THE JOURNAL OF BIOLOGICAL CHEMISTRY VOL. 284, NO. 47, pp. 32651–32661, November 20, 2009


# Interactions between EB1 and Microtubules

## *DRAMATIC EFFECT OF AFFINITY TAGS AND EVIDENCE FOR COOPERATIVE BEHAVIOR*[*S]

Received for publication, April 29, 2009, and in revised form, September 21, 2009 Published, JBC Papers in Press, September 23, 2009, DOI 10.1074/jbc.M109.013466

**Zhiqing C. Zhu[‡], Kamlesh K. Gupta[‡], Aranda R. Slabbekoorn[‡], Benjamin A. Paulson[‡], Eric S. Folker[§], and Holly V. Goodson[‡1]**

*From the [‡]Department of Chemistry and Biochemistry, University of Notre Dame, Notre Dame, Indiana 46556 and the [§]Department of Anatomy and Cell Biology, Columbia University, New York, New York 11032*

**Plus end tracking proteins (+TIPs) are a unique group of microtubule binding proteins that dynamically track microtubule (MT) plus ends. EB1 is a highly conserved +TIP with a fundamental role in MT dynamics, but it remains poorly understood in part because reported EB1 activities have differed considerably. One reason for this inconsistency could be the variable presence of affinity tags used for EB1 purification. To address this question and establish the activity of native EB1, we have measured the MT binding and tubulin polymerization activities of untagged EB1 and EB1 fragments and compared them with those of His-tagged EB1 proteins. We found that N-terminal His tags directly influence the interaction between EB1 and MTs, significantly increasing both affinity and activity, and that small amounts of His-tagged proteins act synergistically with larger amounts of untagged proteins. Moreover, the binding ratio between EB1 and tubulin can exceed 1:1, and EB1-MT binding curves do not fit simple binding models. These observations demonstrate that EB1 binding is not limited to the MT seam, and they suggest that EB1 binds cooperatively to MTs. Finally, we found that removal of tubulin C-terminal tails significantly reduces EB1 binding, indicating that EB1-tubulin interactions are mediated in part by the same tubulin acidic tails utilized by other MAPs. These binding relationships are important for helping to elucidate the complex of proteins at the MT tip.**

Microtubules (MTs)[2] are a major component of the cytoskeleton, the network of proteinacious fibers that endow the cell with structural integrity, motile properties, and internal organization (1–4). MTs play a particularly important role in cell organization: they help to pull the chromosomes apart at mitosis, act as a "railway" for intracellular transport, and define the localization and structure of internal membrane systems (5–11).

The cellular functions of MTs are highly dependent on their dynamic nature, which is regulated by a number of microtubule associated proteins (MAPs) (3, 9, 12, 13). The plus end tracking proteins (+TIPs) are an unusual group of MAPs that preferentially localize to growing MT plus ends (13–15). A large number of proteins have now been identified as +TIPs, but one of the most important is EB1 (end binding protein-1) (4, 13, 16, 17). EB1 was initially discovered as a binding partner of the adenomatous polyposis coli protein, but it is becoming apparent that EB1 binds to an astonishing array of other proteins including other +TIPs (CLIP-170, p150, and CLASPs), molecular motors (Tea2 and kinesin), signal transduction proteins (Rho-GEF2), and cytoskeletal scaffolding proteins (spectraplakins and formins) (4, 13, 15). As might be expected from a protein with so many interactions, EB1 is strikingly well conserved across eukaryotes (18–21). These observations suggest that EB1 is the structural and evolutionary core of a complex of proteins that regulates the behavior of MT plus ends (4, 13–15). However, its activities and the mechanisms of its activities are still poorly defined.

One reason for the difficulty in establishing EB1 function and activity is that the existing work on EB1 is contradictory. For example, Tirnauer *et al.* (22) reported that the $K_d$ of the EB1-MT interaction is 0.44 $\mu$M, whereas the behavior of EB1 (or its relatives) in other experiments has implied a much weaker interaction (23–25). Some studies have reported that EB1 can induce the polymerization of low concentrations of tubulin (26), whereas others report that EB1 by itself has no effect on MT polymerization; EB1 activity requires the removal of its autoinhibitory tail (aa 249–268) or activation by proteins that bind this tail (19, 27–29). Although one might predict that the greater activity of the activated EB1 fragment lacking the autoinhibitory tail ($EB1_{1-248}$) is due to its having a higher affinity for MTs, comparative affinities of full-length EB1 and $EB1_{1-248}$ have not yet been measured.

One explanation for the inconsistencies in reported EB1 activity is that the EB1 proteins used in these studies are not identical. All of the existing studies have used EB1 proteins purified by the use of affinity tags (*e.g.* histidine or glutathione *S*-transferase), but the tags and purification strategies differ. Some studies used proteins with the tags left intact (26–28), whereas others used tag-cleaved proteins (19, 24, 29, 30). In some cases where the tags were cleaved off, several amino acids still remained after the main tag was removed. Although it is often assumed that tags are benign, tags and tag remnants could alter inter- or intramolecular interactions, thus changing activity. Another potential explanation for inconsistency in reported EB1 activity is that EB1 might bind MTs cooperatively.

* This work was supported, in whole or in part, by National Institutes of Health Grant R01 GM065420 (to H. V. G.). This work was also supported by a Notre Dame Grace Fellowship (to Z. Z.).

[S] The on-line version of this article (available at http://www.jbc.org) contains supplemental Table S1 and Figs. S1–S6.

[1] To whom correspondence should be addressed: 439 Stepan Hall of Chemistry and Biochemistry, University of Notre Dame, Notre Dame, IN 46556. Tel.: 574-631-7744; Fax: 574-631-6652; E-mail: hgoodson@nd.edu.

[2] The abbreviations used are: MT, microtubule; PIPES, piperazine-*N*,*N*′-bis(2-ethanesulfonic acid); MAP, microtubule-associated protein; aa, amino acid(s); ST-MT, subtilisin-treated MT.

Researchers would observe different affinities and activities depending on how much EB1 they used.

To investigate these questions, we have characterized the MT binding behaviors of His-tagged, His-tag-cleaved, and untagged (classically purified) full-length EB1($EB1_{FL}$). We have compared these behaviors with each other and with those of similar constructs lacking the autoinhibitory tail ($EB1_{1-248}$). First, we found that the commonly utilized N-terminal His tags dramatically increase the activity of both $EB1_{FL}$ and $EB1_{1-248}$ as measured by both MT binding and polymerization assays. Second, removal of the C-terminal tail does increase the apparent affinity of EB1 for MTs (as expected). However, we were intrigued to observe that the binding behavior of both $EB1_{FL}$ and $EB1_{1-248}$ is complex. Therefore, the affinities of these proteins for MTs cannot be measured accurately from simple binding curves. Third, saturation studies confirm the complex MT binding behavior of both $EB1_{FL}$ and $EB1_{1-248}$ and indicate that $EB1_{1-248}$ can bind MTs at a ratio of greater than one EB1 dimer to one tubulin dimer. This binding ratio demonstrates that EB1 binding is not limited to the MT seam (seam-only binding predicts saturation at a ratio of 1 EB1 dimer to 13 tubulin dimers) (24) and suggests that EB1 molecules might bind to MT-bound EB1. Finally, we also found that small amounts of His-tagged EB1 proteins act synergistically with untagged EB1 proteins. Because it is difficult to fully cleave off the His tag without causing over-digestion of EB1, this observation suggests that some of the results reported for "tag-cleaved" EB1 may have been influenced by contaminating tagged EB1.

The sum of our observations indicates that native $EB1_{FL}$ and even native $EB1_{1-248}$ have intrinsically weak activity, contrary to the strong MT binding and polymerization activities previously reported with tagged proteins (22, 26). Our results also suggest that $EB1_{FL}$ and $EB1_{1-248}$ can bind to MTs cooperatively, thus increasing their activity. The apparent cooperativity is weak, but such weak cooperativity could be significant in the context of the complex of proteins at the MT tip.

## EXPERIMENTAL PROCEDURES

*Cloning, Expression, and Purification of EB1 Proteins*—The cDNA sequences of human $EB1_{FL}$ and $EB1_{1-248}$ were amplified by PCR and cloned into pET15b (Invitrogen, for His-tagged constructs) and pET21b (Invitrogen, for untagged constructs), and all of the constructs were verified by DNA sequencing. The proteins were expressed in the *Escherichia coli* strain BL21 (DE3). Bacteria were grown at 37 °C in LB medium containing 100 $\mu$g/ml ampicillin. After induction with isopropyl $\beta$-D-1-thiogalactopyranoside for 4 h, the cells were pelleted and lysed by sonication at 0 °C. His-tagged proteins were purified on a nickel-nitrilotriacetic acid column at 4 °C following the manufacturer's protocol (Novagen). To produce proteins with the His tag cleaved off ("His-cut" proteins), ~5 units/mg of human thrombin (Sigma-Aldrich) was added to His-tagged proteins and incubated for ~ 5 h at 4 °C. Subsequently, the proteins were passed through *p*-aminobenzamidine-agarose (Sigma-Aldrich) and nickel-nitrilotriacetic acid columns (Novagen) columns to remove the thrombin and excess His-tagged proteins. For purification of untagged EB1 proteins, the cell lysates were passed through sequential cation and anion exchange columns prepacked with DE52 and P11 resins (Whatman) at pH 7.9 and 6.8, respectively, and finally eluted with 100–300 mM NaCl.

