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Erythrocyte protein 4.1 associates with tubulin

Isabel CORREAS* and Jesús AVILA
Centro de Biología Molecular (CSIC-UAM), Universidad Autónoma, Canto Blanco, 28049 Madrid, Spain

Protein 4.1 binds to tubulin, as determined by sedimentation and immunoelectron-microscopy analyses, at a molar ratio similar to that described for brain microtubule-associated proteins. The binding site appears to be located at the *C*-terminal region of tubulin. Experiments performed *in situ* by adding exogenous protein 4.1 to permeabilized 3T3 cells show that this protein binds to microtubule and nuclear components.

INTRODUCTION

Protein 4.1 is a cytoskeletal protein originally identified in non-nucleated cells, and for many years it was considered exclusive to erythrocytes. This protein plays an important role in the organization of the erythrocyte cytoskeleton, as it strengthens the association between two other cytoskeletal proteins, spectrin and actin [1], and links the spectrin–actin network to the plasma membrane by interactions with two glycoproteins, glycophorin [2] and band 3 [3]. The functional domains of protein 4.1 involved in these interactions have been identified. An 8 kDa peptide located within the α-chymotryptic 10 kDa domain of protein 4.1 is responsible for spectrin–F-actin associations [4], and its complete amino acid sequence has been reported [5]. The 30 kDa *N*-terminal domain links protein 4.1 to the membrane [6]. The involvement of protein 4.1 as a stabilizer of the membrane skeleton is further supported by the facts that defective binding of protein 4.1 to spectrin [7] and protein 4.1 deficiency [8,9] are associated with different types of haemolytic anaemia.

The interest in protein 4.1 and its role in non-erythroid cells has increased notably since immunoreactive forms of protein 4.1 have been identified in fibroblasts, platelets, neutrophils, monocytes, neurons and lenticular and endothelial cells [6,10]; however, little is known about its role in these cells. It has recently been found that antibodies raised against different functional domains of the human erythroid protein 4.1 stain the mitotic spindle of cells in division, suggesting an interaction between protein 4.1 or immunologically related proteins and microtubules (I. Correas, R. A. Anderson, C. W. Mazzucco, W. Knowles & V. T. Marchesi, unpublished work).

In this work we have studied whether this association takes place through the major component of microtubules, tubulin.

MATERIALS AND METHODS

Microtubule protein was obtained from porcine brain by two temperature-dependent assembly–disassembly cycles [11] and stored as pellets at −70 °C. Immediately before use, the microtubule pellets were resuspended in 0.1 M-Mes/0.5 mM-$MgCl_2$/2 mM-EGTA/1 mM-GTP, pH 6.4 (buffer A) and a third cycle of assembly–disassembly was performed. Tubulin depleted of microtubule-associated proteins (MAPs) was obtained by phosphocellulose chromatography as described by Weingarten *et al.* [12]. The protein concentration was determined from A_{280} as 1.15 mg/ml [13]. Tubulin was digested for 30 min at 30 °C with subtilisin at an enzyme-to-substrate ratio of 1:100, as previously indicated [14].

Protein 4.1 was obtained from porcine erythrocytes by the method described by Tyler *et al.* [15], with slight modifications [16]. The method involved a high salt extraction of spectrin-depleted erythrocyte membranes, followed by ion-exchange chromatography of the extract. Protein 4.1 was biotinylated following the method of Stähli *et al.* [17]. Affinity-purified polyclonal antibodies against human erythrocyte protein 4.1 were used in some experiments. These antibodies have previously been characterized [5].

Tubulin or subtilisin-digested tubulin was polymerized at 37 °C for 20 min in the presence of 10 μM-taxol. Protein 4.1 was added to either of these proteins and incubated at 37 °C for 20 min. In some experiments, isolated high M_r MAPs were also added. Samples were applied to a 0.1 ml cushion of 10% sucrose in buffer A and centrifuged at room temperature for 5 min at 150000 *g* in a Beckman Airfuge. Supernatant and pellet protein fractions were analysed by electrophoresis on SDS/polyacrylamide slab gels [18].

To measure the amount of either tau factor, albumin or protein 4.1 bound to microtubules, increasing amounts of those proteins (1 to 10 μg) were mixed with 50 μg of tubulin in a final volume of 30 μl. Taxol was added to a final concentration of 10 μM. After 20 min at 30 °C the mixture was centrifuged on a carbon/collodion-coated grid in an EM-90 Beckman Airfuge rotor for 2 min at maximum speed. The grid was washed for 10 min in 0.2% glutaraldehyde in buffer A, followed by an incubation in 0.4% glycine in buffer A for 10 min. Polyclonal antibodies against erythrocyte protein 4.1, tau factor or bovine serum albumin (BSA) were added at 1:100 dilution for 1 h. Protein A labelled with 12 μm gold particles was added at 1:100 dilution for 30 min, and samples were analysed in a Jeol 100B electron microscope.

