the nucleic acid behaves as a microtubule associated molecule. The nucleic acid fragments which preferentially bind to microtubules have been isolated and characterized. These fragments contain DNA regions enriched in repetitive sequences that hybridize preferentially to the pericentromeric zone of metaphase chromosomes. These results give further support to the model of interaction microtubule-chromosome based upon the mediator function of the microtubule associated proteins.

INTRODUCTION

During mitosis the eukaryotic chromosome interacts with the microtubules through its pericentromeric region. It has been shown that the chromosome can promote the assembly of microtubules in vitro and that polymerization of microtubules is compatible with its linkage to the chromosome (1,2). The kinetochore is the site for attachment and, presumptive, assembly of microtubules in the eukaryotic chromosome (3). The kinetochore has been morphologically characterized but very little is known about its nature, although it could contain nucleic acid and protein, like the rest of the chromosome. Recent studies indicate that: a) DNA could be involved in the chromosome-microtubule interaction (4), b) high molecular weight microtubule associated polypeptides bind to DNA (5), and c) those polypeptides bind preferentially to DNA regions found near the centromere (6). However DNA inhibits microtubule polymerization (4,7) although this effect
could depend on the sequence of the nucleic acid tested (4). These results suggest that DNA is involved in the microtubule-chromosome interaction but do not explain the initiation of microtubule assembly in the presence of DNA. To interpret the nucleation of microtubule polymerization on chromosomes, it is necessary to hypothesize one or both of the following explanations: a) the existence of a DNA sequence which does not decrease the ability of microtubular protein for assembly; b) the existence of another kinetochore component, different from DNA, which promotes microtubule polymerization. In this report we explore these possibilities.

METHODS

Preparation and fractionation of microtubule proteins

Microtubule proteins were purified from porcine brain by three cycles of assembly-disassembly by the procedure of Shelanski et al (8). Depolymerized microtubules were chromatographed on DEAE-Sephadex according to Murphy et al (9) under the conditions previously described (4), to obtain HMW polypeptides, tau fraction and tubulin.

Microtubule associated protein (MAP2) was obtained as previously described (10) following the method of Herzog and Weber (11).

DNA preparation

Radioactive DNA was isolated from 3T6 mouse fibroblasts cells after labelling exponentially growing cultures with 3.5 µCi/ml of $^{32}$P-phosphate or 2 µCi/ml of $^3$H-thymidine (20 Ci/mmol) for 50 h at 37°C. The cells were lysed as previously described (5) and the DNA was purified by the procedure of Botchan et al. (12). The isolated DNA was chromatographed on a Sepharose-4B column equilibrated in 15 mM NaCl, 1.5 mM Na citrate, to isolate high molecular weight fraction. The purified $^{32}$P-DNA was resistant to alkali or protease treatment but sensitive to DNase digestion.

To test the affinity of microtubule protein for different mouse DNA sequences, high molecular weight $^{32}$P-DNA was digested with EcoRI restriction nuclease (100 µg/ml) in 100 mM Tris
(pH 7.5), 50 mM NaCl, 10 mM MgCl₂, for 3 hours at 37°C. Digested DNA was electrophoresed in 0.3% agarose gels in an horizontal slab gel system as described by McDonell et al (13). 217 μg of mouse DNA were electrophoresed in a gel 12 cm long for 1 hour at 20 V followed by 12 hours at 50 V. The agarose gel was fractionated into fragments of 4 mm and the DNA was extracted by three cycles of freezing and thawing in 0.5 ml of 20 mM Tris (pH 7.0), 1 mM EDTA. The extracted DNA was sheared by passing it through a thin hypodermic needle (as indicated below) to obtain a homogeneous size distribution. The recovery was followed by measuring Cerenkov radiation.

To isolate mouse DNA fragments showing higher affinity for microtubule protein, high molecular weight DNA was sheared by passing it through a 20 gauge needle about 10 times. The obtained DNA fragments have an average molar mass of 3 x 10⁶, as determined by sedimentation in sucrose gradient. This fragmented DNA was added to a depolymerized microtubule protein solution and three cycles of assembly-disassembly were done in the presence of DNA. After the third cycle, depolymerized microtubule protein was chromatographed on a Sepharose 4B column equilibrated in 0.1 M MES (pH 6.4), 0.5 mM MgCl₂, 2 mM EGTA. The excluded material was mixed with a 40-fold excess of φ29 phage DNA and filtered through a nitrocellulose filter (100 μg of protein per 2.5 cm diameter filter) as described below. DNA was extracted from the filter by addition of 1% sodium dodecyl sulfate, 5 mM EDTA, and it was deproteinized by two phenol extractions. After sonication, to obtain shorter DNA fragments, this material was used for Cot analysis.