After fractionation, all of the proteins were loaded onto columns prepacked with Bio-Gel P-6DG (Bio-Rad) where the buffer was changed to PEM (100 mM PIPES, 1 mM EGTA, 1 mM $MgCl_2$, 5 mM $\beta$-mercaptoethanol, pH 6.8). All of the proteins were flash frozen and stored at −80 °C. The concentrations of all proteins were measured using the Bradford protein assay (Bio-Rad) with bovine serum albumin as standard. Although EB1 is expected to be a dimer under most of the concentrations used in this work, the concentrations are presented as the EB1 monomer concentration, consistent with common practice in the EB1 literature.

*Preparation of Tubulin and Microtubules*—Tubulin was isolated from porcine brain by two cycles of polymerization and depolymerization as described (31). Taxol (paclitaxel; Sigma-Aldrich) MTs (32), Taxol-MT seeds (33), rhodamine-labeled tubulin (34), and subtilisin-treated Taxol-MTs (35) were prepared as described previously. Western blots were also used to verify that the subtilisin treatment fully removed the C-terminal tails from both $\alpha$- and $\beta$-tubulin (supplemental Fig. S3) (35–37).

*Microtubule Cosedimentation Assays*—To investigate the binding affinities between EB1 proteins and MTs, 2 $\mu$M of purified EB1 proteins (calculated as monomer concentration as is standard in the EB1 literature) were incubated with increasing amounts of Taxol-MTs (0–20 $\mu$M, calculated as tubulin dimer concentration) in PEM buffer at 37 °C for 30 min. After centrifugation (165,000 × *g*) at 37 °C for 15 min, separated supernatant and pellet fractions were loaded onto SDS-PAGE, and the band intensities were analyzed by Image J (National Institutes of Health). An EB1 standard was also loaded and used in quantifying the bound fractions of EB1. For initial attempts at estimating a $K_d$, data were fit to a bimolecular binding equation ($Y = (B_{max} * X)/(K_d + X)$, where $Y$ is the fraction of bound EB1 proteins, and $X$ is the concentration of free MTs) using Prism 5.0 (GraphPad) under nonlinear regression conditions. Except when otherwise indicated, all of the analyses were performed under the assumption of 1:1 stoichiometry (EB1 monomer: tubulin dimer), and the $B_{max}$ values were set to 1.0. Supplemental Fig. S1 shows that estimated $K_d$ values do not change significantly when 2:1 stoichiometry is used.

For the MT saturation assays, 2.5 $\mu$M of Taxol-MTs (calculated as tubulin dimer) were incubated with increasing amounts of EB1 proteins (0–20 $\mu$M, calculated as EB1 monomer). The theoretical curves (see Fig. 6, *dashed* and *dotted lines*) predicted for these MT saturation assays were obtained by considering the reaction as a simple 1:1 binding equilibrium E + M ↔ EM, where "E" represents EB1 dimer and "M" represents polymerized tubulin dimer (in this case, we used a 2:1 EB1 monomer: tubulin dimer stoichiometry because it fit better than 1:1; data not shown). The theoretical curves were produced by calculating $[E]_{bound}$ as a function of $[E]_{total}$ using the equation $[K_d = \{([E]_{total} - [E]_{bound}) * ([M]_{total} - [E]_{bound})\}/[E]_{bound}]$ (38) and used the assumed $K_d$ values extracted from the data in supplemental Fig. S1. Moreover, supplemental Fig. S2 shows that the data do not fit this equation regardless of the $K_d$ assumed.

*Tubulin Polymerization Assay*—Tubulin polymerization was measured by light scattering assay as previously described (39). Briefly, the reaction was started by adding tubulin (12 $\mu$M) into PEM buffer (final GTP concentration, 1 mM) containing EB1 protein (10 $\mu$M) in the absence or presence of MT seeds as indicated. Turbidity was recorded as the absorbance of light at 350 nm as measured by a spectrophotometer (Perkin Elmer Lamda 2). The measurements were taken at 6-s intervals, and the reaction solution was held at 37 °C.

*Light and Electron Microscopy*—For light microscopy, the reactions were initiated by the addition of 12 $\mu$M mixed tubulin (labeled:unlabeled = 1:10) into PEM (final GTP concentration, 1 mM) buffer in the presence of the various EB1 proteins (10 $\mu$M) or a buffer control. After 30 min of incubation, the samples were fixed with prewarmed 0.5% glutaraldehyde and then diluted 25× in PEM buffer before being placed onto the coverslips. Imaging was performed on a TE2000 inverted microscope (Nikon). Polymer structures were visualized using a 60× 1.4NA objective. The data were acquired from a cooled back-thinned CCD camera (Photometrics) controlled by Metamorph software (Molecular Devices). The images were further processed using Photoshop 7.1 (Adobe). For electron microscopy, the samples were prepared as described in the tubulin polymerization assay. After 30 min of incubation, the samples were fixed with prewarmed 0.5% glutaraldehyde and then diluted 25× before being applied to carbon-coated electron microscopy grids (300 mesh). The grids were subsequently negatively stained with 1% uranyl acetate solution. The images were acquired using an H-600 transmission electron microscope (Hitachi) and further processed by Photoshop 7.1 (Adobe).

*Proteolytic Digestion*—EB1 proteins were digested with proteinase K (EB1:protease = 500:1) in PEM buffer supplemented with 7.5 mM $CaCl_2$ at room temperature. Proteolysis was stopped by heating the samples in SDS sample buffer for 5 min. The digested fragments were resolved by 12% SDS-PAGE and stained with Coomassie Blue. An additional band at ~30 kDa appears immediately after the commencement of digestion in samples containing His-tagged proteins (see Fig. 7, *A* and *D*) and is due to a loss of the His tag, as shown by Western blots (supplemental Fig. S4).

*Western Blotting*—For Western blot analysis, the proteins were transferred from SDS-PAGE to nitrocellulose membrane (Whatman), blocked with milk solution, and then probed by anti-histidine-tagged protein (Novagen), anti-EB1 (BD Biosciences; immunogen aa 107–268), monoclonal anti-$\alpha$-tubulin 1A2 (a gift from Kreis's lab), or anti-$\beta$-tubulin DM1B (Sigma-Aldrich). Antibody detection was performed using anti-mouse horseradish peroxidase secondary antibody (BD Biosciences) followed by chemiluminescent substrate (Pierce).

## RESULTS

*Preparation of His-tagged, His-Tag-cleaved, and Untagged EB1 Proteins*—To clarify whether the commonly utilized hexahistidine affinity tag influences the activity of EB1, we purified three bacterially expressed variants of the full-length EB1 protein: 1) "His $EB1_{FL}$" is the $EB1_{FL}$ protein directly acquired from the nickel-nitrilotriacetic acid column. This protein has the purification tag containing six histidine residues at the N terminus. The exact composition of the tag can be found in supplemental Table S1. 2) "His-cut $EB1_{FL}$" is the product of His $EB1_{FL}$ treated with thrombin. Three extra (non-native) amino acids remain on the N terminus of this protein after thrombin treatment (supplemental Table S1). As discussed more under "Experimental Procedures," obtaining pure His-cut $EB1_{FL}$ requires using a post-digestion nickel-nitrilotriacetic acid column to remove the remaining His-tagged protein. 3) "untagged $EB1_{FL}$" is the native $EB1_{FL}$ sequence expressed in bacteria and purified by classical methods utilizing ion exchange chromatography. There are no non-native amino acids at either the N or C termini of this protein.

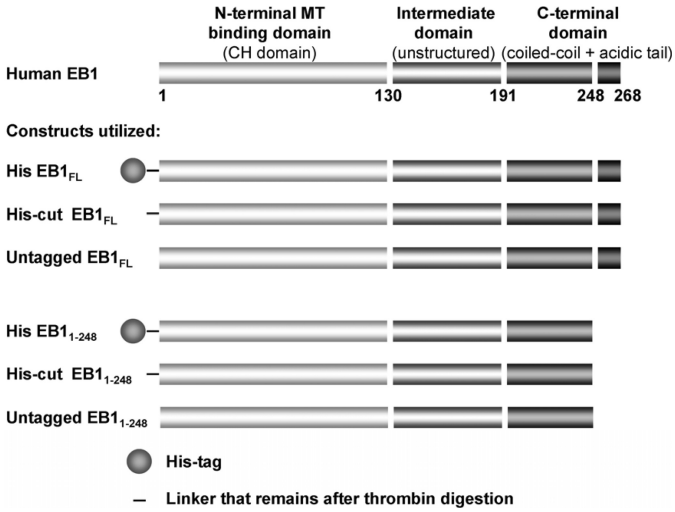


FIGURE 1. **Schematic representation of EB1/EB1 fragments used in our study.** His $EB1_{FL}$ and His $EB1_{1-248}$ have an N-terminal His tag. His-cut $EB1_{FL}$ and His-cut $EB1_{1-248}$ are the proteins that result from purification of thrombin-treated His $EB1_{FL}$ and His $EB1_{1-248}$, respectively. Untagged $EB1_{FL}$ and untagged $EB1_{1-248}$ have the native $EB1_{FL}$/$EB1_{1-248}$ sequences and are purified by classical methods. All of these EB1 proteins are expressed in bacteria. EB1 proteins truncated at aa 248 have been previously reported to be activated via a loss of tail-mediated autoinhibition (19, 24).

