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Binding of Heterochromatin Protein 1 to the Nuclear Envelope Is Regulated by a Soluble Form of Tubulin*

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We have previously shown that the mouse heterochromatin protein 1 homologue M31 interacts dynamically with the nuclear envelope. Using quantitative *in vitro* assays, we now demonstrate that this interaction is potently inhibited by soluble factors present in mitotic and interphase cytosol. As indicated by depletion and order-of-addition experiments, the inhibitory activity co-isolates with a 55-kDa protein, which binds avidly to the nuclear envelope and presumably blocks M31-binding sites. Purification of this protein and microsequencing of tryptic peptides identify it as α2/6:β2-tubulin. Consistent with this observation, *bona fide* tubulin, isolated from rat brain and maintained in a nonpolymerized state, abolishes binding of M31 to the nuclear envelope and aborts M31-mediated nuclear envelope reassembly in an *in vitro* system. These observations provide a new example of "moonlighting," a process whereby multimeric proteins switch function when their aggregation state or localization is altered.

Heterochromatin protein 1 (HP1)[1] represents the founding member of a large protein family, which includes the *Polycomb* group and other gene regulators (for recent reviews see Refs. 1 and 2). This molecule possesses a dimeric, quasi-symmetrical structure and contains two sequence-related and similarly folded domains: the N-terminal chromodomain (3, 4), and the C-terminal chromo shadow domain (5). These two domains consist of anti-parallel, three-stranded β-sheets packed against one or two α-helices and are separated from one another by a flexible hinge region. Dimerization of HP1 involves intermolecular interactions between chromo shadow domains that tether two polypeptide chains at their C-terminal ends but leave the chromodomains unconstrained (6, 7).

A single HP1 species was originally identified in *Drosophila melanogaster* (8). However, subsequent studies have revealed multiple variants of this protein in higher eukaryotes. Mammalian HP1 includes three distinct isotypes termed hHP1α, β, and γ in humans and mHP1α, M31, and M32 in mice (3, 9–11). Although these proteins are structurally similar, they are distributed in different territories of the cell nucleus (12–15).

HP1 binds to several chromatin-remodeling factors and transcriptional regulators. Among these are CAF-1, BRG1/SNF2β, and the transcriptional intermediary factors α and β (7, 10, 15, 16). Physical or spatial associations between HP1 and elements of the origin recognition complex, actin-related proteins (Arp4), and SET or chromodomain-containing proteins, such as Su(var)3–9 and Su(var)3–7 have also been described (14, 17–19). Finally, interactions with the centromeric protein inner centromere protein, the nuclear autoantigen SP100, and the inner nuclear membrane protein LBR have been reported recently (10, 11, 20–23).

The interactions between HP1 proteins and elements of the nuclear envelope are particularly intriguing. First, peripheral heterochromatin is in physical contact with the inner nuclear membrane during interphase and could be directly linked to the nuclear envelope; second, transient associations involving components of condensed chromatin and inner nuclear membrane proteins are thought to provide the basis for nuclear envelope reassembly at the end of mitosis. In previous studies (24) we have found that all three variants of mouse HP1 target the nuclear periphery when injected into living cells and bind to isolated nuclear envelopes under *in vitro* conditions.

To further explore these interactions, we focused on M31. In this work, we demonstrate that *in vitro* binding of M31 to isolated nuclear envelopes is potently inhibited by soluble factors present in mitotic and interphase cytosol. Using biochemical methods, we have characterized this inhibitory activity as a soluble form of α2/6:β2-tubulin. The functional implications of these findings are discussed below.

EXPERIMENTAL PROCEDURES

Antibodies and Plasmids—A previously characterized monoclonal antibody directed to M31 (MAC 353; Refs. 13 and 14) was used throughout this study. Fusion proteins were detected by polyclonal, affinity-purified antibodies against recombinant glutathione *S*-transferase (GST). Anti-tubulin antibodies were obtained from Sigma. The characterization of anti-lamin B and anti-LAP2B antibodies has been reported previously (25, 26). M31 (full length) was expressed as a fusion protein with GST using pGEX1 and as a His_6-tagged protein employing pET-25b.

Cell Culture—Human endometrial carcinoma cells (Ishikawa) were maintained in minimum Eagle's medium, whereas HeLa cells and Chinese hamster ovary cells were grown in Dulbecco's modified Eagle's medium. All media contained 10% fetal bovine serum and antibiotics. Synchronization in mitosis was achieved by treating the cells with 40–120 ng/ml nocodazole for 18 h.

Indirect Immunofluorescence and Immunoblotting—Conventional

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[1] The abbreviations used are: HP1, heterochromatin protein 1; GST, glutathione *S*-transferase; PMSF, phenylmethylsulfonyl fluoride; DTT, dithiothreitol; SDS-PAGE, SDS-polyacrylamide gel electrophoresis; Pipes, 1,4-piperazinediethanesulfonic acid; NE, nuclear envelope vesicle; LBR, lamin B receptor.