Fragments with a higher affinity for MAP₂ were obtained by binding, in a mass ratio one : one (see 4), the purified polypeptide to whole labelled mouse DNA, previously sheared by sonication. Mouse DNA fragments bound to MAP₂ were isolated by filtration on nitrocellulose membranes and prepared for Cot analysis as indicated above.

Chromatin preparation

3T6 cell nuclei were prepared by the method of Kumar and Lindberg (14). Nuclei were resuspended in 10 mM Tris (pH 7.4),
10 mM NaCl, 3 mM MgCl₂ and 1 mM CaCl₂, at a DNA concentration of 1.5 mg/ml (30 A₂₆₀ units/ml) and digested for 30 min at 37°C with micrococcal nuclease (Worthington, 10 units/ml). The reaction was terminated by chilling the tubes on ice followed by centrifugation at 1000 x g in the Sorvall HB-4 rotor. The precipitate was resuspended by gentle homogenization in 0.22 mM EDTA, pH 7.5, lysed and centrifuged again at 16,000 x g for 10 min. The supernatant was then centrifuged in a 5 to 20% sucrose gradients, containing 10 mM Tris (pH 7.5), 0.7 mM EDTA. Centrifugation was at 30,000 rpm for 13 hours in the SW40 rotor (15). The gradient fractions, corresponding to hexanucleosomes, were used as chromatin in the binding assays.

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Complex formation was measured using nitrocellulose filters according to Jones and Berg (16) in the conditions previously described (5).

**DNA reassociation analysis**

³²P-DNA was sonicated and afterwards it was denatured and reassociated in 0.57 M NaCl, 1 mM EDTA, 0.05% SDS and 10 mM Tris (pH 7.5) at 65°C. The renaturation reactions were stopped by immersing the reaction mixtures into dry ice-acetone. The samples were then added to a column of hydroxylapatite (BioRad) at 60°C. Single stranded DNA was eluted by washing the column with 0.12 M phosphate buffer (pH 7.0) and double stranded DNA by washing with 0.4 M of the same buffer. The amount of eluted DNA was determined by measuring Cerenkov radiation.

**Nucleic acid hybridization to the DNA of metaphase chromosomes**

*In situ* hybridization was done essentially following the procedures of Pardue and Gall (17) and Jones (18). Exponentially growing 3T6 cells were treated for 10 h with 0.2 μg/ml of vinblastine. Mitotic cells were collected by treatment with trypsin and gentle centrifugation. Afterwards the cells were fixed in cold 50% acetic acid for 30 min and squashes were made from a drop of a dense cell suspension. The cover slips were flipped off after freezing the squashes in liquid nitrogen and the preparation was maintained in 95% ethanol for 10 min, air dried and
stored at 4°C.

For the hybridization procedure chromosomal DNA was denatured by addition of 0.07 N NaOH at room temperature for 5 min. The same denaturing treatment was followed for mouse $^3$H-DNA (0.1 mg/ml, $10^7$ cpm/ml) isolated as indicated above for Cot analysis. Hybrid formation was performed at 65°C in a moist chamber humidified with 0.3 M NaCl, 0.03 M Na citrate. 0.1 ml of DNA solution was added to the chromosome preparation. After incubation, the cover slips were washed and dehydrated and afterwards they were covered with Kodak AR10 stripping film for radioautography. Before being stored at 4°C the covered slides were exposed to vapours of hydrogen peroxide to eradicate background.

Other methods

The size distribution of DNA molecules was analyzed electrophoretically on 1% agarose gels as described (13). Fragments of $\phi$29 DNA (kindly supplied by Dr. C. Escamís) prepared by digestion with EcoRI restriction endonuclease, were run in parallel as size markers. Proteins were analyzed by sodium dodecyl sulfate/polyacrylamide gel electrophoresis according to Laemmli (19). Protein determination was accomplished using the method of Lowry et al (20).