Previous work has indicated that EB1 exists in an autoinhibited form where the EB1 C-terminal tail (aa 249–268) has the ability to negatively regulate its polymerization promoting and MT binding activities (19, 24). To enable us to compare the activity of $EB1_{1-248}$ to that of $EB1_{FL}$, three $EB1_{1-248}$ proteins were produced using approaches analogous to those used to study the $EB1_{FL}$: 1) "His $EB1_{1-248}$" is the His-tagged $EB1_{1-248}$; 2) "His-cut $EB1_{1-248}$" is the $EB1_{1-248}$ with the His tag removed by thrombin digestion, leaving three amino acids at the N terminus; and 3) "untagged $EB1_{1-248}$" is the native $EB1_{1-248}$ sequence expressed in bacteria and purified by classical methods. A schematic representation of all of the EB1 constructs utilized in this work is shown in Fig. 1. The purified proteins are shown in Fig. 2*A*.

*Activities of Full-length EB1: Strong Effect of N-terminal His Tags*—As discussed above, there are major discrepancies in reported EB1-MT binding behaviors. To clarify the affinity of the EB1-MT interaction and test whether these discrepancies could result from differences related to affinity tags, we performed binding assays utilizing MT cosedimentation. Our data indicate that His $EB1_{FL}$ binds Taxol-MTs with much higher affinity than do either of the His-tag-free $EB1_{FL}$ proteins (His-cut or untagged); the $K_d$ values estimated from the best fit

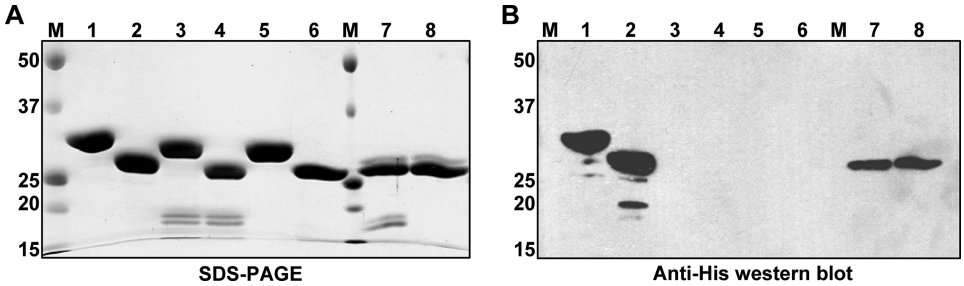


FIGURE 2. **SDS-PAGE and anti-His Western blot of purified EB1 proteins.** *A*, SDS-PAGE. *B*, Western blot. Molecular mass markers (*lane M*) and their molecular masses are indicated to the *left* of the figures in kDa. The samples are in the same order in both panels. *Lane 1*, His $EB1_{FL}$; *lane 2*, His $EB1_{1–248}$; *lane 3*, His-cut $EB1_{FL}$; *lane 4*, His-cut $EB1_{1–248}$; *lane 5*, untagged $EB1_{FL}$; *lane 6*, untagged $EB1_{1–248}$; *lane 7*, a mixture of 10% His $EB1_{1–248}$ + 90% His-cut $EB1_{1–248}$; *lane 8*, a mixture of 10% His $EB1_{1–248}$ + 90% untagged $EB1_{1–248}$.

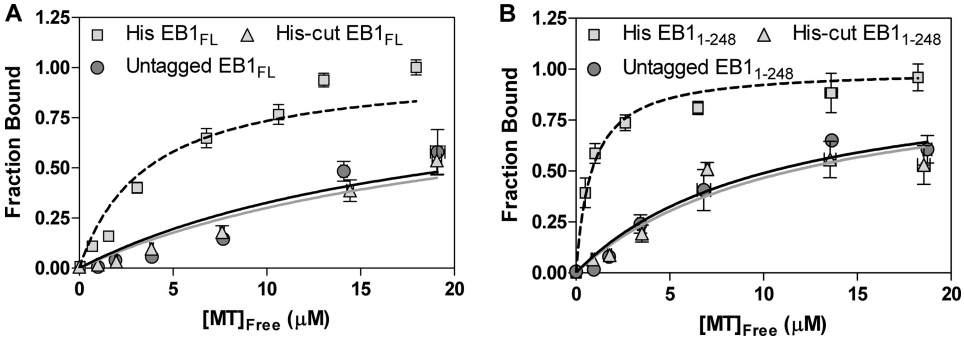


FIGURE 3. **The His tag influences $EB1_{FL}$ and $EB1_{1–248}$ activity in MT cosedimentation assays.** *A*, behavior of full-length EB1 proteins. *B*, behavior of the activated $EB1_{1–248}$ fragments. In both *A* and *B*, 2 $\mu$M (calculated as EB1 monomer) of the indicated EB1 proteins were incubated with varying concentrations of Taxol-stabilized MTs (0–20 $\mu$M, calculated as tubulin dimer) and centrifuged, and the resulting supernatants and pellet fractions were analyzed by SDS-PAGE. The concentration of free polymerized tubulin (*x* axis) was calculated from this data, assuming a 1:1 ratio (EB1 monomer:tubulin dimer). The "best fit" standard binding curves are shown for each protein; the poor fit of these curves to the data is discussed in the text. *A* shows that His $EB1_{FL}$ ($K_d$ = 3.6 ± 0.4 $\mu$M as estimated from the best fit curve) binds MTs more strongly than either His-cut $EB1_{FL}$ ($K_d$ = 23.3 ± 2.4 $\mu$M) or untagged $EB1_{FL}$ ($K_d$ = 20.6 ± 2.3 $\mu$M). *B* shows that His $EB1_{1–248}$ ($K_d$ = 0.8 ± 0.1 $\mu$M) binds MTs much more strongly than either His-cut $EB1_{1–248}$ ($K_d$ = 11.4 ± 1.4 $\mu$M) or untagged $EB1_{1–248}$ ($K_d$ = 10.4 ± 1.2 $\mu$M). Comparison of *A* and *B* indicate that $EB1_{1–248}$ constructs interact with MTs more strongly than do the analogous $EB1_{FL}$ constructs, supporting the idea that the flexible EB1 tail (aa 249–268) negatively regulates EB1 protein activity (19). Data points are the averages of three or more independent experiments and are plotted as the means ± S.E. (*vertical bars* represent error in the fraction bound, and *horizontal bars* represent error in $[MT]_{Free}$; where *error bars* are not visible, the error is smaller than the symbol for the data point).

standard binding curves were ~3 and ~20 $\mu$M, respectively (Fig. 3*A*). Thus, the hexahistidine affinity tag does influence the activity of $EB1_{FL}$, greatly increasing its ability to bind to MTs. However, it is important to note that it was difficult to derive accurate $K_d$ values from these experiments, because the data sets for the untagged and His-tag-cleaved EB1 proteins reproducibly fit poorly to the standard binding curve (Fig. 3*A*; discussed more below).

As mentioned above, it has been suggested that full-length EB1 can promote MT growth *in vitro* (26), but this conclusion remains controversial (19, 28, 29). To clarify whether EB1 has the ability to promote MT polymerization and whether EB1 polymerization promoting activity is also influenced by the presence of His tags, we performed tubulin polymerization (light scattering) assays with all three full-length EB1 proteins (His $EB1_{FL}$, His-cut $EB1_{FL}$, and untagged $EB1_{FL}$). We found that only His $EB1_{FL}$ promotes MT assembly at the concentrations used (10 $\mu$M EB1, 12 $\mu$M tubulin); His-cut and untagged $EB1_{FL}$ both have minimal influence (Fig. 4*A*). Even when the polymerization was performed in the presence of MT seeds, only His $EB1_{FL}$ promoted polymerization in a detectable way (Fig. 4*B*). These observations confirm that full-length EB1 has negligible effects on MT polymerization as measured by light scattering, even when it is present at high concentrations (19, 29). Combined with our previous results, these observations demonstrate that in addition to promoting binding of $EB1_{FL}$ to MT, the hexahistidine affinity tag also promotes $EB1_{FL}$ MT polymerization activity.

To verify that the observed changes in light scattering were due to the formation of MTs and not non-MT aggregates, we used both light and electron microscopy to investigate the morphologies and structures of the polymerized products. As expected, only a few short individual (not bundled) MTs were seen by light microscopy in controls containing tubulin alone in the absence of seeds (Fig. 5*A*, *inset a*). The addition of His $EB1_{FL}$ not only increased the number of MTs but also induced some bundling (Fig. 5*A*). We were surprised to see that the His-tag-free $EB1_{FL}$ proteins (both His-cut and untagged) caused a qualitative increase in the amount of polymer (Fig. 5, *B* and *C*) (it was difficult to measure this increase quantitatively). This observation suggests that these constructs do have some polymerization promoting activity and that light scattering assays are relatively insensitive to these small increases in polymer mass. To complement the light microscopy experiments, electron microscopy was used to investigate MT structure. We found that none of the three $EB1_{FL}$ proteins induced obvious protein aggregates, and the MTs formed in the presence of all three appeared to be normal (data not shown).