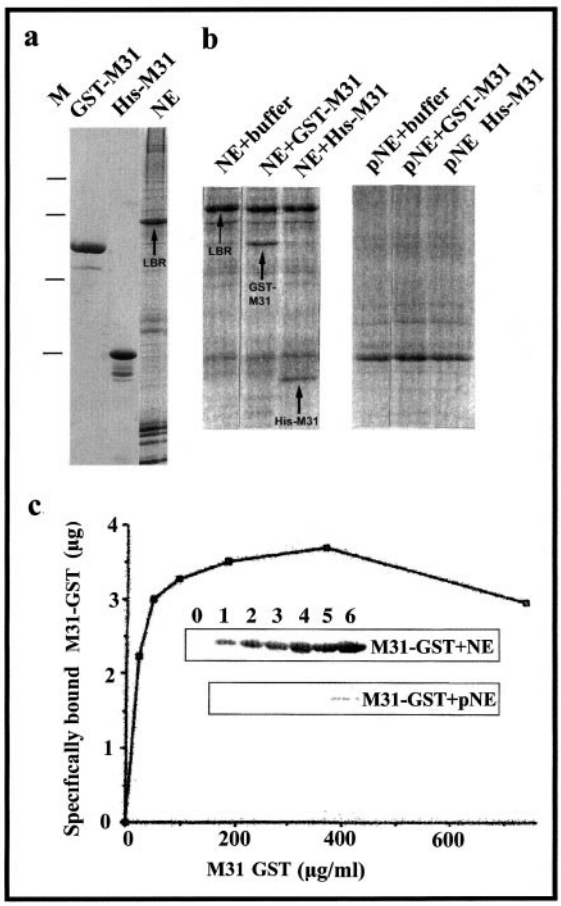

FIG. 1. **Assay system.** *a*, SDS-PAGE profiles of urea-stripped turkey erythrocyte nuclear envelopes (25 μg) and recombinant M31 (*GST-M31*, *His-M31*; 5 μg) used in *in vitro* binding assays. The *arrow* points to LBR, the major integral protein of the inner nuclear membrane. *Bars* denote molecular weight values of 97,000, 68,000, 45,000, and 31,000. *M*, molecular weight markers. *b*, binding of recombinant M31 to the nuclear envelopes, as detected by SDS-PAGE and Coomassie Blue staining (only the relevant area of the gels is shown). The positions of LBR, GST-M31, and His-M31 are indicated by *arrows*. The input of M31 and nuclear envelopes was as in *a*. For more details see "Experimental Procedures." *pNE*, proteolyzed NE. *c*, binding of increasing amounts of GST-M31 to intact (NE) and proteolyzed nuclear envelopes, as detected by Western blotting. *Lane 1*, 0 μ g; *lane 2*, 0.5 μ g; *lane 3*, 1.0 μ g; *lane 4*, 2 μ g; *lane 5*, 4 μ g; *lane 6*, 8 μg. The blots were developed by alkaline phosphatase-conjugated secondary antibodies.

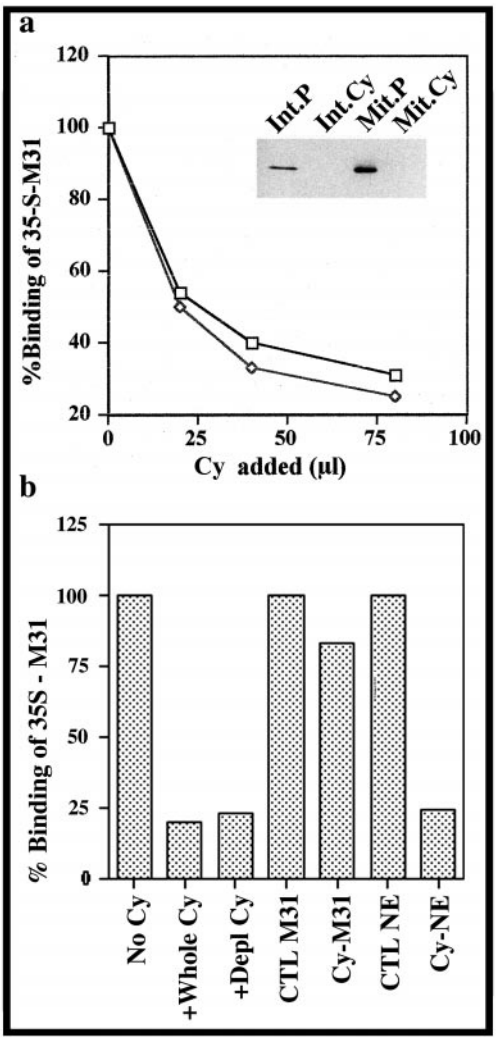

FIG. 2. **Cytosolic factors inhibit M31 binding to the nuclear envelopes.** *a*, relative binding of [^{35}S]M31-GST to nuclear envelopes in the presence of increasing quantities of interphase (*squares*) or mitotic (*diamonds*) cytosol. The protein concentration of the cytosol was 5 mg/ml; each assay mixture contained 40 μg of nuclear envelopes and 5 μg of [^{35}S]M31-GST in a reaction volume of 120 μl. When cytosol was omitted, the samples were supplemented with gelatin (5 mg/ml) to compensate for the absence of soluble protein. "100%" binding corresponds to a value of 30 μg of [^{35}S]M31-GST/mg of nuclear envelopes, *i.e.* about one-half of the maximal binding capacity under our standard assay conditions (65 μg of [^{35}S]M31-GST/mg of membranes). Each point represents averages of four independent observations, with variation not exceeding 10%. *Inset*, Western blot indicating the partitioning of endogenous M31 in mitotic and interphase cells. *Int.P*, insoluble material (100,000 $\times$ *g* pellet) from interphase cells; *Int.Cy*, soluble material (cytosol, 100,000 $\times$ *g* supernatant) from interphase cells; *Mit.P*, insoluble material from mitotic cells; *Mit.Cy*, soluble material from mitotic cells. *b*, binding of [^{35}S]M31-GST to the nuclear envelopes under various assay conditions. *No Cy*, no cytosol added; + *Whole Cy*, addition of whole cytosol (80 μl); + *Depl Cy*, addition of cytosol (80 μl; ~400 μg of protein) that had been pre-incubated with 2 mg of immobilized M31-GST; *CTL M31*, assay with [^{35}S]M31-GST, pre-incubated with gelatin/KHM and reisolated by affinity chromatography; Cy-M31, assay with [^{35}S]M31-GST pre-incubated with 80 μl of cytosol and reisolated by affinity chromatography; *CTL NE*, assay with nuclear envelopes pre-incubated with gelatin/KHM and washed with buffer; *Cy-NE*, assay with nuclear envelopes pre-incubated with cytosol and washed with buffer. The experiments were done in triplicate under the conditions specified in *a*.

and confocal immunofluorescence, as well as Western blotting, were performed as described previously (25, 27, 28). Staining of the cells with propidium iodide was accomplished after a 30-min incubation with 200 units/ml RNase A and subsequent incubation with 1 μg/ml of this dye for 5 min.

Expression, Purification, and Metabolic Labeling of Recombinant Proteins—GST fusion proteins and His_6-tagged polypeptides were expressed in BL21 (DE3) cells and purified from bacterial lysates according to standard procedures (29). For metabolic labeling, the cells were grown in methionine-free medium (M9-based) to an OD of 0.9. Isopropyl-1-thio-β-D-galactopyranoside (0.1 mM) and [^{35}S]methionine (200–300 μCi) were added, and incubation ensued for 3 h at 37 °C. After that, the bacteria were collected, and the recombinant proteins were purified as usual.

