Interaction of Kinesin Motor Domains with $\alpha$- and $\beta$-Tubulin Subunits at a Tau-independent Binding Site

REGULATION BY POLYGLUTAMYLATION*

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Interaction of rat kinesin and Drosophila nonclaret disjunctional motor domains with tubulin was studied by a blot overlay assay. Either plus-end or minus-end-directed motor domain binds at the same extent to both $\alpha$- and $\beta$-tubulin subunits, suggesting that kinesin binding is an intrinsic property of each tubulin subunit and that motor directionality cannot be related to a preferential interaction with a given tubulin subunit. Binding features of dimeric versus monomeric rat kinesin heads suggest that dimerization could drive conformational changes to enhance binding to tubulin. Competition experiments have indicated that kinesin interacts with tubulin at a Tau-independent binding site. Complementary experiments have shown that kinesin does not interact with the same efficiency with the different tubulin isoforms. Masking the polyglutamyl chains with a specific monoclonal antibody leads to a complete inhibition of kinesin binding. These results are consistent with a model in which polyglutamylation of tubulin regulates kinesin binding through progressive conformational changes of the whole carboxyl-terminal domain of tubulin as a function of the polyglutamyl chain length, thus modulating the affinity of tubulin for kinesin and Tau as well. These results indicate that microtubules, through tubulin polymorphism, do have the ability to control microtubule-associated protein binding.

The various structures and functions of microtubule (MT) networks are mediated by many different structural and motor microtubule-associated proteins (MAPs) (for review see Refs. 1-4). To coordinate these functions, the major problem in cells is to regulate not only the expression of the different MAPs but, more precisely, their differential interactions with MTs. Regulation of MAP binding through phosphorylation has been extensively documented (5-7), although little information is as yet available concerning motor proteins (8-11). However, several reports have clearly shown that part of this regulation had to be supported by the MTs themselves. For instance, it has been shown that in lobster axons, only a subset of MTs was competent for vesicle transport (12), suggesting that the MT surface available for interaction with motors was not homogeneous along all of the axonal MTs. The same relationship between distinct MT subsets and specific binding of kinesin-like proteins has also been observed in the mitotic spindle apparatus (13-15) and in the Chlamydomonas flagellum where Kip1 was shown to interact selectively with the central pair C2 MT (16). The chemical diversity of the MT surface could provide such specific positional and binding information in cells for many MT-interacting proteins. The large genetic and post-translational heterogeneity of the $\alpha$- and $\beta$-tubulin subunits (for review see Refs. 17-19) can represent the molecular bases for such information. Indeed, we recently showed that polyglutamylation, the oligomeric post-translational modification of tubulin (20-23), was involved in the regulation of Tau and MAP2 interaction with tubulin through a modulation of affinity as a function of the polyglutamyl chain length (24). Because, on one hand, the extension of the polyglutamyl chain from one to six units is thought to induce conformational changes in the carboxyl-terminal domain of both tubulin subunits, leading to the observed modulation of affinity for structural MAPs, and, on the other hand, all of the known MT-based motors (kinesin, kinesin-related proteins, and cytoplasmic and flagellar dyneins) have been reported to bind also in this carboxyl-terminal domain (25, 26), it follows that their binding could also be under the same control as the structural MAPs.

We have analyzed the binding of different kinesin head motor domains (conventional rat kinesin (RK) and the Drosophila minus-end-directed kinesin-related protein (ncd, for nonclaret disjunctional)) to tubulin and investigated the influence of the degree of polyglutamylation of the various $\alpha$- and $\beta$-tubulin isoforms on motor binding. In this paper, we report that the two types of motors bind in vitro to both $\alpha$- and $\beta$-tubulin subunits and that this interaction appears to be controlled by polyglutamylation in the same manner as for Tau or MAP2. In addition, competition experiments carried out with several monoclonal antibodies and with kinesin heads and heat-stable MAPs indicate that the respective binding sites of structural and motor MAPs are independent from each other, although located in a sufficiently close vicinity on the tubulin molecule to undergo the same conformational control by polyglutamylation.

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The abbreviations used are: MT, microtubule; PAGE, polyacrylamide gel electrophoresis; MAP, microtubule-associated protein; MES, 2-(N-morpholino)ethanesulfonic acid; RK, rat kinesin; ncd, nonclaret disjunctional; PIPES, 1,4-piperazinediethanesulfonic acid; MEM, MES-EGTA-MgCl2 buffer; OV, overlay buffer; HPLC, high pressure liquid chromatography.