*Activities of the "Activated" Fragment $EB1_{1–248}$: Strong Effect of N-terminal His Tags*—Previous work has indicated that full-length EB1 is autoinhibited and that removal of the EB1 C-terminal tail (aa 249–268) relieves this autoinhibition (19, 27). Consistent with this work, recent studies have indicated that the activated fragment ($EB1_{1–248}$) binds MTs more strongly than does the full-length protein, although affinities were not measured (24). To quantify the MT binding behavior of $EB1_{1–248}$ and test whether the His tag affects this activated fragment in the same way that it affects full-length EB1, we conducted MT binding assays with His $EB1_{1–248}$ and the two His-tag-free $EB1_{1–248}$ proteins (His-cut and untagged). Using the same binding assays as described above, we found that His $EB1_{1–248}$ binds Taxol-MTs very strongly, whereas the other two $EB1_{1–248}$ proteins bound MTs weakly (the $K_d$ values estimated from the best fit curves were

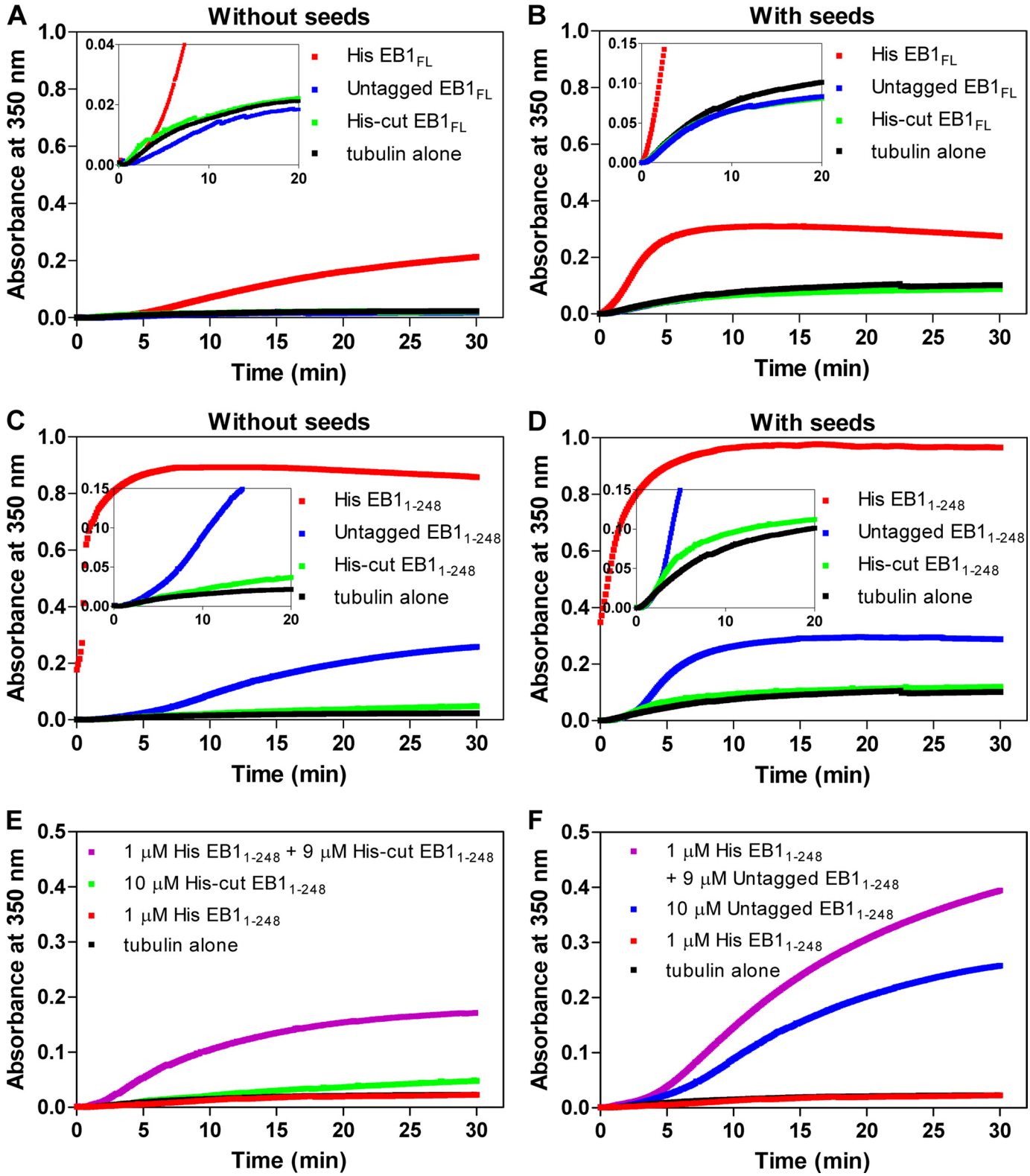


FIGURE 4. **Effect of EB1 constructs on tubulin polymerization (light scattering assays).** *A–D*, behavior of $EB1_{FL}$ (*A* and *B*) and $EB1_{1-248}$ (*C* and *D*) constructs. In each experiment, 10 $\mu$M EB1 protein as indicated (or buffer) was added to 12 $\mu$M cold tubulin and shifted to 37 °C, and the resulting change in absorbance was recorded at 6-s intervals. Higher absorbance indicates more polymerization, more bundling, or a combination of the two. The assays were conducted with and without MT seeds (*right* and *left panels*, respectively) to test for the possibility that the proteins can promote polymerization but not nucleation. The *insets* show the same data with an expanded *y* axis. These experiments show that 1) the His tag dramatically increases the activity of both $EB1_{FL}$ and $EB1_{1-248}$; 2) full-length EB1 constructs that lack the His tag (both His-cut and untagged) have no detectable activity in these assays; and 3) His-cut $EB1_{1-248}$ has much less polymerization promoting activity than untagged $EB1_{1-248}$ even through these proteins had similar MT binding activity as shown in Fig. 3*B*. *E* and *F*, synergistic effects of mixing small amounts of His-tagged EB1 proteins with larger amounts of His-tag-free EB1 proteins. Representative experiments with His-cut $EB1_{1-248}$ are shown in *E*, whereas analogous experiments with untagged $EB1_{1-248}$ are show in *F*. In both cases, the absorbance obtained from the mixed EB1 proteins is greater than the sum of the absorbance of each EB1 protein alone.

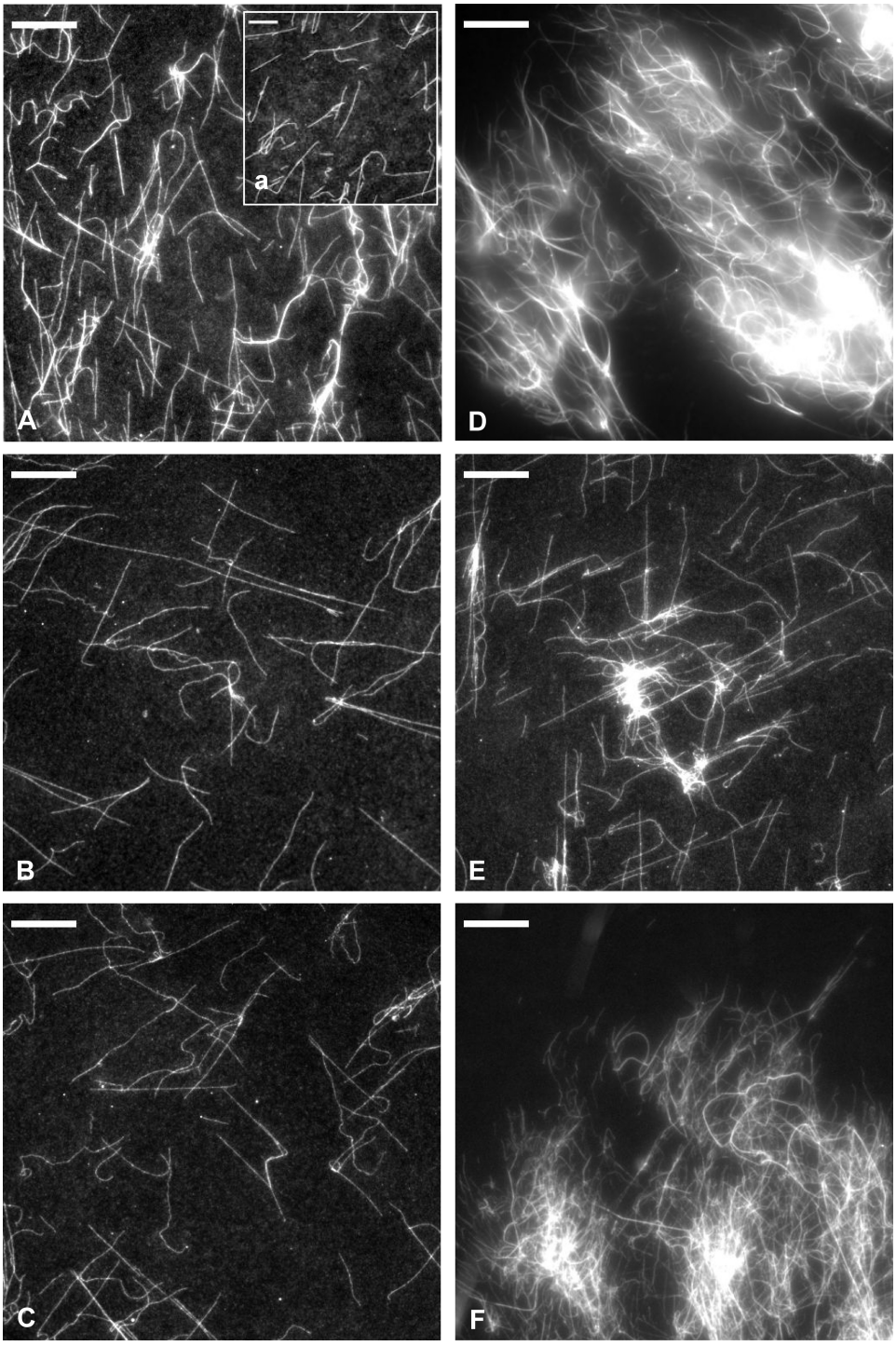


FIGURE 5. **Light microscopy of the polymers assembled in the presence of tubulin and EB1 proteins as indicated.** Samples were prepared as described under "Experimental Procedures." The images were acquired at 60× magnification, and the scale *bars* represent 20 $\mu$m. *A*, His $EB1_{FL}$. *inset a* shows the tubulin-only control, which has only a few short MTs. *B*, His-cut $EB1_{FL}$. *C*, untagged $EB1_{FL}$. *D*, His $EB1_{1-248}$. *E*, His-cut $EB1_{1-248}$. *F*, untagged $EB1_{1-248}$. *A–C* and *E* are adjusted to one consistent level chosen to optimize visualization of the single MTs in these images, whereas *D* and *F* are adjusted to a different level chosen to optimize visualization of the MT bundles seen in these experiments.