Preparation of Tubulin—Tubulin was isolated from rat brain tissue according to published methods (30).

Partial Purification of the 55-kDa Protein—To isolate the 55-kDa protein, 500 μl of a thick suspension of urea-extracted nuclear envelopes (concentration of 5–10 mg/ml in KHM buffer (78 mM KCl, 50 mM Hepes-KOH, pH 7.0, 4 mM $MgCl_2$, 8 mM $CaCl_2$, 10 mM EGTA, 1 mM DTT, 1 mM PMSF), 1% gelatin, and protease inhibitors) was combined with 700 μl of freshly prepared Ishikawa cytosol (5 mg/ml). After a 45-min incubation at room temperature, the membranes were pelleted (12,000 $\times$ *g*, 30 min, 4 °C) and resuspended in 800 μl of the same buffer. Following another centrifugation, the nuclear envelopes were eluted with 600 μl of buffer E (250 mM NaCl, 30 mM Tris-HCl, pH 8.0, 1 mM EDTA, 1 mM DTT, and 0.2 mM PMSF). The eluate collected was concentrated by ethanol precipitation or kept in its original state at −70 °C.

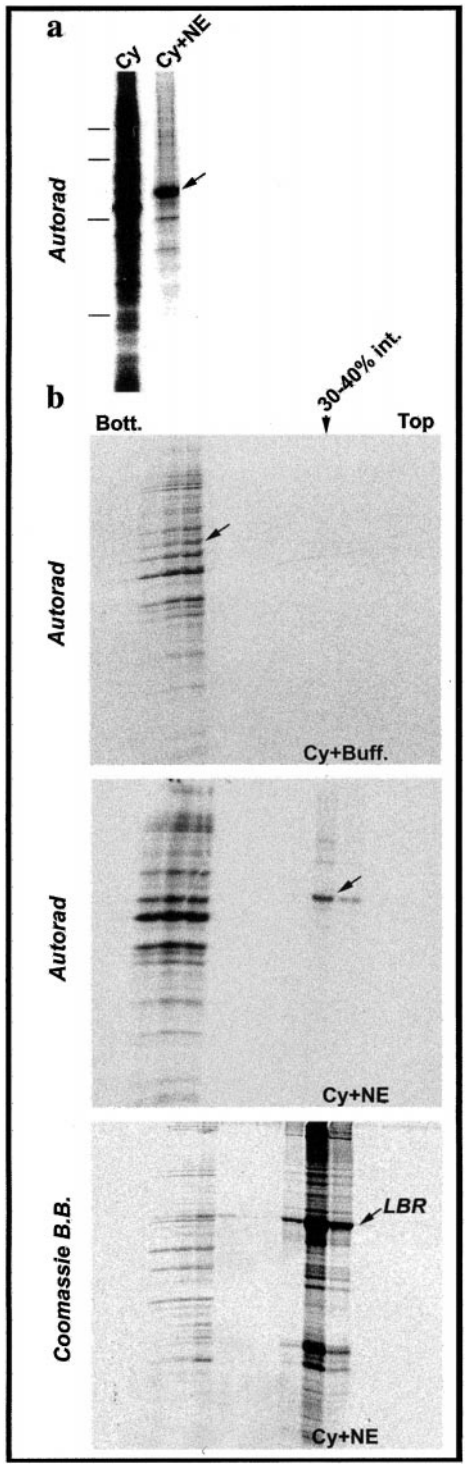

FIG. 3. **Binding of the 55-kDa protein to nuclear envelopes as detected by flotation in sucrose gradients and direct sedimentation assays.** *a*, direct sedimentation assays. One hundred μl of [^{35}S]methionine-labeled cytosol was co-incubated for 45 min with 100 μg of purified nuclear envelopes and centrifuged at 12,000 $\times$ *g*. One-quarter of the solubilized pellet (*Cy+NE*) and a sample of whole cytosol (*Cy*, 10 μl) were analyzed by SDS-PAGE and autoradiographed. The *arrow* indicates the position of the 55-kDa protein. *b*, flotation assays. One hundred μl of [^{35}S]methionine-labeled cytosol was mixed with an equal volume of KHM/1% gelatin or 100 μg of purified nuclear envelopes (1 mg/ml). Following a 45-min incubation, the samples were combined with 600 μl of 80% sucrose in KHM, loaded at the bottom of gelatin-coated tubes, and overlaid with sucrose (4 ml of 40%, 4 ml of 30%, and 3.2 ml of 20% in KHM). After ultracentrifugation (100,000 $\times$ *g*, 18 h, 4 °C) and collection of fractions, aliquots were analyzed by SDS-PAGE and autoradiography. *Autorad*, autoradiogram; *Cy+Buff.*, cytosol and buffer; *Cy+NE*, cytosol and nuclear envelopes; *Coomassie B.B.*, Coomassie Blue-stained gel. *Arrows* denote the position of the 55-kDa protein. The position of the major nuclear envelope protein LBR is also indicated. The *bottom* (*Bott.*) of the gradients is to the *left*, and the *top* is to the *right*. Note that all cytosolic proteins remain at the loading zone when the membranes are omitted. However, the 55-kDa protein migrates to the 30–40% sucrose interface when nuclear envelopes are included in the reaction. The membranes also float to the 30–40% sucrose zone, as documented in the Coomassie Blue-stained gel.

Isolation of Nuclear Envelopes—Turkey erythrocyte nuclear envelopes were isolated as specified in Ref. 31. The membranes were washed sequentially with 2 M KCl, 50 mM Tris-HCl, pH 7.5, 1 mM DTT, 1 mM PMSF, and protease inhibitors (2 μg/ml each leupeptin, pepstatin, aprotinin, and antipain); double distilled water; and 8 M urea, 10 mM Tris-HCl, pH 8.0, 4 mM EDTA, and 1 mM PMSF (when specified). Before use, nuclear envelopes were washed in assay buffer (see below) and thoroughly resuspended in the same buffer by mild sonication. To prepare proteolyzed membranes, 0.8–2.0 mg/ml of nuclear envelopes were incubated with a mixture of trypsin and chymotrypsin (0.16 mg/ml) for 30 min at room temperature. Digestion was stopped by adding PMSF (1.3 mM), protease inhibitors (see above), and 1% fish skin gelatin (scavenger). The membranes were washed with assay buffer containing PMSF/protease inhibitors/gelatin and resuspended in the same buffer.