RESULTS

Binding of microtubule associated proteins (MAPs) to chromatin

In previous reports (4-6) we have described the interaction of MAPs with DNA, suggesting that it could explain the interaction between chromosome and microtubule. However the chromosome is composed of proteins and DNA, therefore, chromosomal proteins could also be implicated in the chromosome-microtubule interaction. To test the influence of chromosomal proteins in the binding of MAPs to DNA we have obtained sheared mouse chromatin by digestion of nuclei with micrococcal nuclease, and tested its binding to MAPs. Figure 1a shows the sedimentation profile of the mouse nuclei digest, on a sucrose gradient. The size of the DNA from the different gradient fractions and the protein composition of sheared chromatin were determined by electrophoresis (Fig. 1b and 1c). Fractions corresponding to mono, di, ..
Fig. 1. Binding of microtubule protein to chromatin. 32p- or 3H-labelled mouse cell nuclei, at a DNA concentration of 1.5 mg/ml (30 A260 units/ml), were digested with micrococcal nuclease (10 units/ml). The solubilized chromatin fragments were centrifuged as indicated in Methods. The sedimentation pattern is shown (a). The size of the DNA from the different gradient peaks of Fig. 1a, was analyzed electrophoretically on 1% agarose gels (b). EcoRI digestion fragments from phage Φ29 DNA were used as size markers. Protein composition (c) of the fractions from the gradient was analysed by electrophoresis on 12% acrylamide gels as described (22). Fig. 1d shows the binding of tubulin (△–△), HMWs (●–●), MAP2 (○–○) and tau factor (▲–▲) to sheared chromatin (0.5 μg of DNA) characterized as indicate in the top of this figure. Electrophoresis gels indicate the polypeptide composition of the preparations of tubulin (1), HMWs (2), MAP2 (3) and tau factor (4) used in the binding assay.
and heptanucleosomes were filtered on nitrocellulose membranes to test whether or not they are quantitatively retained. Our results indicate a 20% retention for every fraction tested, which allowed us to use the nitrocellulose binding assay to analyze the interaction of sheared chromatin with MAPs. Our previous results suggested that high molecular weight MAPs, essentially MAP2, showed a preferential binding to specific DNA sequences (4) while tau factor did not. Similar results were obtained when the binding to chromatin of HMWs, MAP2, tau factor and tubulin was tested (Fig. 1d). This figure suggests a preferential binding of MAP2 to certain regions of sheared chromatin, an unspecific binding of tau factor to the whole chromatin and the absence of binding of tubulin to chromatin. From the results of Figure 1 we conclude that chromosomal proteins do not substantially alter the interaction of MAPs with DNA. In a previous report (4) we have focused our study on the interaction of high molecular weight MAPs (HMWs) to DNA, because it appears to be preferential for certain sequences, as also confirmed above. Our results have indicated that such DNA regions could be enriched in satellite DNA (6) when mouse DNA was used. However from such results it was difficult to conclude whether satellite regions contains, exclusively, the DNA sequences whose interaction with HMWs is preferential.

HMWs bind preferentially to certain DNA sequences

To test whether there is one or several, non clustered, DNA sequences in mouse DNA to which high molecular weight microtubule associated polypeptides bind preferentially, we have digested high molecular weight mouse DNA with EcoRI restriction nuclease and have fractionated the resultant fragments by electrophoresis. Botchan et al (12) have previously shown that EcoRI digested mouse DNA results in different sized fragments, and that the larger ones (about 10 millions of molar mass) are enriched in satellite DNA. Figure 2 shows a size distribution for DNA fragments similar to that reported by Botchan et al. for EcoRI digested mouse DNA. We have extracted the DNA from different gel electrophoresis fractions and used it to test its binding to HMW polypeptides, tau factor and tubulin. The result in
Fig. 2. Length distribution of the DNA fragments obtained by digestion of mouse 32P-DNA with EcoRI, after electrophoresis in agarose gels. 217 µg of 32P-labelled mouse DNA were digested with EcoRI nuclease for 3 h at 37°C and the digested mixture layered onto a 0.3% agarose gel and electrophoresed, as indicated in Methods. DNAs of phages λ, Φ29 and EcoRI Φ29 DNA fragments were used as markers of molar mass. The agarose gel was cut into 4 mm slices and the DNA extracted from the gel. A fraction of the extracted DNA was used to determine radioactivity (o—o) and the rest was sheared by passing it through a 20 gauge needle to be used for binding experiments. The binding test of (●—●) HMW polypeptides, (▲—▲) tau factor and (△—△) tubulin to DNA fragments, was done in 0.02 M Tris-HCl (pH 7.0), 0.05 M NaCl, using a constant amount of protein (0.3 µg for HMW and 5 µg for tau factor and tubulin) and DNA (0.1 µg) per assay.