Cells grown on coverslips were permeabilized and

Abbreviations used: MAPs, microtubule-associated proteins; PBS, phosphate-buffered saline; BSA; bovine serum albumin.

* To whom reprint requests should be sent.

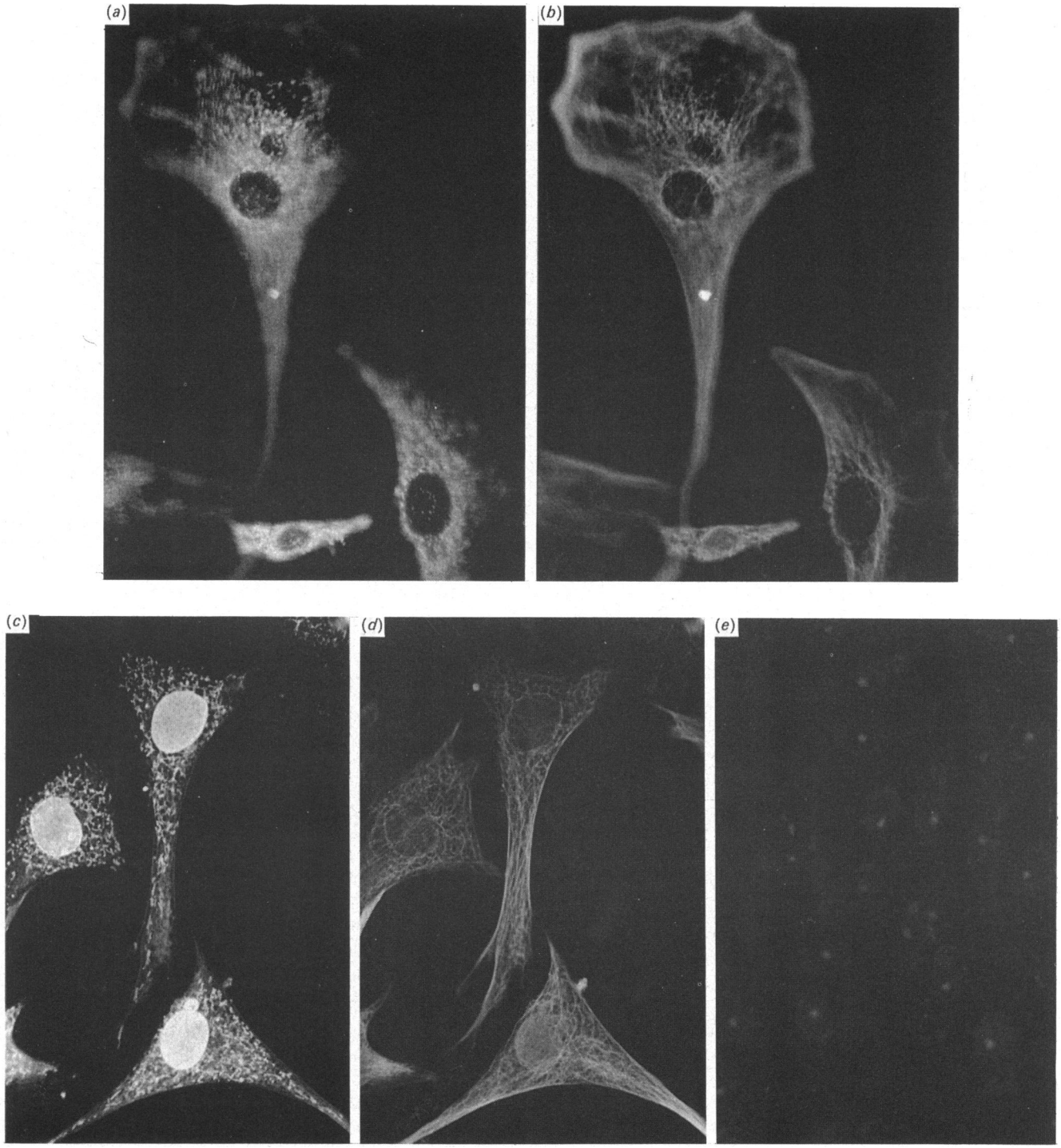

Fig. 1. Binding of exogenous protein 4.1 to 3T3 microtubules

Permeabilized 3T3 cells were double stained with biotinylated protein 4.1 (*a*), biotinylated MAP2 (*c*) or biotinylated BSA (*e*) followed by fluorescein-conjugated avidin, and with a monoclonal anti-tubulin antibody, YL1/2, followed by rhodamine-conjugated goat anti-(rat IgG) antibody (*b* and *d*).