Preparation of Cytosol—Cytosol was prepared from interphase or nocodazole-arrested Ishikawa cells according to the following method. The cells were detached by trypsinization, pelleted at 300 $\times$ *g*, resuspended in culture medium containing 20 μM cytochalasin B, and incubated at 37 °C for 45 min. After washing twice with phosphate-buffered saline and KHM buffer, the pellet was resuspended in one volume of KHM/cytochalasin B and Dounce-homogenized (300 strokes). The homogenate was ultracentrifuged at 100,000 $\times$ *g* for 1 h at 4 °C, and the supernatant (cytosol) was collected. When mitotic homogenates were prepared, 1 mM MgATP, 20 mM creatine phosphate, 400 μg/ml creatine kinase, 80 mM β-glycerophosphate, 50 mM NaF, and 1 μM microcystin LR were added to the media to preserve the mitotic state.

In Situ Assays—Unsynchronized cells grown on coverslips were washed three times with phosphate-buffered saline and permeabilized with 0.2% Triton X-100 in KHM buffer/protease inhibitors (5 min, room temperature). The cells were rinsed two times with KHM, blocked with KHM, 1% gelatin, 1 mM DTT for 10 min, and incubated with 10 μg of recombinant proteins for 30 min at room temperature. After rinsing with KHM, 0.02% Triton X-100, washing with KHM, 1% gelatin, 1 mM DTT (two times, 2 min), and rinsing again with plain KHM, the cells were fixed with 4% formaldehyde (5 min, room temperature) and processed for indirect immunofluorescence.

Binding Assays—All reactions were carried out in Eppendorf tubes coated with 1% boiled/filtered fish skin gelatin. 10–50 μg of nuclear envelopes were combined with increasing amounts of labeled or unlabeled M31-GST and M31-His$_6$ reconstituted in assay buffer (150 mM NaCl, 20 mM Tris-HCl, pH 7.4, 2 mM $MgCl_2$, 0.1 mM EGTA, 1 mM DTT, 0.2 mM PMSF, 10% sucrose, and 0.1% gelatin) and adjusted in volume to 100 μl. After a 45-min incubation at room temperature (mixing by rotation), the samples were spun in a Microfuge (12,000 $\times$ *g*, 30 min), and the pellets were washed with 300 μl of assay buffer. Following another centrifugation (15 min, in the same fashion), the supernatants were carefully aspirated, and the walls of the tubes were wiped with cotton swabs. The final pellets, representing nuclear membranes and associated material, were either solubilized in Laemmli buffer or dissolved in scintillation fluid. Binding was detected by SDS-PAGE/Western blotting (unlabeled proteins) or by β-counting (^{35}S-labeled probes). Quantitative measurements were always done in duplicate or triplicate.

In Vitro Reassembly Assays—Chinese hamster ovary cells were synchronized in mitosis with 120 ng/ml nocodazole (18 h, 37 °C). After shake-off, the cells were washed three times with cold Pipes buffer (50 mM Pipes-KOH, pH 7.4, 50 mM KCl, 5 mM $MgCl_2$, 2 mM EGTA, and 1 mM PMSF) and resuspended at a density of 10^6 cells/ml in the same medium plus 1 mM DTT and protease inhibitors (2 μg/ml each leupeptin, pepstatin, aprotinin, and antipain). Digitonin was added from a 10 mg/ml stock to a final concentration of 50 μg/ml, and the suspension was left on ice for 5 min. The lysate was divided in equal aliquots ($\sim$2 $\times$ 10^5 cells). One sample (control) was diluted to 300 μl with cold Pipes buffer and processed immediately after addition of 2 mM MgATP, 20 mM creatine phosphate, 400 μg/ml creatine kinase, 80 mM β-glycerophosphate, 50 mM NaF, and 3 μM microcystin LR. The rest were combined with various peptides or exogenous proteins (12–120 μg), adjusted in volume to 300 μl, and incubated at 33 °C for 2 h. Half of each reaction mixture was loaded onto a cushion of 20% sucrose and spun (4 °C) for 10

min at 1,000 × *g* on glass coverslips. Material adhering to glass was washed two times with cold Pipes buffer and fixed with 4% formaldehyde for 10 min. Replicas were stained with anti-LAP2B and anti-lamin B antibodies as specified above. Morphometric analysis involved detailed examination of at least 50 cells in the confocal microscope.

RESULTS

Cytosolic Factors Inhibit M31 Binding to the Nuclear Envelope—To measure binding of HP1 proteins to the nuclear envelope, we used a simple cosedimentation assay. Recombinant M31 (Fig. 1*a*, *GST-M31* or *His-M31*) was co-incubated with nuclear envelope vesicles (NEs) that had been stripped of peripheral proteins by urea extraction (Fig. 1*a*, *NE*; for more information see Refs. 31–33). As shown in Fig. 1, *b* and *c*, GST-M31 and His_6-M31 co-pelleted with intact NEs but did not bind to protease-digested membranes. Furthermore, as indicated by quantitative immunoblotting, the binding of M31-GST was concentration-dependent and saturable (Fig. 1*c*).

To identify cellular factors involved in M31-nuclear envelope interactions, we performed binding experiments in the presence and absence of cytosol. For higher sensitivity, in these experiments we used a metabolically labeled probe ([^{35}S]GST-M31) and worked under nonsaturating conditions (for details see "Experimental Procedures"). As shown in Fig. 2*a*, mitotic and interphase cytosol from Ishikawa cells inhibited M31 binding in a dose-dependent fashion. This inhibition was specific and did not occur when cytosol was substituted by concentrated solutions of bovine serum albumin or gelatin (see legends to Figs. 2 and 4*c*). Furthermore, the decrease of M31 binding did not reflect competition by endogenous M31 contaminating the cytosol preparations; as could be confirmed by Western blotting, the endogenous protein partitioned exclusively with a 100,000 × *g* pellet and was undetectable in the cytosolic fraction (Fig. 2*a*, *inset*).