EXPERIMENTAL PROCEDURES

Antibodies—The monoclonal antibodies DM1A and DM1B were purchased from Amersham Corp., Tau-1 was from Boehringer Mannheim, and anti-MAP2 was from Sigma. The monoclonals YL1/2 and YOL1/34 were generous gifts of Dr. J. Kilmarin (M.R.C. Laboratory of Molecular Biology, Cambridge, UK). The polyclonal anti-HIPYR (14) was kindly provided by Dr. T. Mitchison (Department of Pharmacology, University of California, San Francisco, CA). The GT335 monoclonal antibody was...
produced in our laboratory (27).

Recombinant Proteins—The recombinant motor domain constructs of rat kinesin (RK375 and RK329) and Drosophila ncd (HA-N333) were kindly provided by E.-M. Mandelkow and F. Kozierski (Max-Planck Institute for Structural Molecular Biology, Hamburg, Germany). RK375 and RK329 correspond to amino acids 1-379 and 1-329 of the rat kinesin sequence, respectively. HA-N333 corresponds to amino acids 333-700 of the Drosophila claret segregational protein sequence.

Protein Purification—Tubulin was prepared from a 100,000 × g supernatant of adult mouse brain by two cycles of assembly disassembly and further purified by phosphocellulose column chromatography (Pharmacia, Uppsala). The rat kinesin (RK375 and RK329) and Drosophila ncd were purified by phosphocellulose column chromatography.

Subtilisin Digestion—Purified brain tubulin (1 mg/ml) was digested in MEM buffer with 1.25 or 5 μg/ml subtilisin (Carlsberg, Sigma; 7-15 units/mg) for 45 min at 37 °C, leading to a limited or a total digestion of tubulin, respectively. Reaction was stopped by the addition of 1 mM phenylmethylsulfon fluoride, and aliquots were processed by one-dimensional PAGE.

One-dimensional and Two-dimensional PAGE—Protein separation by one-dimensional or two-dimensional PAGE and immunodetection was carried out as described previously (24, 27) except that amphotypes 5-6 (Serva) were used in the isoelectric focusing dimension. Antibody binding was revealed by the ECL system (Amersham Corp.), and autoradiograms were quantified by scanning using a Vernom integrating densitometer.

Blot Overlay Assay—Binding of kinesin heads to tubulin separated by one-dimensional or two-dimensional PAGE and transferred onto nitrocellulose membranes (Hybond C, Amersham Corp.) was performed essentially as described previously (24). Briefly, after monitoring of blotted tubulin by Ponceau red staining, strips corresponding to lanes of one-dimensional gels (or rectangles corresponding to tubulin regions of two-dimensional gels) were cut from the blots and placed into the grooves of a hand-made, Plexiglas incubation device adjusted to the size of the strips. Nitrocellulose pieces were blocked overnight in overlay buffer (OV = MEM buffer containing 1 mM dithioretilol, 0.1% (v/v) Tween 20, and 0.1% (w/v) gelatin), incubated for 1 h at 20 °C with the overlaying protein fraction, and then washed 5 × 10 min. Protein interactions were then stabilized with formaldehyde (24) before the blots were equilibrated in TBS-T (20 mM Tris, pH 7.5, 136.8 mM NaCl, 0.1% Tween 20) and processed for immunodetection.

Kinesin Binding to Coated Tubulin Heterodimers—Phosphocellulose-purified tubulin (1 μg in 100 μl) was coated onto nitrocellulose immunosorbent assay multiwell microtiter plates (Nunc) in MEM buffer at 37 °C. We checked by immunoassay that a constant amount of 500 ng was actually coated in each well according to the manufacturer. Wells were equilibrated and blocked overnight in OV then incubated with various concentrations of kinesin heads in 100 μl for 60 min at 25 °C. Wells were rinsed with OV (5 × 5 min), and kinesin-tubulin interactions were stabilized with formaldehyde (24). After equilibration in TBS-T, tubulin-bound kinesin was detected with anti-HIPYR 2 h at 37 °C and a β-galactosidase-conjugated secondary anti-rabbit IgG (Bio-sys, France) 1 h at 37 °C, using α-nicotinamide-β-galactosidase (Sigma) as chromogen. Reactions were quantified automatically at 490 nm.