~0.5 and ~11 $\mu$M for the His-tagged and His-tag-free proteins respectively) (Fig. 3*B*).

Thus, the affinity tag has a strong influence on the MT binding behavior of the activated (tailless) EB1 fragment $EB1_{1-248}$. However, there are two other aspects of these experiments worth noting. First, as with $EB1_{FL}$, it was difficult to extract $K_d$ values from these experiments because the data sets for the His-tag-free proteins again fit poorly to the standard binding equation. The deviation from the curves was especially apparent for the His-tag-free $EB1_{1-248}$ proteins (both His-cut and untagged) at low tubulin concentrations (Fig. 3*B*). Second, although all versions of $EB1_{1-248}$ bound MTs more strongly than did the analogous full-length proteins, deletion of the autoinhibition domain seemed to affect the His-tagged protein more than its His-tag-free counterparts (Fig. 3, compare *A* and *B*).

To further investigate the activity of $EB1_{1-248}$, we also tested these three constructs in tubulin polymerization assays. His $EB1_{1-248}$ has a dramatic effect on tubulin polymerization (Fig. 4*C*). The addition of MT seeds causes little change, indicating that it is a strong nucleator at this concentration (10 $\mu$M) (Fig. 4*D*). untagged $EB1_{1-248}$ does induce MT polymerization, but it does so with much slower kinetics and achieves a lower maximum value (Fig. 4*C*). The kinetics of assembly in the presence of untagged $EB1_{1-248}$ are enhanced by the addition of MT seeds, indicating that this protein is a relatively poor nucleator (Fig. 4*D*). It is interesting to note that the His-cut $EB1_{1-248}$ has less polymerization promoting activity than untagged $EB1_{1-248}$, perhaps because of the remnant tag (3 amino acids). Light and electron microscopy confirmed that the increased signals in the light scattering assays reflect formation and bundling of MT polymer as opposed to formation of non-MT aggregates (Fig. 5, *D–F*, and data not shown).

Thus far, our experiments demonstrated that N-terminal His tags dramatically augment the interaction between EB1 proteins and MTs and that full-length His-tag-free EB1 proteins have little detectable polymerization promoting activity. However, we were puzzled by the poor fit of the binding data to standard binding equation. The slight "S" shape of the binding data for the His-tag-free proteins (Fig. 3) suggested the existence of cooperativ-

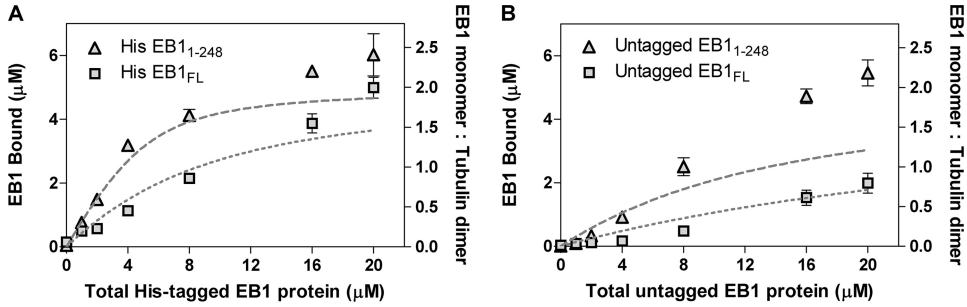


FIGURE 6. **Saturation binding experiments.** MTs (2.5 $\mu$M) were mixed with varying concentrations of EB1 proteins as indicated, and binding was assessed by standard MT cosedimentation assays. *A*, His-tagged EB1$_{FL}$ and EB1$_{1-248}$. *B*, untagged EB1$_{FL}$ and EB1$_{1-248}$. Theoretical curves plotted using a rearranged version of the standard binding equation (see "Experimental Procedures") and the $K_d$ values estimated from supplemental Fig. 1 are shown for each data set as *dotted* (EB1$_{FL}$) or *dashed* (EB1$_{1-248}$) *lines*. Inspection of these data and theoretical curves shows that EB1 saturation behavior conforms poorly to the expectations of a standard 1:1 EB1 dimer:tubulin dimer binding model.

ity, but it was not immediately apparent how binding of EB1 to MTs could depend cooperatively on the concentration of MTs as is implied by this data. To investigate the EB1-MT interaction in more depth, we examined MT saturation by EB1.

*Saturation of MTs by EB1: Evidence for EB1 Cooperative Binding*—Recently, cryo-electron microscopy work has provided evidence that the fission yeast EB1 homolog, Mal3p, binds preferentially to the seam of the MT lattice (25). Consequently, if Mal3p binds primarily to the seam, one would expect that binding of Mal3p to MTs would saturate at a ratio of 1 Mal3p dimer to 13 tubulin dimers. Consistent with this idea, MT binding studies of both Mal3p and human EB1 have been interpreted as supporting the idea that Mal3p and EB1 saturate MTs at a 1:13 ratio (24, 25). However, a more recent study observed that Mal3 saturates MTs at a 1:1 ratio (EB1 dimer: tubulin dimer) (40).

To resolve this discrepancy and test whether human EB1 can bind to sites on the MT other than the seam, we performed MT saturation assays in which we held the MT concentration constant and varied the concentration of EB1 protein. What we saw was surprising. First, untagged EB1$_{1-248}$ accumulates on MTs at a ratio of at least 1 EB1 dimer:1 tubulin dimer (Fig. 6*B*). This observation demonstrates conclusively that human EB1 can bind to the regular MT lattice, not just the seam. Second, the curve shows no sign of saturation at high EB1$_{1-248}$ concentrations, suggesting that EB1$_{1-248}$ can accumulate to ratios surpassing 1:1. Third and perhaps most importantly, the observed binding behavior is inconsistent with a simple binding model; the data fit poorly to the rearranged version of the standard binding equation regardless of the binding affinity or stoichiometry chosen (Fig. 6*B*, supplemental Figs. S1 and S2, and data not shown).

Similar observations were made for the other constructs analyzed. His EB1$_{FL}$ and His EB1$_{1-248}$ also accumulated to ratios at or above 1:1 (EB1 dimer:tubulin dimer). Interestingly, the behaviors of His-tagged and untagged EB1$_{1-248}$ were most different at low EB1 concentrations and became more similar at high EB1 concentrations (Fig. 6; this is discussed more later). Untagged EB1$_{FL}$ bound MTs too weakly to achieve saturation at the concentrations used but did accumulate to concentrations much higher than the 1:13 ratio expected for a "seam-only" binding model (Fig. 6*B*). The saturation behavior of all constructs was inconsistent with the simple binding equation regardless of the binding affinity or stoichiometry assumed (Fig. 6, supplemental Figs. S1 and S2, and data not shown). For most constructs, deviations from the predicted theoretical curves are particularly obvious at high EB1 concentrations (the data points fall above the predicted curves).

The incompatibility of the EB1 binding behaviors with standard binding models suggests that EB1 binds MTs cooperatively. The observation that EB1 dimers can bind polymerized tubulin dimers at a ratio approaching or exceeding 1:1 suggests that the mechanism of the cooperativity is binding of EB1 to MT-bound EB1. Alternatively, binding of EB1 to MTs could induce a conformational change in tubulin that increases EB1 binding, but under this scenario, it is difficult to explain how the binding ratio can exceed 1:1 as seen in Fig. 6.

*Synergism between His-tagged and His-tag-free EB1 Proteins*—To further investigate the idea that EB1 molecules interact with each other, we tested the effect of mixing His-tagged and His-tag-free proteins in polymerization assays. Strikingly, we found that when we mixed small amounts of His EB1$_{1-248}$ with larger amounts of His-tag-free proteins (either His-cut or untagged EB1$_{1-248}$), the light scattering signal increased dramatically above that obtained from either protein alone (Fig. 4, *E* and *F*). The observation that the signal is much greater than the sum of the signals obtained from the proteins by themselves indicates that small amounts of His EB1$_{1-248}$ interact synergistically with larger amounts of His-tag-free proteins.

The observation that His-tagged EB1 proteins can synergistically promote the activity of tag-free EB1 proteins supports the idea that EB1 proteins act cooperatively. It also suggests that some activities previously reported for His-tag-cleaved EB1 proteins may result in part from small amounts of contaminating His-tagged EB1 proteins. One could argue that this apparently synergistic behavior results from heterodimerization between the His-tagged and His-tag-free proteins, effectively increasing the concentration of the His-tagged proteins from 1 to 2 $\mu$M. We cannot rule out this explanation, but we find it unlikely because 2 $\mu$M His EB1$_{1-248}$ alone has an activity that is significantly less than that observed when 1 $\mu$M His EB1$_{1-248}$ is mixed with 9 $\mu$M His-tag-free EB1$_{1-248}$ proteins (supplemental Fig. S5).