The Inhibitory Activity Co-isolates with a 55-kDa Nuclear Envelope-binding Protein—We reasoned that the factors responsible for the inhibitory effect should act either by "neutralizing" M31 or by blocking M31-binding sites at the nuclear envelope. To differentiate between these two possibilities, we proceeded to depletion and order-of-addition experiments. Cytosol depleted from M31-binding proteins after a pre-incubation with immobilized GST-M31 contained as much inhibitory activity as mock-depleted cytosol (Fig. 2*b*, compare *columns No Cy*, *+Whole Cy*, and *+Depl Cy*). In line with this, [^{35}S]GST-M31 that was pre-incubated with cytosol before incubation with the NEs did not show any significant difference from the corresponding control (Fig. 2*b*, compare *columns CTL M31* and *Cy-M31*). However, when the NEs were pre-incubated with cytosol before incubation with [^{35}S]GST-M31, a dramatic decrease in the binding was detected (Fig. 2*b*, compare *columns CTL NE* and *Cy-NE*). From these observations it can be inferred that the inhibitory factors associate primarily with the NEs.

To confirm this idea, we mixed NEs with metabolically labeled cytosol, centrifuged the samples at 12,000 × *g*, and analyzed the pellet fraction by SDS-PAGE and autoradiography. As shown in Fig. 3*a*, a major 55-kDa protein and several minor species co-sedimented with the membranes. This did not reflect aggregation or nonspecific "sticking," because the 55-kDa protein floated up to the 30–40% sucrose interface and co-migrated exactly with the NEs upon analysis in sucrose density gradients (Fig. 3*b*, compare panels *Cy+NE* and *Cy+Buff.*).

The 55-kDa Protein Represents a Soluble Form of Tubulin—The 55-kDa polypeptide did not dissociate from the NEs after washing with assay buffer but was partially extracted by distilled water and high salt (Fig. 4*a*). Exploiting this, we isolated mg amounts of the 55-kDa protein from lysates of interphase and mitotic cells (Fig. 4*b*; for details see "Experimental Procedures"). The partially purified polypeptide abolished binding of [^{35}S]GST-M31 to the NEs (Fig. 4*c*), suggesting a close relationship with the inhibitory factor we were seeking.

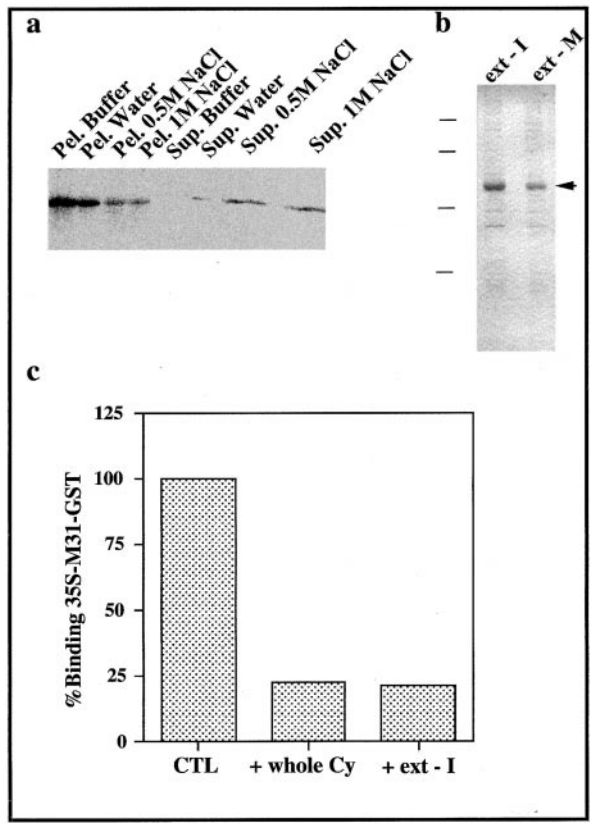

FIG. 4. **Isolation and functional testing of the 55-kDa protein.** *a*, partial extraction of the 55-kDa protein from the nuclear envelopes. Samples containing 40 μl of [^{35}S]methionine-labeled cytosol and 50 μg of nuclear envelopes were incubated for 45 min and spun at 12,000 × *g* for 30 min. After resuspension of the pellets, the membranes were extracted with KHM buffer, distilled water, and KHM containing 0.5 M or 1.0 M NaCl and recentrifuged. The panel shows an SDS-PAGE/autoradiographic profile of the material that remains bound to nuclear envelopes (*Pel.*) or is released into the supernatant (*Sup.*) after the various treatments. Only the relevant area of the gel is depicted here. *b*, material highly enriched in the 55-kDa protein (*arrow*) that has been isolated by incubating nuclear envelopes with interphase (*ext-I*) or mitotic (*ext-M*) cytosol, and subsequent extraction with salt (for details see "Experimental Procedures"). An SDS gel is shown after staining with Coomassie Blue. The *lines* on the *left* indicate molecular weight values (97,000, 68,000, 45,000, and 31,000 from *top* to *bottom*). *c*, binding of [^{35}S]M31-GST in the absence of cytosol (*CTL*), in the presence of whole cytosol (+ *whole Cy*; 45 μl of 8 mg/ml), and in the presence of the partially purified 55-kDa protein (+ *ext - I*; 11 μg of protein). The assay was performed as specified in the legend to Fig. 2.

To establish the identity of this protein, we excised the 55-kDa band from SDS gels, digested the material with trypsin, and determined the amino acid sequence of nine internal peptides. Analysis by mass spectrometry revealed that the 55-kDa band includes two different protein chains, corresponding to β2- and α2/6-tubulin (Fig. 5*a*). This result was fully confirmed by Western blotting; when whole cytosol was co-incubated with NEs, soluble tubulin bound to the membranes and partitioned with the pellet fraction (Fig. 5*b*).