Microtubule Sedimentation Assay—MTs were polymerized from a whole brain supernatant in the presence of 20 μM Taxol and washed with 0.5 mM NaCl to remove bound MAPs. MTs were pelleted, resuspended in MEM buffer, and used in micro-assays of kinesin head binding. MTs (60 pmol) were mixed with kinesin heads (50 pmol) in the absence or the presence of different nucleotides and salt in a final volume of 25 μl. After centrifugation (10 min at 25 p.s.i. in the A100 Airfuge rotor, Beckman), the whole pellet and supernatant proteins were analyzed by one-dimensional PAGE.

Size Exclusion HPLC—RK329 and RK375 were suspended in MEM buffer (50 mM MES, pH 6.8, 2 mM EGTA, 2 mM MgCl2) containing 1 mM Mg-GTP and a mixture of protease inhibitors (10 μg/ml aprotinin, 10 μg/ml leupeptin, 1 mM phenylmethylsulfon fluoride). Heat-stable MAPs (essentially Tau and MAP2) were routinely prepared from once cycled MTs, which were depolymerized at 4 °C, cleared by centrifugation, brought to 0.5 mM NaCl, and boiled for 5 min.

RESULTS

Interaction of Rat Kinesin Heads (RK375) with α- and β-Tubulin Subunits—Increasing concentrations of RK head were overlaid onto a constant amount of tubulin separated by one-dimensional PAGE and immobilized onto nitrocellulose. Fig. 1A shows that RK head binding, detected on both α- and β-tubulin subunits with the specific anti-kinesin peptide (HIPYR) antibody, increased proportionally with RK concentration up to a saturation value. RK375 binding to both α- and β-tubulin was
directed motor of Ncd also bound to both the tubulin-kinesin interactions occurring during the blot overlay assay. Brain (Fig. 1B, right panel). These results indicate that the tubulin-kinesin interactions occurring during the blot overlay procedure were specific. Likewise, the reverse, minus-end-directed motor head of ncd also bound to both α- and β-tubulin subunits (Fig. 1C), suggesting that the directionality of a kinesin motor protein along a MT is not determined by its selective interaction with a given α- or β-tubulin subunit.

To assess the in vitro binding assay used in the preceding experiments, RK head binding was tested in the presence of nucleotides. Kinesin binding was progressively inhibited by increasing concentrations of GTP or of its hydrolyzable form, Mg-GTP (Fig. 2A). The effect of nucleotides on RK head binding was also tested using heterodimeric tubulin coated onto multiwell enzyme-linked immunosorbent assay plates and taxol-stabilized MTs by a sedimentation assay. Fig. 2 (B and C) shows that in both cases the binding of RK heads was weakened by GTP, indicating that monomeric tubulin in the blot overlay assay behaves as heterodimeric or MT-assembled tubulin with respect to kinesin head binding.

Formation of RK head-tubulin complexes was also readily prevented by raising the salt concentration in the incubation medium (Fig. 2, C and D). No interaction could be detected beyond 75 mM NaCl. Formation of kinesin-tubulin complexes was still effective, however, in the presence of 1 or 2 M urea (data not shown). These results indicate that kinesin-tubulin interactions occur mainly through electrostatic bonds.

It is interesting to note that when MES was substituted by PIPES, the binding efficiency of kinesin to tubulin dropped severalfold. At 100 mM PIPES, a widely used buffer molarity, however, binding was faintly detectable, at levels 5–10 times lower than with 50 mM MES (data not shown).

In all of these experiments where nucleotides, salt, chaotropic agent, or buffers were tested, the variation in the binding of RK heads was always similar for α- and β-tubulin subunits, indicating that the same basic binding features for kinesin were shared by both subunits, which in turn suggests the involvement of related sequences in orthologous domains in both subunits.

Binding of a Truncated Rat Kinesin Motor Domain—When a truncated derivative of RK head, RK329, was overlaid onto immobilized tubulin (Fig. 3A), the resulting binding was estimated to be 5–10-fold lower than that obtained with the whole RK375 head. Although less efficiently, RK329 bound to both tubulin subunits at similar levels. The same difference in tubulin binding was observed when increasing concentrations of RK375 and RK329 were added to microwells coated with heterodimeric tubulin (Fig. 3B). These results suggest that amino acids 330–375 of RK are important for enhancing the interaction with tubulin.