fixed with methanol at −20 °C for 15 min, extensively washed with phosphate-buffered saline (PBS) and treated with 0.2% Triton X-100 in PBS for 10 min at 0 °C. Biotinylated 4.1, MAP2 or BSA proteins were added to the cells in PBS/1% BSA for 1 h, followed by a fluorescein-conjugated avidin solution (Sigma, St. Louis, MO, U.S.A., 1:200 dilution), which was incubated for 30 min at room temperature. Cell microtubules were located by incubation with a rat monoclonal anti-tubulin antibody, YL1/2 (Sera Laboratories, Sussex, U.K.; 1:100 dilution) for 1 h and then with a secondary rhodamine-conjugated goat anti-(rat IgG) antibody (Cappell Laboratories, Cochranville, PA, U.S.A.; diluted 1:200) for 1 h. Between each incubation, cells were washed extensively with PBS.

Immunoblot analyses of proteins transferred from

SDS/polyacrylamide gels to nitrocellulose sheets were carried out following the method described by Towbin *et al.* [19].

RESULTS AND DISCUSSION

Binding of protein 4.1 to cell microtubules was tested by experiments *in situ* on fixed 3T3 cells after 0.2% Triton X-100 treatment. An overlay technique was performed using biotin-labelled protein 4.1 and staining the biotin–4.1 complex with fluorescein-conjugated avidin. This method will only detect exogenous biotinylated protein. Using this procedure, exogenous protein 4.1 was bound to cytoplasmic filamentous structures which were identified as microtubules by a double-immunofluorescence assay using an anti-(tubulin) antibody (Figs. 1*a* and *b*). Protein 4.1 was also found in the nucleus as punctuate staining. To test for the presence and localization of proteins immunologically related to 4.1 in the cell, polyclonal anti-(4.1) antibodies were used to stain 3T3 cells. The fluorescent distribution pattern observed was similar to that described above for exogenous protein 4.1. It is of interest that protein 4.1 contains a sequence, KKKRERLD [5], which correlates with a kariophylic signal previously found in other nuclear proteins [20]. Since this sequence may be present in the immunologically related 4.1 proteins endogenously found in 3T3 cells, we used an antibody against a synthetic peptide which contained this sequence [5]. In this case, nuclear staining was also observed (results not shown).

Since a similar fluorescent distribution pattern of both intranuclear speckles and cytoplasmic and mitotic microtubules has been described for some MAPs [21], we have tested by the method described above the binding of an authentic MAP (brain MAP2), as a positive control and that of albumin, a protein that is unable to bind to microtubules (negative control). Fig. 1(*c*) shows the interaction of MAP2 together with the immunofluorescence pattern obtained when an anti-(tubulin) antibody was used (Fig. 1*d*). As indicated in Fig. 1(*c*), MAP2 was bound to cytoplasmic structures (microtubules) and to the nucleus. The latter interaction may be due to the reported interaction of MAP2 with DNA [22]. Finally, in Fig. 1(*e*) it is shown that biotinylated BSA does not bind to cytoplasmic filaments. Given this noteworthy similarity in the distribution of protein 4.1 and MAPs, we analysed whether protein 4.1 binds to the major component of microtubules, tubulin, as MAPs do. When purified porcine erythrocyte protein 4.1 was incubated with taxol in the absence of porcine brain tubulin, protein 4.1 remained primarily in the supernatant fraction (Fig. 2, lane 1). In the presence of MAPs-depleted tubulin and of taxol, both proteins 4.1 and tubulin copolymerized and were found in the assembled protein pellet (Fig. 2, lane 2). By densitometry analysis, we found that 1 mol of protein 4.1 bound to 6 mol of tubulin, a result in the same range as those described for MAPs [23].