Figure 2 indicates a preferential binding of HMW polypeptides, or MAP2 alone (not shown), to the slow migrating DNA fragments, a similar binding of the tau factor to the different DNA fractions and the absence of binding of tubulin to DNA. These results confirm the previous report of a preferential binding of HMW polypeptides to certain eukaryotic DNA regions and support the hypothesis that HMW polypeptides (6), possibly MAP2 (4), could be the bridge between the chromosome and the microtubule.
in mitosis, through their binding to centromeric DNA.

**Partial isolation and characterization of mouse DNA sequences showing a preferential affinity to microtubule protein.**

We and others have indicated (4,7) that the addition of DNA to microtubule protein results in a decrease of its ability to polymerize. This has been interpreted as a trapping of MAPs by the nucleic acid, which results in an increase of the critical concentration that tubulin requires to polymerize. It is not known whether after addition of DNA, the nucleic acid remains associated to the assembled microtubules or not. To test it we have polymerized microtubules in the presence of labelled mouse DNA through successive cycles. About 43%, 14% and 6% of the total labelled mouse DNA added is found in the pellet of the first, second and third polymerization cycle respectively. The high proportion of DNA found in the first pellet could be due to a preferential but not exclusive binding of HMWs to certain DNA sequences, as previously shown in Figure 2, and to the existence in the microtubule solution of other proteins as, for example, tau factor, which bind without any specificity to the whole DNA (see Figure 2 and (4)).

Figure 3 shows the ratio of DNA to protein found in the pellet fraction after successive polymerization cycles. The DNA/protein ratio decreased gradually until a plateau was reached. The figure shows that, when the nucleic acid is in a mass ratio to protein of about 1/150, the nucleic acid behaves as a microtubule associated molecule, pelleting at a constant ratio to microtubule protein. On the other hand, the decrease in the amount of DNA associated to microtubules after several polymerization cycles could suggest an increase in the specificity of the bound nucleic acid. We have isolated the protein-bound DNA to characterize it. Microtubules assembled in the presence of DNA (3 cycles, Figure 3), were depolymerized and the nucleic acid-protein mixture was filtered through nitrocellulose membranes to measure the amount of DNA associated to protein. About 85% of the nucleic acid was retained on the filter, indicating that DNA is bound to protein in that proportion. This result was confirmed when the protein-DNA mixture was chromatographed on a Sepharose
Fig. 3. Amount of mouse DNA found in microtubules pellets after successive polymerization-depolymerization cycles. a) 15.5 mg of microtubule protein was mixed with 0.26 mg of $^{32}$P-labelled mouse DNA (1500 cpm/μg). The mixture was polymerized-depolymerized for several times. After each polymerization step the pellet was resuspended and the protein content and radioactivity associated was determined. From these data the DNA/protein ratio in each pellet was calculated. The fraction of the genome bound to microtubule pellet was 43%, 14%, 6%, 4.5%, 3.9% and 3.2% after the first, second, third, fourth, fifth and sixth polymerization cycles respectively.

4B column. The nucleic acid, along with protein, was excluded from the column, while DNA was included in the column, in the absence of protein (not shown).

To determine whether or not the interaction microtubule protein-DNA takes place through certain DNA fragments, we mixed the excluded material from the Sepharose 4B chromatography with a large excess of cold φ29 phage DNA, to compete with the labelled mouse DNA for the microtubule protein. The mixture was filtered through nitrocellulose membranes and the labelled DNA, retained on the filter, characterized on the basis of its reassociation kinetics.