*Investigating the Mechanism of the His-tag Effect*—The strong effect of His tag on EB1 behavior suggested that investigation of the origin of this effect might provide insight into the behavior of native EB1. One possibility is that the His tag causes a conformational change in EB1 that favors the EB1-MT interaction, perhaps by interfering with additional uncharacterized autoinhibition mechanisms. To test this idea, we performed proteinase K digestion assays. It is well established that proteinase K digestions provide a sensitive assay for differences in protein structure (41, 42). The expectation is that if the His-tag

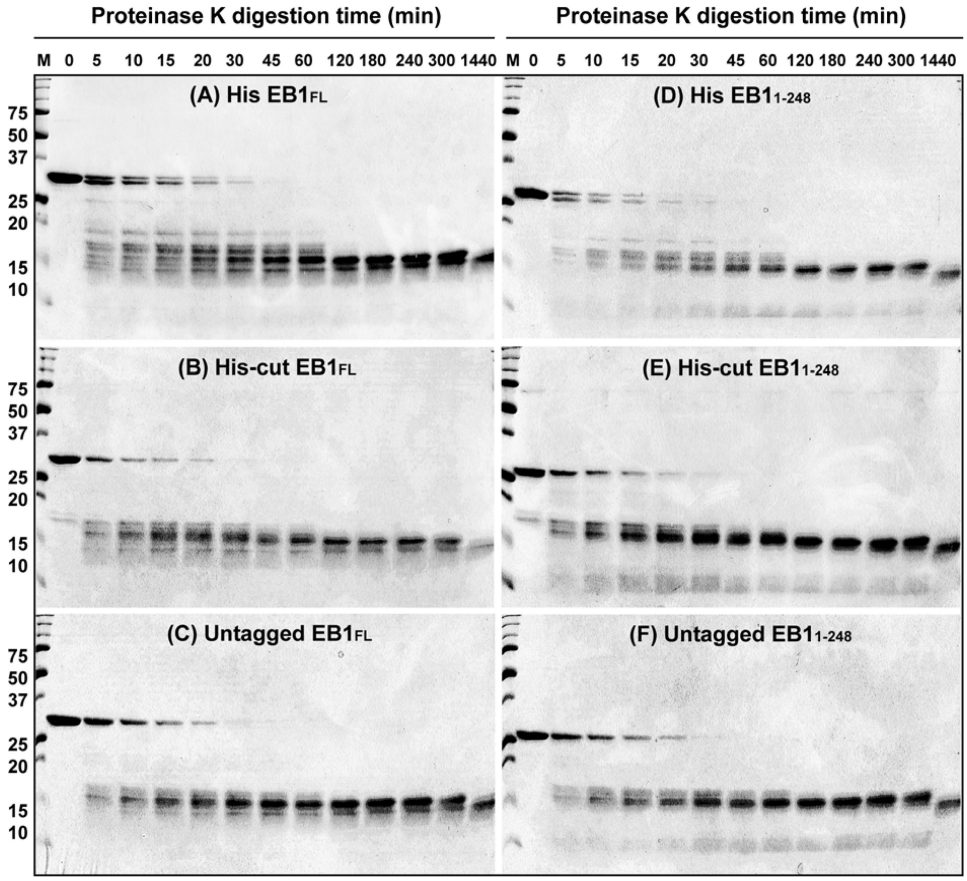


FIGURE 7. **Examination of possible structural differences between EB1 proteins by proteinase K digestion.** *A–F*, EB1 proteins (as indicated) were digested by proteinase K (1:500) for times as indicated. To gain information about the origins of the digested bands, anti-His and anti-EB1 Western blots were performed on the protein samples shown in *A* (supplemental Fig. S4).

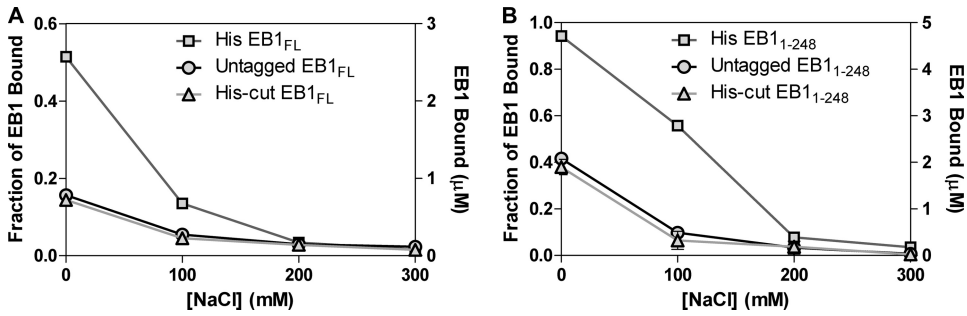


FIGURE 8. **The effect of salt on interactions between EB1 proteins and MTs.** EB1 proteins as indicated (5 $\mu$M) were incubated with MTs (5 $\mu$M) in the presence of varying concentrations of salt, and the fraction of EB1 bound was determined by MT cosedimentation. *A*, Full-length EB1 constructs. *B*, $EB1_{1-248}$ constructs. The data points represent the averages of three independent experiments and plotted as the means ± S.E.

induces a change in EB1 conformation, the His-tagged and His-tag-free EB1 proteins will have different proteinase K digestion patterns.

When we analyzed the proteinase K-treated samples, we found that the digestion patterns are indistinguishable (Fig. 7). This observation shows that the His tag does not dramatically alter the EB1 conformation and demonstrates that the lower activity of the His-tag-free proteins is not due to purification-induced unfolding. However, we cannot conclusively rule out the possibility that the His tag alters EB1 conformation in a minor way that is not detectable by proteinase K digestion assays. Further investigation is required to resolve this issue more decisively.

A second potential mechanism for the His-tag effect is that the tag could increase the affinity of EB1 for the MT surface. Because the His tag is partially positively charged in our assays (43), it is reasonable to propose that the His tag interacts with the negatively charged MT surface (15, 44). As a first test of this idea, we compared salt sensitivity of both His-tagged and His-tag-free EB1 proteins. First, we observed that the interaction between the His-tag-free EB1 proteins (both $EB1_{FL}$ and $EB1_{1-248}$) and MTs is abolished at moderate salt concentrations (100 mM; Fig. 8), consistent with previous work showing that the EB1-MT interaction is at least partially electrostatic (18). Further experiments showed that binding of the His-tagged EB1 proteins ($EB1_{FL}$ and $EB1_{1-248}$) is also abrogated by salt, but a complete loss of binding requires higher concentrations (Fig. 8). These observations suggest that the His tag enhances the EB1-MT interaction through an additional interaction that is also electrostatic.

*EB1 Binds to the Acidic C-terminal Tail of Tubulin*—The observation that binding of the His-tagged EB1 proteins to MTs is at least partially electrostatic suggests that the His tag might bind to the acidic C-terminal tail of tubulin (44–46). To test this hypothesis, we examined the ability of the various EB1 proteins to bind to subtilisin-treated MTs (ST-MTs). ST-MTs are Taxol-MTs that have been treated with subtilisin to remove the C-terminal tails of both $\alpha$- and $\beta$-tubulin (see supplemental Fig. S3 for characterization of the ST-MTs). Because removal of the C-terminal tyrosine of tubulin has no effect on EB1 binding *in vivo* (47), we expected that subtilisin treatment would have no effect on MT binding of untagged EB1. However, our data show that removal of the acidic tail greatly decreases the ability of both His-tagged and untagged $EB1_{FL}$/$EB1_{1-248}$ to bind to MTs (Fig. 9). This observation demonstrates that the acidic tail is involved in binding of native EB1 to MTs, as was originally proposed by Hayashi and Ikura (18). However, the observation that both His-tagged and untagged $EB1_{FL}$/$EB1_{1-248}$ proteins still retain some ability to bind ST-MTs (~30%) reveals that other parts of

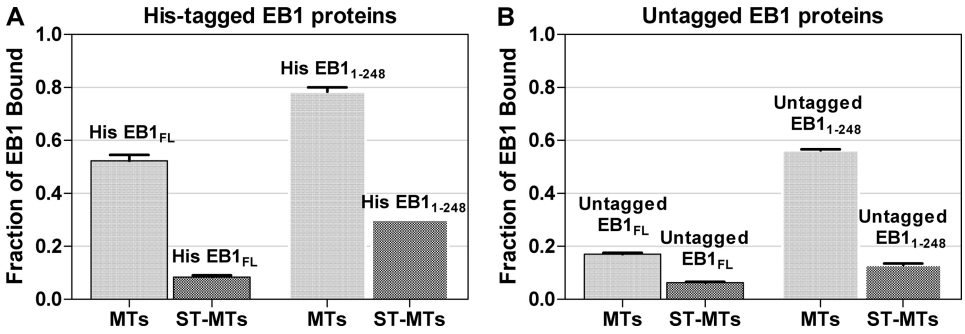


FIGURE 9. **Efficient binding of EB1 to MTs requires the tubulin C-terminal tail.** In these experiments, EB1 constructs as indicated (2 $\mu$M) were incubated with either standard Taxol-stabilized MTs (*MTs*, 10 $\mu$M) or Taxol-stabilized MTs treated with subtilisin to remove the C-terminal tails of both $\alpha$- and $\beta$-tubulin (*ST-MTs*, 10 $\mu$M). The fraction of EB1 bound to these MTs was then analyzed by MT cosedimentation. The data represent the averages of three independent assays and are plotted as the means ± S.E.

the tubulin dimer contribute to the EB1-MT interaction as well. The observation that the His-tagged EB1 proteins bind better to ST-MTs than do their untagged counterparts proteins suggests that the His tag binds to a part of the MT other than the acidic tail.