The binding of protein 4.1 to tubulin was also demonstrated by immunoelectron-microscopy analysis after polymerization of MAPs-depleted tubulin in the presence of purified protein 4.1. Increasing amounts of protein 4.1 or tau factor, a MAP with a molecular mass related to protein 4.1, were mixed with 50 μg of tubulin and the mixture was incubated in the presence of taxol to polymerize microtubules. To test the binding of the

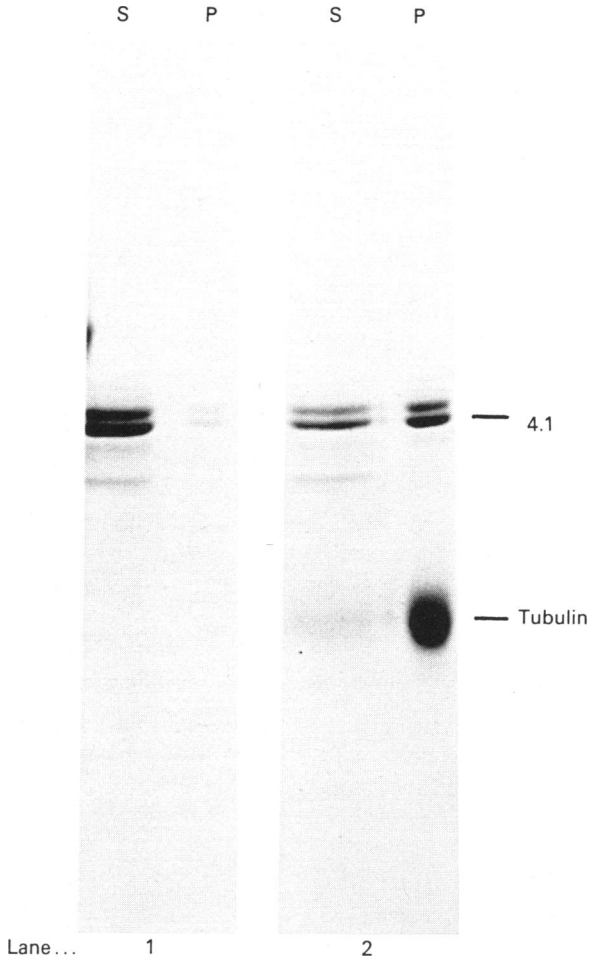

Fig. 2. Sedimentation analysis of protein 4.1 binding to tubulin

MAPs-depleted tubulin (30 μg) was polymerized for 20 min at 37 °C in the presence of 10 μM-taxol. Protein 4.1 (7 μg) was added and incubated for 20 min at 37 °C, followed by sedimentation as described in the Materials and methods section. Supernatant protein fractions (S) and pellets (P) were electrophoresed on SDS/7.5% polyacrylamide gels. Protein 4.1 alone (lane 1), protein 4.1 and tubulin (lane 2).

putative MAPs to microtubules, antibodies against protein 4.1 or tau factor were added and afterwards the preparation was incubated with Protein A–colloidal gold particles (an example is given in Fig. 3). Since an excess of antibodies and protein A were used, the number of gold particles will increase with the amount of bound MAP. We found that it was the case for protein 4.1 that reaches a maximum of 12 ± 2 gold particles/0.1 μm. This value was reached by adding 7 μg of protein 4.1. Fig. 3 also shows the result obtained for tau. In that case, a maximum of 17 ± 4 gold particles/0.1 μm was obtained. This value was reached by adding 4 μg of tau factor. The distribution pattern of protein 4.1 on assembled tubulin resembles that of MAP2 and tau [24].

MAP2 and tau bind to the *C*-terminal end of tubulin [25,26]. Experiments performed with subtilisin-cleaved tubulin, which lacks the 4 kDa *C*-terminal peptide, have shown that the binding of MAP2 and tau to this molecule is almost completely abolished [25]; we have obtained similar results when using protein 4.1 (Fig. 4).