RK329 and RK375 were then analyzed by gel filtration chromatography to check the assembly state of each motor. Fig. 3, C and D shows that RK329 (calculated mass, 36.8 kDa) and RK375 (calculated mass, 42.7 kDa) eluted as a single peak (retention times, 9.3 and 7.8 min, respectively) corresponding to globular polypeptides of M, 37,200 and 120,000, respectively. Given the steric occupancy of a nonspherical, two-headed protein, these values are compatible with a dimerized state of RK375. When the column was run in 500 mM NaCl, RK375 eluted at the same position (Fig. 3C, dotted line). The salt resistance of RK375 dimers suggests that α-helical coiled-coil interactions could be involved between amino acids 330–375 of kinesin sequences (see Ref. 28 and “Discussion”).

Regulation of Kinesin–Tubulin Interaction by Polyglutamylation—Because polyglutamylation was previously shown to modulate the affinity of tubulin for the structural MAPs Tau and MAP2 (24), we researched whether it could also be involved in the control of the binding of kinesin. Brain tubulin was separated by resolutive two-dimensional PAGE to physically space the different isoforms carrying an increasing number of glutamyl units (22, 24) and transferred onto nitrocellulose. The different isotubulins were then probed for their ability to interact with kinesin. By comparison with the whole set of tubulin isoforms available on the nitrocellulose membrane (Fig. 4A), as detected with general anti-tubulin antibodies, Fig. 4B shows that RK375, immunodetected with anti-HIPYR, does not interact with the same efficiency with all of the different forms of tubulin. As previously observed for Tau (24), kinesin interacted principally with moderately modified α- and β-isotubulins. The unmodified, more basic, primary translation products as well as the fully modified, more acidic isoforms only bound weakly, if at all, to kinesin. Because the type of response was similar for α- and β-tubulin and involved post-translationally derived tubulin isoforms, polyglutamylation appeared as the only obvious candidate responsible for this differential kinesin binding; it is the only modification that occurs on both tubulin subunits (Fig. 4C), and its oligomeric structure, which confers one to six negative charges to tubulin (20–23), can explain the progressive variation of RK binding observed as a function of tubulin acidification. It appears that addition of the first three glutamyl units increases the affinity of tubulin for kinesin, whereas further lengthening of the post-translational chain decreases it.

Competition with Monoclonal Anti-tubulin Antibodies—To explain the influence of polyglutamylation on kinesin binding, an attempt was made to block the post-translational chains
with a monoclonal antibody raised in our laboratory, GT335, which specifically recognizes the polyglutamylated motif on α- and β-tubulin (27). As a control, four other anti-tubulin monoclonal antibodies were used in parallel to block their respective epitopes, all located also in the carboxyl-terminal domain (Fig. 5F): the anti-tyrosinated α-tubulin YL1/2, the anti-α-tubulin YOL1/34, and two general anti-α- and anti-β-tubulin, DM1A and DM1B, respectively (29, 30). Purified brain tubulin was separated by one-dimensional PAGE, transferred onto nitrocellulose, and progressively saturated by increasing concentrations of each monoclonal antibody. After extensive washing to eliminate unbound antibodies, membrane strips were overlaid with RK375, and tubulin-bound kinesin was detected with the polyclonal anti-HIPYR antibody and a peroxidase-labeled anti-rabbit IgG, to avoid any cross-reaction with the previously bound mouse IgG (Fig. 5A–E).

Increasing fixation of GT335 on the polyglutamyl chains progressively and rapidly inhibited kinesin binding on both tubulin subunits (Fig. 5A). A similar binding inhibition by GT335 was previously observed for Tau (24). Fixation of YL1/2 on its carboxyl-terminal tyrosylated epitope of α-tubulin did not significantly affect the binding of RK375 (Fig. 5B). Fixation of DM1A and DM1B interfered with kinesin binding on the α- and the β-tubulin subunit, respectively (Fig. 5, C and D). DM1A being much more efficient than DM1B in binding inhibition. In the presence of YOL1/34, a significant inhibition of RK binding to α-tubulin was also observed (Fig. 5E). In these experiments, binding of RK to β-tubulin in the presence of YL1/2, YOL1/34, or DM1A and to α-tubulin in the presence of DM1B served as internal controls.