## DISCUSSION

*Variability in Reported EB1 Behaviors and the Effect of His Tag on EB1 Activity*—EB1 appears to be both the evolutionary and structural core of the complex of proteins at the MT plus end (4, 13, 16, 17), but it remains poorly understood, in part because of variability in reported activities (18–30). All of the existing studies of EB1 have used proteins that were purified via affinity tags. We find that N-terminal histidine tags dramatically increase both EB1-MT affinity and polymerization promoting activity (Figs. 3 and 4) and that small amounts of contaminating His-tagged proteins act synergistically with larger amounts of His-tag-free proteins (Fig. 4, *E* and *F*). Moreover, it is hard to remove the His tags by thrombin without cleaving EB1 itself or leaving contaminating His-tagged proteins (data not shown). Therefore, we propose that part of the reason for inconsistency in the EB1 literature is the variable presence of His-tagged proteins. A second reason for the variability in reported EB1 activity, discussed more below, is that both His-tagged and untagged EB1 proteins bind MTs with complex, apparently cooperative behavior.

*Activity and Autoinhibition of Native EB1*—It has been suggested that the C-terminal tail of EB1 (aa 249–268) acts as an autoinhibitory domain, promoting interactions between the EB1 C-terminal domain (aa 183–268) and the MT-binding head (aa 1–130) (19). Our data are consistent with this idea; $EB1_{1-248}$ constructs have comparatively more activity than the analogous $EB1_{FL}$ constructs in both microtubule binding and polymerization promoting assays (Figs. 3 and 4).

However, the overall weakness of these activities is surprising: under standard conditions, $EB1_{FL}$ and $EB1_{1-248}$ constructs without His tags are half-saturated by Taxol-MTs at ~20 and ~10 $\mu$M, respectively. One explanation of these observations could be that Taxol-MTs are in the wrong conformation for optimal EB1 binding; it is expected that EB1 binds strongly to tip-like or seam-like conformations and weakly to GDP lattice conformations. However, it is important to note that untagged $EB1_{1-248}$ is also a relatively poor promoter of MT assembly; it robustly induces MT assembly when present at high concentrations (10 $\mu$M; see Fig. 4*C*) but has little effect at nearly physiological concentrations (data not shown, but see Fig. 4 for the weak activity of His $EB1_{1-248}$ at 1 $\mu$M). These observations suggest either that native sequence $EB1_{1-248}$ may require additional factors to be fully activated or that it must work in concert with other proteins to promote MT assembly.

*Evidence for Cooperative Behavior*—An additional observation from the binding experiments is that the data for most EB1 constructs fit poorly to standard binding curves (Fig. 3 and supplemental Fig. S1). This behavior suggested that EB1-MT interactions might be complex and prompted us to perform MT saturation experiments. However, the saturation data diverged even more significantly from standard binding models (Fig. 6 and supplemental Fig. S2), confirming that EB1-MT interactions are not simple.

By themselves, these data reveal little about the mechanism of the complex behavior. However, closer examination of the saturation data suggests that the His $EB1_{1-248}$ construct exhibits two binding phases (Fig. 6*A*): an initial fast phase that approaches saturation at a ratio ~1:1 (EB1 dimer:tubulin dimer) and a later slower phase that does not saturate even as the EB1 dimer:tubulin dimer ratio exceeds 1:1. Other constructs also appear to have two binding phases, although the relative strength of these phases differs. The existence of two phases implies that two separable interactions are involved in binding of EB1 to MTs, whereas failure to saturate at a 1:1 ratio indicates either that one tubulin dimer can directly bind more than one EB1 dimer or that EB1 binds to MT-bound EB1. One could argue that the second phase, which consists of a nearly linear increase in MT binding at high EB1 concentration, is due to nonspecific interactions. However, weak EB1-EB1 interactions could become significant in the context of the complex of proteins at the MT tip.

The observation that $EB1_{1-248}$ constructs can achieve a 1:1 (or greater) ratio is significant for another reason: recent cryo-electron microscopy work has provided evidence that the fission yeast EB1 homolog Mal3p binds preferentially to the seam of the MT lattice (25), and MT binding studies of both Mal3p and human EB1 have been interpreted as supporting the idea that both proteins saturate MTs at a 1:13 ratio (24, 25). However, an alternative way to interpret these data is that Mal3p and EB1 bind MTs with weak affinity. Indeed, a more recent study found that Mal3 can accumulate on MTs at a 1:1 ratio (40). Consistent with this Mal3 observation, our data show that both $EB1_{FL}$ and $EB1_{1-248}$ can accumulate to ratios much higher than 1:13 and so demonstrate conclusively that human EB1 can also bind to the regular MT lattice, not just the seam.

We conclude from these data that binding of EB1 to MTs is complex and likely involves at least two separable interactions.

Although we cannot definitively assign a mechanism for this behavior, we suggest that these data are most consistent with a model where EB1 binds to MTs cooperatively, and does so through a mechanism in which EB1 binds to MTs directly, but also binds to MT-bound EB1. This model would explain sigmoidal aspects of the data in Fig. 3, as well as shape and supersaturation aspects of Fig. 6. We cannot rule out the possibility that EB1 alters MT conformation in a way that promotes EB1 binding, but under this model, it is difficult to explain the supersaturation observed in Fig. 6.

*EB1-binding Site(s) on Tubulin/MTs*—Initial work on the EB1-MT interaction indicated that this interaction is electrostatic in nature and predicted that it is mediated by the interaction between the positively charged EB1 N-terminal MT-binding domain and the negatively charged C-terminal acidic tail of tubulin (18). However, the observation that removal of the C-terminal tyrosine of tubulin has no effect on EB1 binding (47) led researchers away from the idea that the tubulin tail is involved in EB1-MT interactions. Our observation that EB1-MT interactions can be disrupted by salt (Fig. 8) confirms the conclusion that the interaction between EB1 and MTs is mediated by electrostatic interactions. Moreover, we found that removal of the tubulin acidic tail greatly reduces the ability of EB1 to bind MTs (Fig. 9), confirming the original prediction (18) that the tubulin tail mediates part of the interaction with EB1. This also means that EB1 binds to the same part of the MT as do CLIP-170 and many more classical MAPs such as tau (45, 48, 49). However, untagged (native sequence) $EB1_{FL}$/$EB1_{1-248}$ still retains ~30% binding to ST-MTs (10 $\mu$M), indicating that EB1 also interacts with other parts of tubulin in addition to the C-terminal acidic tail.

*Use of Affinity Tags in Future EB1 Work*—Affinity tags greatly facilitate protein purification (50–52). In many cases they are benign, but they can affect protein behavior in complex ways including altering protein dimerization (43, 53), conformation (50), and binding to other proteins (54). In this study, we established that N-terminal His tags dramatically increase EB1 microtubule binding and polymerization promoting activity. It is important to note that the effect of His tags on EB1 is not limited to the pET15b vector used in this study; His-tagged $EB1_{1-248}$ expressed from the pDEST17 vector has even greater activity than His-tagged $EB1_{1-248}$ expressed from the pET15b vector under the same experimental conditions, perhaps because the tag is longer (data not shown).

One might expect that cleavage of the His tag would take care of this problem, but we observed two difficulties with this approach. First, in our hands, over-digestion of EB1 occurred unavoidably before removal of the His tag was complete (data not shown), resulting in contamination of tag-cleaved proteins by tagged proteins. We found that removal of these tagged proteins by an additional "clean-up" nickel column was essential to our characterization because small amounts of contaminating His-tagged proteins can act synergistically with larger amounts of His-tag-free EB1 proteins (Fig. 4*E*). Although we have tested only His-tagged proteins for synergistic effects, we suspect that tags such as GST could have similar effects, especially if they mediate multidimerization.

A second problem with tag-cleaved proteins is that they behaved differently from untagged EB1 proteins in MT polymerization assays (Fig. 4, *C* and *D*). We have not determined the reason for this difference, but we think that it is unlikely to be due to over-digestion or folding problems because the tag-cleaved protein behaved indistinguishably from untagged protein in the proteinase K assays (Fig. 7). The problem may be related to the remnant linker (3 amino acids) left at the EB1 N terminus after the His tag is removed (supplemental Table S1). The idea that such a small tag could be problematic is surprising, but the striking conservation of the EB1 N terminus (18) suggests that the extreme N terminus of EB1 has an important role in EB1 activity.

On the basis of these observations, we suggest that the following points be considered for future analysis of EB1 *in vitro*: 1) When tags of any type are used for purification, it is important to remove them before working with the purified protein; we have demonstrated here that N-terminal tags alter EB1 activity, but we are suspicious that C-terminal tags could have similar effects by interfering with EB1 autoinhibition; 2) it is best to use vector systems that leave no tag remnants behind after cleavage; 3) when tags are removed, Western blot data should be presented to verify that no contaminating tagged proteins remain (Fig. 2*B*); and 4) although we have presented here only data on human EB1, we predict that His tags have similar effects on EB1 proteins from other organisms, given the strong conservation of EB1 structure and function across evolution.