Purified Rat Brain Tubulin Associates with the Nuclear Envelope and Inhibits Binding of M31—Proceeding further, we tested highly purified tubulin preparations isolated from rat brain (SDS-PAGE profile shown in Fig. 6*a*). Nonpolymeric tubulin, maintained in a soluble form by nocodazole/low salt, bound specifically to intact NEs but did not co-sediment with proteolyzed membranes (Fig. 6*b*). Quantitative assays showed that binding was concentration-dependent, exhibited saturable

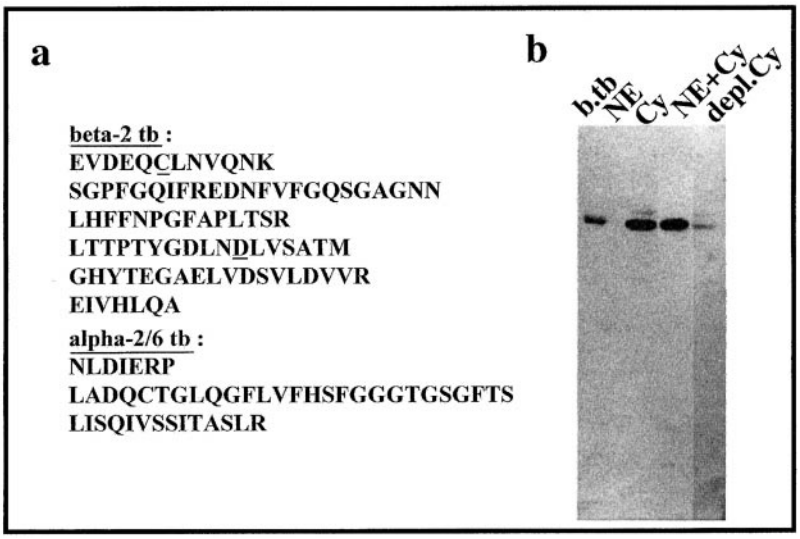

FIG. 5. **Identification of the 55-kDa protein as α2/6:β2-tubulin.** *a*, internal sequences obtained from the 55-kDa band. In all but two positions, the peptides match exactly human β2- and human or mouse α2/6-tubulin. Single amino acid differences in two of the β2 peptides (Cys instead of Met and Asp instead of His) are *underlined*. *b*, binding of soluble tubulin to nuclear envelopes. Nuclear envelopes and interphase cytosol were assayed as specified in the legend to Fig. 2. Samples were analyzed by SDS-PAGE and immunoblotted with anti-tubulin antibodies. *b. tb*, rat brain tubulin; *NE*, turkey erythrocyte nuclear envelopes; *Cy*, whole cytosol; *NE+Cy*, nuclear envelopes after co-incubation with cytosol; *depl.Cy*, material remaining in the supernatant fraction after co-incubating cytosol with the nuclear envelopes.

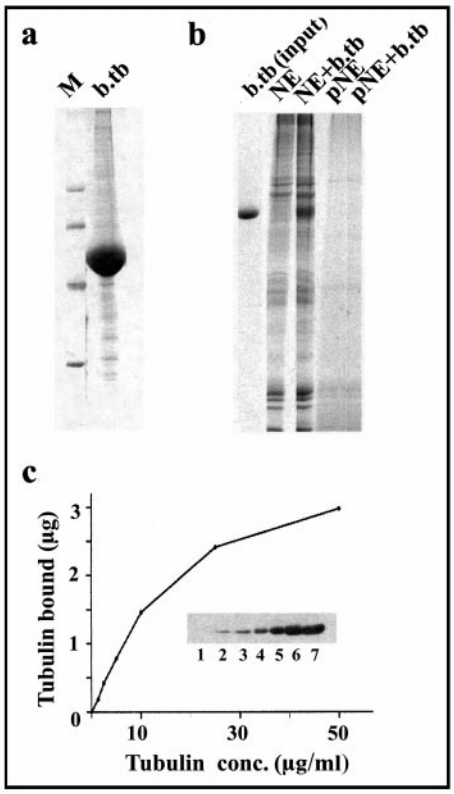

FIG. 6. **Purified rat brain tubulin associates specifically with the nuclear envelope.** *a*, SDS-PAGE profile of purified rat brain tubulin (*b.tb*) and molecular weight markers (*M*) (97,000, 68,000, 45,000, and 31,000 from *top* to *bottom*). The gel was overloaded on purpose. *b*, binding of purified tubulin to nuclear envelopes as detected by a co-sedimentation and SDS-PAGE. *NE+b.tb*, brain tubulin and nuclear envelopes; *pNE*, proteolyzed nuclear envelopes; *pNE+b.tb*, brain tubulin and proteolyzed nuclear envelopes. *c*, quantification of tubulin binding to the nuclear envelopes as assayed by co-sedimentation and quantitative immunoblotting (ECL). The curve shows the amount of nuclear envelope-bound tubulin as a function of its concentration. *Inset*, typical ECL blot from which quantitative data were obtained. *Lane 1* (no signal) corresponds to a sample that received no tubulin, whereas *lanes 2–7* correspond to samples that received 0.1, 0.2, 0.5, 1.0, 2.0, and 5.0 μg, respectively, of tubulin. The assay volume was 100 μl, and all samples in *b* and *c* contained 1 μM nocodazole in 10 mM Pipes-KOH to maintain the tubulin in a nonpolymeric form.

features, and was characterized by a relatively high affinity (apparent $K_d = 10^{-7}$ M; Fig. 6*c*).

In line with the previous results, nonpolymeric tubulin potently inhibited the binding of [^{35}S]GST-M31 to the NEs, whereas a control protein (bovine serum albumin) tested in parallel had no effect (Fig. 7*a*, compare *columns 3* and *4*). When the experiment was performed under conditions allowing tubulin polymerization (isotonic salt/Mg and absence of nocodazole), inhibition of [^{35}S]GST-M31 binding was less pronounced (Fig. 7*a*, compare *columns 2* and *4*), indicating that at the same nominal concentrations nonpolymeric tubulin is a much more effective inhibitor than polymeric tubulin.

From a variety of previous studies it is known that certain isotypes of mammalian tubulin possess a C-terminal tail consisting of 10 tandemly arranged glutamic acids. Furthermore, tubulin is extensively glutamoylated and phosphorylated after biosynthesis, acquiring an even higher negative charge. Because of this peculiarity and considering that M31-GST is also negatively charged at neutral pH (estimated isoelectric point of 5.5), we considered the possibility that tubulin may inhibit M31 binding by mere electrostatic repulsion. Although M31-GST binding to the NEs was not affected significantly by negatively charged ligands (*e.g.* heparin; data not shown), to confront this problem in a more definitive fashion, we repeated the binding/inhibition experiments at a pH of 5.7 at which both tubulin and M31-GST carry minimal charge. At this pH, tubulin inhibited [^{35}S]GST-M31 binding to the same extent as at pH 7.4, ruling out any electrostatic effects (Fig. 7*b*).