Binding of Kinesin to Subtilisin-digested Tubulin—With antibody blocking experiments, it is difficult to know at first whether the observed inhibition is a direct effect, that is, a coincidence of the ligand binding site and the antibody epitope, or is due indirectly to a steric hindrance of the large IgG.

Fig. 4. RK375 binding to the different α- and β-isotubulins: influence of polyglutamation. Tubulin was separated by high resolution two-dimensional PAGE and transferred onto nitrocellulose. A, control. The different α- and β-isotubulins were immunodetected with DM1A then with DM1B, respectively. B, overlay. A parallel blot was overlaid with RK375 (2 μg/ml), and tubulin-bound kinesin was immunodetected with anti-HIPYR. C, the different polyglutamylated α- and β-isotubulins were immunodetected with GT335. 0 and 1 → 6 indicate the number of glutamyl units carried by the post-translationally modified tubulins. 0 corresponds to the unmodified primary translation tubulin products. The brackets are positioned in the same coordinates in the three panels.

Fig. 3. Binding of RK329 versus RK375. A, 2 μg of tubulin separated by one-dimensional PAGE and blotted onto nitrocellulose were overlaid with 2 and 10 μg/ml of RK329 or RK375, and the resulting signals (insert) were quantified (expressed in arbitrary units). B, increasing concentrations (0.5–5 μg in 100 μl) of RK329 or RK375 were added to wells of microtiter plates coated with 500 ng of tubulin. In both types of experiment, kinesin head binding to tubulin was revealed with anti-HIPYR antibody and quantified (expressed in arbitrary units). C, 50 μg of each RK329 and RK375 were mixed in MEM buffer and separated by size exclusion chromatography. Protein elution (1 ml min−1) was monitored at 280 nm (solid line). After equilibration of the column with MEM containing 500 mM NaCl, 75 μg of RK375 in MEM-NaCl were rechromatographed (dotted line). D, aliquots of fractions collected from the first run were analyzed by one-dimensional PAGE. Lane 1, RK329, control; lane 2, RK375, control; lane 3, RK329 + RK375, control; lane 4, peak 1 (7.8 min); lane 5, peak 2 (9.3 min); lane 6, peak 3 (11.3 min). Left panel, Coomassie Blue staining; right panel, anti-HIPYR immunodetection. Peak 3 contained no protein; absorbance at 280 nm was due to the presence of traces of sodium azide, dithiothreitol, and ATP in the loaded samples.

Fig. 2. Interactions between Isotubulins and Kinesin Motor Domains—Binding of RK binding to tubulin subunits. A, binding of RK375 to α-tubulin (A) and β-tubulin (B). Kinesin head binding to tubulin revealed with anti-HIPYR immuno- detection. Peak 3 contained DM1A being much more efficient than DM1B in binding inhibition. In the presence of YOL1/34, a significant inhibition of RK binding to α-tubulin was also observed (Fig. 5E). In these experiments, binding of RK to β-tubulin in the presence of YL1/2, YOL1/34, or DM1A and to α-tubulin in the presence of DM1B served as internal controls.

Binding of Kinesin to Subtilisin-digested Tubulin—With antibody blocking experiments, it is difficult to know at first whether the observed inhibition is a direct effect, that is, a coincidence of the ligand binding site and the antibody epitope, or is due indirectly to a steric hindrance of the large IgG...
molecule, which can block the accessibility of adjacent binding sites. For the inhibition observed with GT335, at least, the corresponding epitopes can be easily removed by digesting tubulin with subtilisin (24, 31, 32). In this case, after blot overlay with RK375 of partially or totally digested tubulin (Fig. 6), it appeared that kinesin still bound to cleaved (αS and βS) tubulin subunits, suggesting that the kinesin binding sites are not located in the extreme carboxyl-terminal domain of tubulin but rather upstream or even close to the subtilisin cleavage sites (Asp438 for α-tubulin and Gln433 for β-tubulin (32)). Altogether, the results from Figs. 4, 5, and 6 suggest that polyglutamylation of tubulin controls kinesin binding through an indirect, conformational change of the carboxyl-terminal domain of tubulin, as previously reported for Tau or MAP2 binding (24).