*Acknowledgments—We thank Susan Skube, Jill Dzurisin, and Julia Philips for lab and materials management and for engaging in insightful discussions; Elena Lastochkin, José Chaverri, Geri Ugrinova, Ivan Gregoretti, Aris Alexandrou, Wenhui Zhang, and Xiaosong Hu for assistance with preparation of materials and helpful discussions; Kurt Piepenbrink and Krastyu Ugrinov for advice on the use of instrumentation; and Mary Prorok for the analytical ultracentrifugation experiments. We are also grateful to Michel Steinmetz and Ewan Morrison for critical analysis and helpful suggestions in the early stages of this work.*

## REFERENCES

1. Desai, A., and Mitchison, T. J. (1997) *Annu. Rev. Cell Dev. Biol.* **13,** 83–117
2. Galjart, N., and Perez, F. (2003) *Curr. Opin. Cell Biol.* **15,** 48–53
3. Howard, J., and Hyman, A. A. (2003) *Nature* **422,** 753–758
4. Lansbergen, G., and Akhmanova, A. (2006) *Traffic* **7,** 499–507
5. Caviston, J. P., and Holzbaur, E. L. (2006) *Trends Cell Biol.* **16,** 530–537
6. Kline-Smith, S. L., and Walczak, C. E. (2004) *Mol. Cell* **15,** 317–327
7. Müsch, A. (2004) *Traffic* **5,** 1–9
8. Zhu, H., Fang, K., and Fang, G. (2009) *Mol. Cells* **27,** 1–3
9. Glotzer, M. (2009) *Nat. Rev. Mol. Cell Biol.* **10,** 9–20
10. Akhmanova, A., Stehbens, S. J., and Yap, A. S. (2009) *Traffic* **10,** 268–274
11. Li, R., and Gundersen, G. G. (2008) *Nat. Rev. Mol. Cell Biol.* **9,** 860–873
12. Gardner, M. K., Hunt, A. J., Goodson, H. V., and Odde, D. J. (2008) *Curr. Opin. Cell Biol.* **20,** 64–70
13. Morrison, E. E. (2007) *Cell Mol. Life Sci.* **64,** 307–317
14. Akhmanova, A., and Hoogenraad, C. C. (2005) *Curr. Opin. Cell Biol.* **17,** 47–54
15. Akhmanova, A., and Steinmetz, M. O. (2008) *Nat. Rev. Mol. Cell Biol.* **9,** 309–322
16. Vaughan, K. T. (2005) *J. Cell Biol.* **171,** 197–200
17. Honnappa, S., Gouveia, S. M., Weisbrich, A., Damberger, F. F., Bhavesh, N. S., Jawhari, H., Grigoriev, I., van Rijssel, F. J., Buey, R. M., Lawera, A.,

Jelesarov, I., Winkler, F. K., Wüthrich, K., Akhmanova, A., and Steinmetz, M. O. (2009) *Cell* **138,** 366–376
18. Hayashi, I., and Ikura, M. (2003) *J. Biol. Chem.* **278,** 36430–36434
19. Hayashi, I., Wilde, A., Mal, T. K., and Ikura, M. (2005) *Mol. Cell* **19,** 449–460
20. Honnappa, S., John, C. M., Kostrewa, D., Winkler, F. K., and Steinmetz, M. O. (2005) *EMBO J.* **24,** 261–269
21. Slep, K. C., Rogers, S. L., Elliott, S. L., Ohkura, H., Kolodziej, P. A., and Vale, R. D. (2005) *J. Cell Biol.* **168,** 587–598
22. Tirnauer, J. S., Grego, S., Salmon, E. D., and Mitchison, T. J. (2002) *Mol. Biol. Cell* **13,** 3614–3626
23. Ligon, L. A., Shelly, S. S., Tokito, M. K., and Holzbaur, E. L. (2006) *FEBS Lett.* **580,** 1327–1332
24. Manna, T., Honnappa, S., Steinmetz, M. O., and Wilson, L. (2008) *Biochemistry* **47,** 779–786
25. Sandblad, L., Busch, K. E., Tittmann, P., Gross, H., Brunner, D., and Hoenger, A. (2006) *Cell* **127,** 1415–1424
26. Vitre, B., Coquelle, F. M., Heichette, C., Garnier, C., Chrétien, D., and Arnal, I. (2008) *Nat. Cell Biol.* **10,** 415–421
27. Ligon, L. A., Shelly, S. S., Tokito, M., and Holzbaur, E. L. (2003) *Mol. Biol. Cell* **14,** 1405–1417
28. Nakamura, M., Zhou, X. Z., and Lu, K. P. (2001) *Curr. Biol.* **11,** 1062–1067
29. Slep, K. C., and Vale, R. D. (2007) *Mol. Cell* **27,** 976–991
30. Dixit, R., Barnett, B., Lazarus, J. E., Tokito, M., Goldman, Y. E., and Holzbaur, E. L. (2009) *Proc. Natl. Acad. Sci. U.S.A.* **106,** 492–497
31. Panda, D., Jordan, M. A., Chu, K. C., and Wilson, L. (1996) *J. Biol. Chem.* **271,** 29807–29812
32. Folker, E. S., Baker, B. M., and Goodson, H. V. (2005) *Mol. Biol. Cell* **16,** 5373–5384
33. Gupta, K. K., Paulson, B. A., Folker, E. S., Charlebois, B., Hunt, A. J., and Goodson, H. V. (2009) *J. Biol. Chem.* **284,** 6735–6742
34. Hyman, A., Drechsel, D., Kellogg, D., Salser, S., Sawin, K., Steffen, P., Wordeman, L., and Mitchison, T. (1991) *Methods Enzymol.* **196,** 478–485
35. Spittle, C., Charrasse, S., Larroque, C., and Cassimeris, L. (2000) *J. Biol. Chem.* **275,** 20748–20753
36. Skiniotis, G., Cochran, J. C., Müller, J., Mandelkow, E., Gilbert, S. P., and Hoenger, A. (2004) *EMBO J.* **23,** 989–999
37. Saoudi, Y., Paintrand, I., Multigner, L., and Job, D. (1995) *J. Cell Sci.* **108,** 357–367
38. Andersen, S. S., Buendia, B., Domínguez, J. E., Sawyer, A., and Karsenti, E. (1994) *J. Cell Biol.* **127,** 1289–1299
39. Gaskin, F., Cantor, C. R., and Shelanski, M. L. (1974) *J. Mol. Biol.* **89,** 737–755
40. des Georges, A., Katsuki, M., Drummond, D. R., Osei, M., Cross, R. A., and Amos, L. A. (2008) *Nat. Struct. Mol. Biol.* **15,** 1102–1108
41. Renn, J. P., and Clark, P. L. (2008) *Biopolymers* **89,** 420–427
42. Junker, M., Schuster, C. C., McDonnell, A. V., Sorg, K. A., Finn, M. C., Berger, B., and Clark, P. L. (2006) *Proc. Natl. Acad. Sci. U.S.A.* **103,** 4918–4923
43. Zhukovsky, E. A., Lee, J. O., Villegas, M., Chan, C., Chu, S., and Mroske, C. (2004) *Nature* **427,** 413–414
44. Lakämper, S., and Meyhöfer, E. (2006) *J. Muscle Res. Cell Motil.* **27,** 161–171
45. Nogales, E., Whittaker, M., Milligan, R. A., and Downing, K. H. (1999) *Cell* **96,** 79–88
46. Lowe, J., Li, H., Downing, K. H., and Nogales, E. (2001) *J. Mol. Biol.* **313,** 1045–1057
47. Peris, L., Thery, M., Fauré, J., Saoudi, Y., Lafanechère, L., Chilton, J. K., Gordon-Weeks, P., Galjart, N., Bornens, M., Wordeman, L., Wehland, J., Andrieux, A., and Job, D. (2006) *J. Cell Biol.* **174,** 839–849
48. Al-Bassam, J., Ozer, R. S., Safer, D., Halpain, S., and Milligan, R. A. (2002) *J. Cell Biol.* **157,** 1187–1196
49. Devred, F., Barbier, P., Douillard, S., Monasterio, O., Andreu, J. M., and Peyrot, V. (2004) *Biochemistry* **43,** 10520–10531
50. Chant, A., Kraemer-Pecore, C. M., Watkin, R., and Kneale, G. G. (2005) *Protein Expr. Purif.* **39,** 152–159
51. Nieba, L., Nieba-Axmann, S. E., Persson, A., Hämäläinen, M., Edebratt, F., Hansson, A., Lidholm, J., Magnusson, K., Karlsson, A. F., and Plückthun, A. (1997) *Anal. Biochem.* **252,** 217–228
52. Paborsky, L. R., Dunn, K. E., Gibbs, C. S., and Dougherty, J. P. (1996) *Anal. Biochem.* **234,** 60–65
53. Amor-Mahjoub, M., Suppini, J. P., Gomez-Vrielyunck, N., and Ladjimi, M. (2006) *J Chromatogr. B Analyt. Technol. Biomed. Life Sci.* **844,** 328–334
54. Goel, A., Colcher, D., Koo, J. S., Booth, B. J., Pavlinkova, G., and Batra, S. K. (2000) *Biochim. Biophys. Acta* **1523,** 13–20