Finally, to confirm the biochemical results by an independent method, we employed a previously established morphological assay, which detects *in situ* binding of M31 to elements of the nuclear periphery (for details see "Experimental Procedures"). As shown in Fig. 8 (panel labeled *GST-M31*), when Triton X-100-permeabilized cells were incubated with GST-M31, the periphery of the nucleus was intensely stained. However, when the same experiment was repeated with a mixture of M31-GST and rat brain tubulin, the decoration of the nuclear periphery was markedly reduced (panel labeled *GST-M31+tb*). This effect was specific, because binding of M31-GST to internal nuclear structures (*e.g.* perinucleolar heterochromatin) was not affected by the presence of tubulin.

Soluble Tubulin Inhibits Nuclear Envelope Reassembly in Vitro—In recent studies, we have shown that M31 mediates recruitment of nuclear envelope precursors around segregated chromosomes and facilitates nuclear envelope reassembly at the end of mitosis (24). Knowing that, we wanted to examine whether soluble tubulin regulates these associations, inhibiting binding of nuclear envelope precursors to chromosome-associated M31 at late phases of mitosis. This interpretation

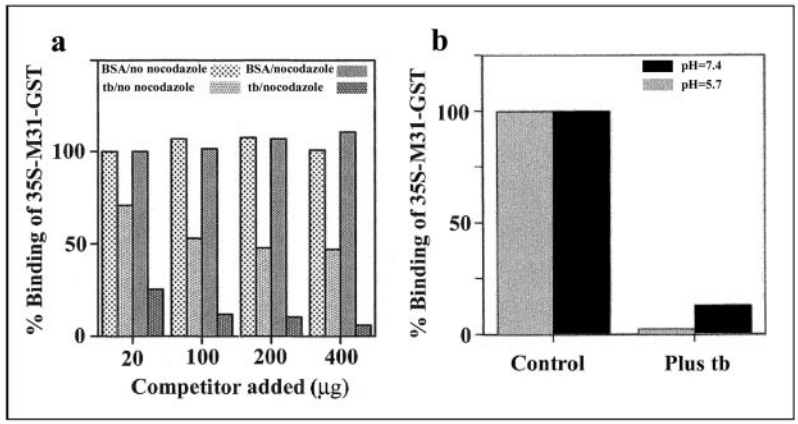

FIG. 7. **Purified tubulin inhibits M31 binding to the nuclear envelope.** *a*, binding of [^{35}S]M31-GST in the presence of bovine serum albumin (*columns 1* and *3*) or purified rat brain tubulin (*tb*) (*columns 2* and *4*). In one set of samples the assay was done in medium (150 mM) salt/Mg at 23 °C (*columns 1* and *2*), whereas in the other case the experiment was done in 10 mM salt at 0 °C and in the presence of 1 μM nocodazole (*columns 3* and *4*). All assays were performed as described in the legend to Fig. 2. *b*, experiments similar to that shown in *a* at two different pH values (*black bars*, pH 7.4; *gray bars*, pH 5.7). In this case 1 mg of exogenous tubulin was added to the assay mixture, and all samples contained 1 μM nocodazole.

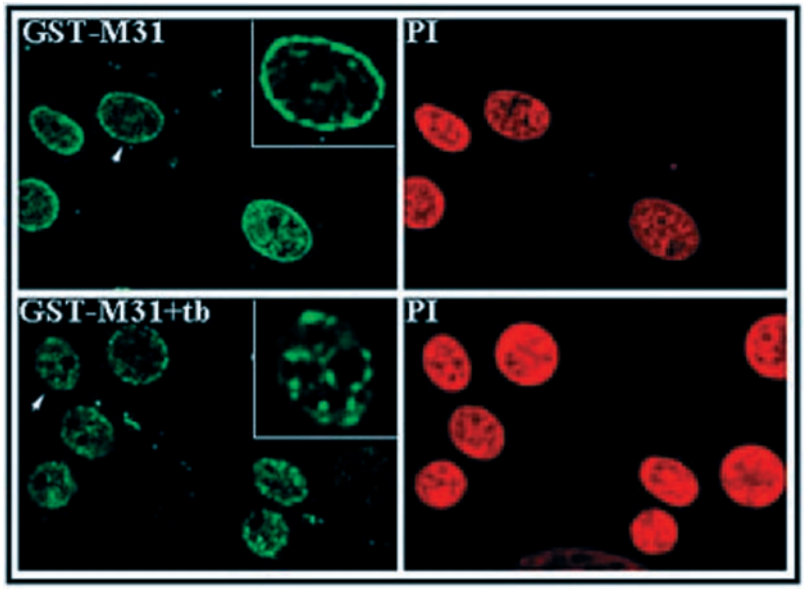

FIG. 8. **Purified tubulin prevents *in situ* binding of GST-M31 with the nuclear envelope.** Confocal sections of permeabilized HeLa cells incubated with M31-GST or M31-GST and soluble rat brain tubulin (*tb*). In the first case the absence of tubulin was compensated by adding an equal amount of bovine serum albumin. All samples contained 1 μM nocodazole. Exogenous M31 was localized using affinity-purified anti-GST antibodies (*green*). *Panels* labeled *PI* (*red*) are the corresponding propidium iodide profiles. The *insets* show larger magnifications of the cells indicated by the *arrowheads*. Note the prominent rim-like pattern in the control and the relatively attenuated staining in the sample containing tubulin.

makes physiological sense, because, upon nuclear envelope breakdown, soluble tubulin is expected to have access to the nuclear interior.

To test this hypothesis, we utilized a novel *in vitro* reassembly assay developed in our laboratory. In this assay, nocodazole-synchronized (prometaphase) cells are first incubated with low concentrations of digitonin, to gently open the plasma membrane and allow addition of exogenous elements. Subsequently, the permeabilized cells are incubated for 2 h at 33 °C to induce destruction of mitotic cyclins and inactivation of the cdc2 kinase. Elimination of this kinase allows dephosphorylation of mitotically modified nuclear envelope proteins and initiation of nuclear envelope reassembly around condensed chromosomes. The progress of this reaction can be easily monitored by spinning the "digitonin ghosts" on glass coverslips and performing indirect immunofluorescence microscopy with anti-lamin B and anti-LAP2B antibodies. These two proteins represent, respectively, peripheral and integral components of the nuclear envelope that disperse during prometaphase and are quantitatively recruited to the surfaces of chromosomes at late anaphase/telophase.