On the other hand, we have isolated the sonicated mouse
DNA bound to purified MAP₂ by filtration on nitrocellulose membranes. This DNA was also characterized by its reassociation kinetics. Figure 4 shows the reassociation analysis of the mouse DNA fragments bound to microtubule protein, the sonicated mouse DNA bound to MAP₂ and the whole mouse DNA used as a control. In the figure we can see an enrichment in repetitive sequences of the DNA fragments that interact with microtubule protein or MAP₂, as compared with the total mouse DNA.

The result of Figure 4 suggests that microtubule protein interact preferentially with repeated sequences. We have localized these repeated sequences by in situ hybridization on metaphase chromosomes. Figure 5 indicates that these sequences are preferentially localized on the centromeric region of the metaphase chromosome. We have analysed the chromosomes containing silver grains and have observed that, from 844 chromosomes counted, 506 contained silver grains over the pericentromeric region. Preliminary data suggests a similar distribution when the sonicated DNA bound to MAP₂ was used.

Fig. 4. Kinetics of renaturation of the DNA which preferentially binds to MAP₂. Total mouse DNA fragments (○—○), DNA fragments which remains associated to microtubule protein after several polymerization cycles (■—■) and DNA fragments which preferentially bind to MAP₂ (△—△) were denatured and reassociated as indicated in Methods.
Fig. 5. Autoradiograph of spread mouse metaphase chromosome preparation after cytological hybridization with $^3$H-labelled mouse DNA fragments isolated as indicated in Methods. Nucleic acid reassociation was done until a Cot/2 of about 0.8 mol x 6/s/liter, (10 µg of DNA and 72 hours of incubation time). This value of Cot/2 is above the calculated Cot/2 for a homogeneous nucleic acid fragment with an average molar mass of $3 \times 10^6$, size of purified DNA fragments.

DISCUSSION

Our results indicate a preferential binding of isolated high molecular weight polypeptides or purified MAP$_2$, to certain regions of mouse DNA. These DNA fragments are enriched in repetitive sequences localized, primarily, in the centromeric region of the chromosome. Binding of HMW polypeptides and MAP$_2$ protein to sheared chromatin was found to be similar to that of DNA previously described (4-6), indicating that some chromosomal polypeptides, like histones, do not affect the interaction of HMW polypeptides or MAP$_2$ to DNA. These results support the previous model for chromosome-microtubule interaction involving the direct binding of HMW polypeptides, possibly MAP$_2$, to the DNA present in the kinetochore (4-6). Such a model, that implicates a
direct binding of microtubule protein to DNA, is not consistent with two observed facts: 1) the inhibitory effect of DNA on microtubule polymerization (4,7) and 2) the microtubule assembly on chromosomes (1,2). To overcome this apparent contradiction we suggest one or several possibilities: a) The implication of some chromosomal proteins such as histones (21), in the polymerization process without interfering in the DNA-microtubule protein binding, b) the existence of certain DNA sequences showing little or no effect on the inhibition of microtubule polymerization (4), c) the existence of enough DNA to allow the interaction with the microtubule but small enough to avoid microtubule polymerization, as suggested in Figure 3, d) if MAP₂ or related proteins are involved in the DNA-microtubule binding process, and also in reducing the critical concentration that tubulin requires to polymerize, these proteins could contain two or more DNA binding domains. One of them could be responsible for the binding to tubulin, and the addition of nucleic acid would result in a competitive inhibition. The other protein domain(s) would bind only to DNA, so that the nucleic acid could bind to it without interfering with the binding of the protein and tubulin.

In this report we have explored some of these possibilities. Our results indicate that histones do not interfere the binding of microtubule protein to DNA. Histones could not be implicated alone in the polymerization of microtubules on chromosomes, since such assembly is site specific and histones are localized along the whole chromosome. Possibilities b), c) and d) could be related if the domain of the microtubule protein (MAP₂), which interact preferentially with certain DNA sequences, is not the same as the protein domain which binds to tubulin. Preliminary results in our laboratory suggest the existence of such different protein domains in MAP₂. This could explain the apparent contradictory effects of DNA on microtubule binding and on microtubule polymerization. Moreover, the results showed in this report indicate the possibility of interaction of microtubules to certain DNA regions enriched in repetitive DNA sequences and localized preferentially in centromeric zones.
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*Present address: The Biological Laboratories, Harvard University, Cambridge, MA 02138, USA

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