Moreover, it is relevant that compared with intact tubulin, kinesin binding is less affected than Tau binding by subtilisin (24, 31, 32). In this case, after blot overlay with RK375 of partially or totally digested tubulin (Fig. 6), it appeared that kinesin still bound to cleaved (αS and βS) tubulin subunits, suggesting that the kinesin binding sites are not located in the extreme carboxyl-terminal domain of tubulin but rather upstream or even close to the subtilisin cleavage sites (Asp438 for α-tubulin and Gln433 for β-tubulin (32)). Altogether, the results from Figs. 4, 5, and 6 suggest that polyglutamylation of tubulin controls kinesin binding through an indirect, conformational change of the carboxyl-terminal domain of tubulin, as previously reported for Tau or MAP2 binding (24).

Moreover, it is relevant that compared with intact tubulin, kinesin binding is less affected than Tau binding by subtilisin cleavage (data not shown). Combined with the observations that DM1A prevented kinesin but not Tau binding and that both are under the same control by polyglutamylation, it can be concluded that the binding sites for kinesin and Tau are distinct, although sufficiently close together within the carboxyl-terminal domain. The Tau binding site would be downstream from that of kinesin, ending, at least, in the vicinity of the subtilisin cleavage site.

Figure 5. RK375 binding to tubulin protected with monoclonal antibodies. Tubulin (2 μg) was separated by one-dimensional PAGE, transferred onto nitrocellulose, and incubated with increasing concentrations of the following monoclonal antibodies: GT335 (A), YL1/2 (B), DM1A (C), DM1B (D), or YOL1/34 (E). F indicates their epitope locations in the carboxyl-terminal domains of α- and β-tubulin subunits. After extensive washing to eliminate unbound antibodies, blot strips were overlaid with RK375 (2 μg ml⁻¹). RK375 binding to α- or β-tubulin subunits was quantified after immunodetection with the polyclonal anti-HIPYR antibody and expressed as a percentage of control binding (without prior blocking antibody) as a function of antibody dilution.

Figure 6. RK375 binding to subtilisin-cleaved tubulin. Tubulin was submitted to a total (lane 2) or a partial (lane 3) digestion with subtilisin and separated by one-dimensional PAGE with undigested tubulin (lane 1) as reference. Left panel, control: double immunodetection with DM1A then DM1B of cleaved (αS and βS) and uncleaved (α and β) tubulin subunits. Right panel, overlay: immunodetection with anti-HIPYR of RK375 bound to the different tubulin products.
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FIG. 7. Competition binding experiments with Tau and kinesin. Tubulin (2 μg) was separated by one-dimensional PAGE, transferred onto nitrocellulose, and overlaid with increasing concentrations of heat stable MAP (0–10 μg·mL⁻¹). This experiment was done in triplicate. Dotted line, control; first series of blot strips immunodetected with Tau-1. Dashed line, second series immunodetected with anti-HIPYR after a second overlay with a constant concentration (2 μg·mL⁻¹) of RK375. Solid line, third series processed in the same manner as the second, except that Tau-tubulin interactions were stabilized with formaldehyde before the second overlay with RK375. The results are expressed in arbitrary units.

Illustrated in Fig. 7 (dashed line), the binding level of RK375 was constant and independent of the amount of Tau previously bound to tubulin, indicating that kinesin head interaction was not impeded by bound Tau. To rule out the possibility that bound Tau could have been displaced by RK375, blot 3 was processed as blot 2 except that tubulin-Tau interactions were stabilized with formaldehyde between the first overlay with Tau and the second with RK375 (Fig. 7, solid line). The comparison of the binding curves shows that stabilization of the tubulin-Tau complex did not modify the constant interaction of RK375 with Tau-saturated tubulin. Altogether, these results strongly suggest that the binding sites for Tau and kinesin on the tubulin subunit molecules are independent.

DISCUSSION

Interactions between Tubulin Subunits and Kinesin—Kinesin is a plus-end-directed microtubule motor that binds to MTs with a longitudinal periodicity of 8 nm, corresponding to that of a tubulin dimer (33, 34), and moves along a single protofilament track (35, 36) with 8-nm steps (37). In agreement with the structural data.