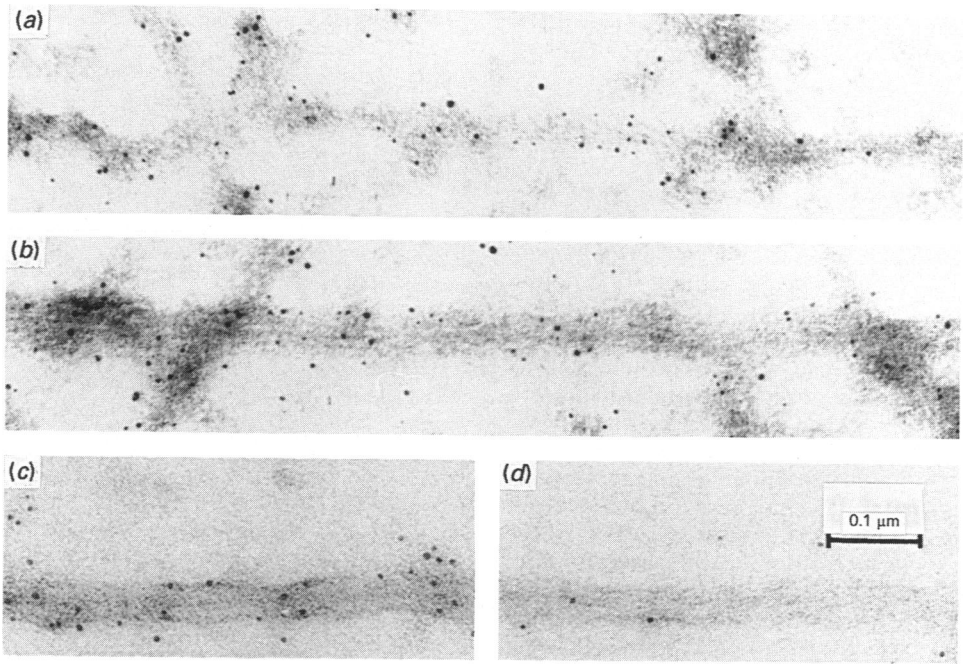

Fig. 3. Immunoelectron-microscopy analysis of protein 4.1 binding to tubulin

Protein samples prepared as described in the Materials and methods section were layered on a grid and incubated with polyclonal antibodies against either protein 4.1 (*a* and *b*) or tau (*c*) for 1 h. Protein A labelled with 12 μm gold particles was then added and incubated for 30 min. (*d*) represents a negative control where no antibodies were added.

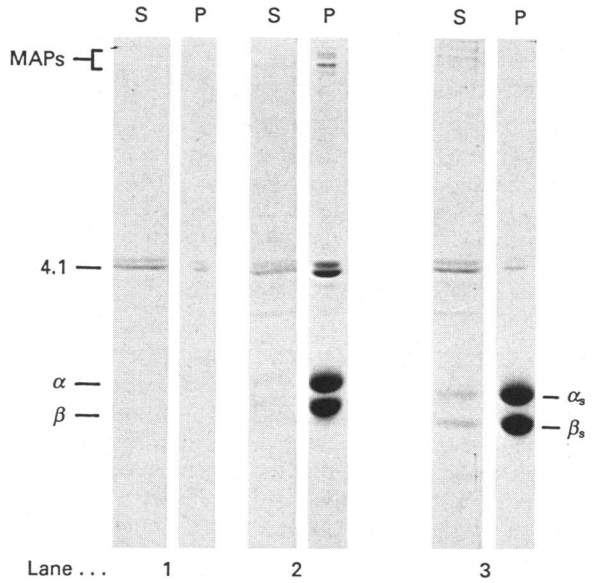

Fig. 4. Incorporation of protein 4.1 into microtubules obtained from digested and undigested tubulin depleted of MAPs

Undigested tubulin (30 μg) and subtilisin-digested tubulin (30 μg) were incubated separately for 20 min at 37 °C in the presence of 10 μM-taxol in buffer A. A mixture of high-molecular-mass MAPs (16 μg) and protein 4.1 (7 μg) was added and incubated for 20 min at 37 °C. Samples were analysed by the sedimentation assay described in the Materials and methods section. Supernatant (S) and pellet (P) fractions were examined on SDS/7.5% polyacrylamide gels. α and β indicate alpha and beta subunits of undigested tubulin. α_s and β_s indicate alpha and beta subunits of subtilisin-digested tubulin.

When protein 4.1, high-M_r MAPs and intact tubulin were incubated together under the conditions described in Fig. 2, both protein 4.1 and MAPs cosedimented with tubulin with similar molar ratios (Fig. 4, lane 2). When subtilisin-digested tubulin was added to protein 4.1 and to high-M_r MAPs, both protein 4.1 and MAPs remained primarily in the supernatant fractions (Fig. 4, lane 3). Protein 4.1 alone also remained in the supernatant fraction (Fig. 4, lane 1). This suggests that the tubulin-binding site for protein 4.1 is located at the *C*-terminal end of the molecule, where MAPs do interact.

Our experiments *in vitro* showing an interaction between purified protein 4.1 and tubulin are consistent with the presence of immunoreactive forms of protein 4.1 associated with spindle microtubules in non-neural cells (I. Correas, R. A. Anderson, C. W. Mazzucco, W. Knowles & V. T. Marchesi, unpublished work) and with brain microtubules. These findings suggest a role for protein 4.1 or related proteins similar to that described for MAPs. Since the 4.1-immunoreactive forms are present mainly in interphase cell nuclei, we suggest that they bind to spindle microtubules after nuclear membrane breakdown, thus having a function similar to that of the spindle associated proteins.

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