As shown in Fig. 9, addition of soluble tubulin completely abolished recruitment of LAP2B and lamin B to the surfaces of chromosomes, whereas addition of bovine serum albumin (control) had no effect.

DISCUSSION

Recent studies have shown that HP1 proteins interact dynamically with the nuclear envelope. The mouse HP1 homologues mHP1α, M31, and M32 accumulate at the nuclear periphery when injected into interphase cells and decorate the nuclei of detergent-permeabilized cells in a characteristic, rim-like fashion. Furthermore, all HP1 variants exhibit saturable and site-specific binding to isolated nuclear envelopes (24).

Nuclear envelope association may involve a specific interaction with the integral membrane protein LBR, which has been

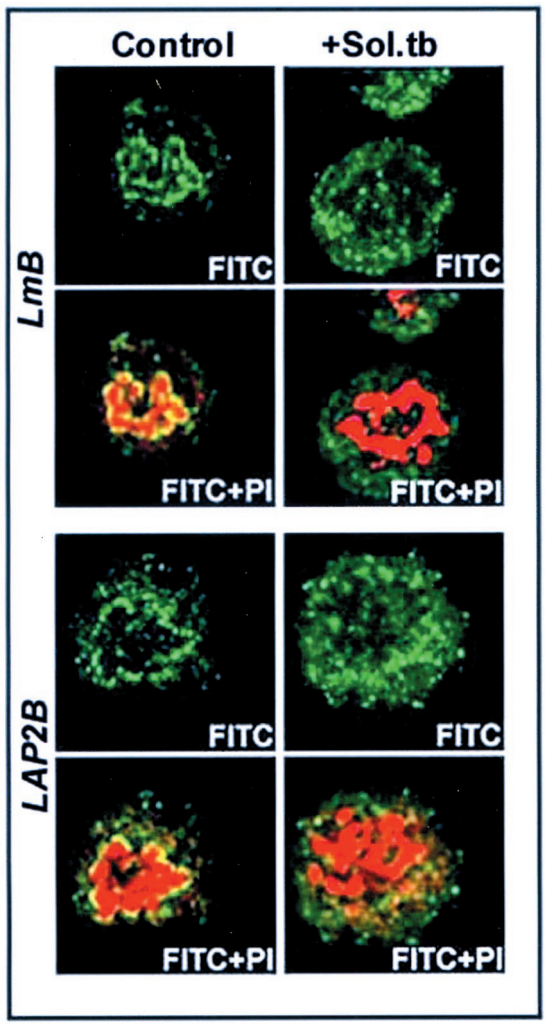

FIG. 9. **Soluble tubulin inhibits nuclear envelope reassembly *in vitro*.** In vitro reassembly assays were performed as described under "Experimental Procedures." *Panels* labeled *Control* show permeabilized mitotic cells incubated for 2 h at 33 °C in the presence of bovine serum albumin. *Panels* labeled *+Sol.tb* correspond to analogous samples incubated in the presence of soluble tubulin. Assembly of nuclear envelope proteins was assessed by staining the particles with anti-LAP2B or anti-lamin B antibodies (*green*) and propidium iodide (*PI*; *red*). *FITC*, fluorescein isothiocyanate

shown to bind the human HP1 homologues hHP1α and hHP1γ in two-hybrid assays (11, 20), or constituents of peripheral heterochromatin (34). Acetylation and other posttranslational modifications undoubtedly play a role in HP1 dynamics, because targeting of M31 and M32 to the nuclear envelope is abolished upon treatment with deacetylase inhibitors (24). Nonetheless, because HP1 proteins oligomerize and are capable of interacting with a wide variety of cellular components (see the Introduction), it would be reasonable to assume that their molecular associations are regulated by auxiliary factors and molecular chaperones. In this study, we have attempted to identify and characterize such factors using quantitative *in vitro* assays in combination with subcellular fractionation. Our results show that M31 binding to the nuclear envelope is potently inhibited by nonpolymeric tubulin. This is a surprising finding, because soluble tubulin has always been considered to be a mere "reservoir" of microtubule subunits.

The biochemical and morphological data presented here make it clear that the inhibitory effect of tubulin is not due to a chaotropic or purely electrostatic effect. First, nonpolymeric tubulin effectively competes with M31 under pH conditions at which the total charge of the molecule is nearly zero. Second, at the same nominal concentration nonpolymeric tubulin is a much better inhibitor than polymeric tubulin, although the charge of the protein is similar in both aggregation states.

Soluble, nonpolymeric tubulin is an abundant constituent of the cytoplasm. Earlier studies indicate that the average hepatocyte contains 3.1×10^7 $\alpha\beta$ dimers, of which only 15% are incorporated into microtubules (35). Although the proportion of polymeric tubulin could be much higher in cultured cells (up to 62% in porcine kidney epithelial cells, according to Zhai and Borisy (36)), the intracellular concentration of unassembled tubulin is on the order of 1 mg/ml (37), *i.e.* well above the critical concentration for microtubule assembly. A transient increase in the concentration of soluble tubulin should be expected at prophase-prometaphase, when the network of interphase microtubules has been destroyed, and the mitotic spindle has not been fully assembled. At that point, soluble tubulin may gain access to internal nuclear structures, because the nuclear envelope is disrupted by a combination of mitotic hyperphosphorylation and mechanical force (for a review see Ref. 38). Tubulin binding to the disassembling nuclear envelope might prevent premature interactions between fragments of the nuclear membrane and M31 at early phases of cell division. Reversal of this process, *i.e.* dissociation of tubulin from nuclear envelope-derived membranes, could occur at subsequent stages of mitosis, when the spindle fully develops, the mass of polymerized tubulin increases to interphase levels (36), and the concentration of soluble tubulin presumably drops.

Be it as it may, tubulin would not be the only example of a protein that participates in more than one function inside eukaryotic cells. As it turns out, a wide variety of cellular proteins, from oligomeric enzymes to crystallins and ion channels, switch partners and become involved in different functions when their aggregation state or localization is changed. These are the so-called "moonlighting proteins," a class of molecules that supposedly evolved when "unused," surface-exposed regions of pre-existing molecules became useful for the global physiology of the cell (for a review see Ref. 39).

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