FIG. 8. Binding sites for Tau and kinesin in the carboxyl-terminal domain of tubulin. A, carboxyl-terminal domains of α- and β-tubulin. The last 60 amino acids of both subunits are represented. I, II, and III correspond to the three regions described by Goldsmith et al. (26) and are delimited by boxed amino acid numbers. I, conserved between both subunits; II, conserved within each subunit only; III, isotype-specific. Epitope locations of DM1A, DM1B, and YOL1/34 are noted, as well as the subtilisin cleavage sites. E represents the polyglutamylated sites. The black boxes represent the α 430-441 and the β 422-434 peptides previously reported as the putative Tau binding sites (58). The shaded box represents the β 400–436 peptide reported to encompass the general motor binding site (26). Striped boxes represent the locations of predicted α-helices (see text). B, schematic representation of the carboxyl-terminal domain of α-tubulin from residues 410 to 451. The second 413-434 α-helix is shown as a cylinder with the monodonal YOL1/34 and DM1A bound, one above the other, on its outer surface; the deduced Tau and kinesin binding sites are positioned on the inner face of the helix (see text). Downstream are indicated the subtilisin cleavage site and the bound monodonal GT335 and YL1/2.

remains, however, that our results show that each α- or β-tubulin monomer has the intrinsic property to interact with kinesin. Moreover, all of the effectors tested in the binding assays modulate in the same way and at the same extent the binding to both tubulin subunits, indicating that both monomers share the same binding features for kinesin. Inhibition of kinesin binding on α- and β-tubulin by competitive free tubulin added in the overlay solution as well as the selective binding of RK to tubulin in a whole protein extract (see Fig. 1B) indicate further
that RK binding was specific.

Interaction of Tubulin with Plus- or Minus-end-directed Kinesin—Comparison of binding properties of the conventional RK motor domain with those of the reverse, minus-end-directed Drosophila kinesin-related protein ncd showed no significant quantitative or qualitative difference. In particular, both tubulin subunits were bound at similar levels by the two motors. Moreover, when analyzed by blot overlay after separation of tubulin by two-dimensional PAGE, the ncd motor bound differentially to the diverse isotubulins as a function of their degree of polyglutamylation (data not shown) following a pattern quite similar to that observed with RK375. Thus, the vectoral specificity of the motors within the kinesin superfam-

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Moreover, it has been reported that taxol-stabilized MTs cut with subtilisin (instead of soluble tubulin dimers, as in this paper) retained the property to bind kinesin but lost completely (48, 51) or partially (52, 53) the property to bind Tau, due to the cleavage of the α-subunit in the latter case.

It remains difficult, however, to assign more precise locations for the kinesin and Tau binding sites on linear representations of tubulin molecules (Fig. 8A). All of the results from the literature point to region II of the carboxyl-terminal domain of the tubulin subunits described by Goldsmith et al. (26), corresponding to sequences highly conserved within each tubulin subunit (Fig. 8A). Secondary structure predictions (54, 55) have suggested that most of the sequences in region II form an anionic α-helix (from amino acids 413–418 to amino acids 434–436 in α-tubulin and from amino acid 407 to amino acid 426 in β-tubulin), which likely corresponds to the ~25-Å-long density observed by Nogales et al. (55). It is interesting to note that in agreement with the putative structure we published recently (24), the DM1A and YOL1/34 epitopes are superimposed on the external face of this helix (Fig. 8B), leaving enough room for protein binding sites on the internal, anionic face. We suggest that the accessibility of these internal binding sites is controlled by the polyglutamyl chains located downstream (anchored at residues Glu445 in α-tubulin and Glu435 in class II β-tubulin; see Refs. 20, 32, and 56). In this view, the accessibility of this anionic face would be blocked in the nonglutamy-

Thus, it remains difficult to assign more precise locations for the kinesin and Tau binding sites on linear representations of tubulin molecules. It is relevant that GT335, which readily inhibits both Tau and kinesin binding, has also been shown recently to strongly inhibit dynein-based flagellar motility (57), suggesting that the polyglutamyl chains could play a crucial role in controlling the whole carboxyl-terminal domain of tubulin. Experiments are in progress to test and complete the data with other structural and motor MAPs.

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Interaction of Kinesin Motor Domains with α- and β-Tubulin Subunits at a Tau-independent Binding Site: REGULATION BY POLYGLUTAMYLLATION

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