The FKBP12-rapamycin-associated protein (FRAP) is a CLIP-170 kinase

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CLIP-170/Restin belongs to a family of conserved microtubule (MT)-associated proteins, which are important for MT organization and functions. CLIP-170 is a phosphoprotein and phosphorylation is thought to regulate the binding of CLIP-170 to MTs. However, little is known about the kinase(s) involved. In this study, we show that FKBP12-rapamycin-associated protein (FRAP, also called mTOR/RAFT) interacts with CLIP-170. CLIP-170 is phosphorylated *in vivo* at multiple sites, including rapamycin-sensitive and -insensitive sites, and is phosphorylated by FRAP *in vitro* at the rapamycin-sensitive sites. In addition, rapamycin inhibited the ability of CLIP-170 to bind to MTs. Our observations suggest that multiple CLIP-170 kinases are involved in positive and negative control of CLIP-170, and FRAP is a CLIP-170 kinase positively regulating the MT-binding behavior of CLIP-170.

INTRODUCTION

The FKBP12-rapamycin-associated protein (FRAP, also called mTOR/RAFT) in mammals and its orthologs Tor1/2 in yeast are highly conserved regulators of cell growth and proliferation (Abraham, 1998; Dennis *et al.*, 1999; Kuruvilla and Schreiber, 1999; Schmelzle and Hall, 2000; Raught *et al.*, 2001; Rohde *et al.*, 2001). Rapamycin is an antibiotic currently undergoing advanced clinical trials for cancer treatment and is a highly specific inhibitor of FRAP/TOR. FRAP/TOR is a protein serine/threonine kinase whose activity is crucial for cell proliferation and signaling to p70S6K1, 4EBP1/PHAS-I and Gln3 (Brown *et al.*, 1995; Zheng *et al.*, 1995; Brunn *et al.*, 1997; Burnett *et al.*, 1998; Bertram *et al.*, 2000).

Microtubules (MTs) play critical roles for diverse cellular functions (Desai and Mitchison, 1997; Cassimeris, 1999; Wittmann *et al.*,

2001). CLIP-170 belongs to a family of conserved MT-associated proteins (MAPs) called CLIPs that regulate MT dynamics and functions (Rickard and Kreis, 1991; Sawin, 2000; McNally, 2001; Schuyler and Pellman, 2001). CLIPs include CLIP-170 and CLIP-115 in vertebrates (Pierre *et al.*, 1992; De Zeeuw *et al.*, 1997), CLIP-190 in *Drosophila* (Lantz and Miller, 1998), Bik1 in budding yeast (Berlin *et al.*, 1990) and Tip1 in fission yeast (Brunner and Nurse, 2000). CLIPs usually contain MT-binding head domains at their N-termini, heptad repeats and putative metal-binding motifs at their C-termini (Scheel *et al.*, 1999; Hoogenraad *et al.*, 2000). CLIPs move along the growing ends of MTs (Perez *et al.*, 1999; Brunner and Nurse, 2000; Hoogenraad *et al.*, 2000). They also appear to be necessary for maintaining normal MT structures since depletion of Bik1 in yeast leads to shorter and thinner cytoplasmic MTs and spindles (Berlin *et al.*, 1990). There are two possible mechanisms by which CLIPs regulate MTs. CLIP-associated proteins (CLASPs) control MT orientation and dynamics (Akhmanova, 2001). In addition, cell-free extracts contain a kinase activity that inhibits the ability of CLIP-170 and CLIP-115 to bind to MTs, suggesting that phosphorylation is involved (Rickard and Kreis, 1991; Hoogenraad *et al.*, 2000).

Recent biochemical and genetic evidence indicates that TOR binds to and acts as an upstream regulator of Bik1 in yeast (Choi *et al.*, 2000). Here we show that FRAP binds to CLIP-170 via its N-terminal HEAT repeats in human cells. FRAP phosphorylates CLIP-170 *in vitro* at sites that are sensitive to rapamycin *in vivo*, and regulates the binding of CLIP-170 to MTs *in vitro*. These data suggest that FRAP is a CLIP-170 kinase positively regulating the ability of CLIP-170 to bind to MTs.

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RESULTS AND DISCUSSION

We have shown previously that the yeast target of rapamycin proteins Tor1 and Tor2 interacted with Bik1 (Choi *et al.*, 2000). Because the FRAP/TOR and CLIP-170–Bik1 protein pairs are conserved, we investigated a possible interaction between FRAP and CLIP-170 in human embryonic kidney 293 (HEK293) cells by co-immunoprecipitation (IP). We found that CLIP-170 was present in the anti-FRAP IP, but not in the IP with a preserum, indicating a specific interaction between the two proteins (Figure 1A). To confirm this result, we expressed FLAG-FRAP and MYC-CLIP-170 in HEK293 cells. We found that the FLAG antibody precipitated MYC-CLIP-170 only in the presence of FLAG-FRAP, but not in its absence (Figure 1B). Conversely, the MYC-specific antibody precipitated FLAG-FRAP only in the presence of MYC-CLIP-170 (Figure 1B). We further studied whether the proteins could interact directly. We generated bacterially produced His₆-FRAP(aa 501–930) and GST–CLIP-170(aa 1–348). These regions of FRAP and CLIP-170 are sufficient to interact in the cell (see Figure 2). We found that His₆-FRAP(aa 501–930) bound to GST–CLIP-170(aa 1–348), but not to GST (Figure 1C). Therefore, FRAP and CLIP-170 appear to interact in a direct fashion.

We next characterized the regions involved in the interaction between the two proteins. By sequential deletion, we found that the N-terminal 348 amino acids of CLIP-170 were sufficient to bind FRAP in a co-IP experiment (Figure 2A). The same region of Bik1 also interacted with Tor1 in a yeast two-hybrid interaction assay (Figure 2B). Interestingly, when a fragment of Bik1 that contained the head domain, but lacked a serine-rich motif was used, no interaction was observed (Figure 2B). Hence, the MT-binding head domain alone appears to be insufficient for interacting with TOR proteins. On the other hand, we found that the amino acids 501–930 of FRAP, which contains the second cluster of HEAT repeats, were capable of interacting with CLIP-170 (Figure 2C; Supplementary data available at *EMBO reports* Online). Like the A subunit of PP2A, the FRAP HEAT domains are involved in protein–protein interaction.

Because FRAP is a kinase and CLIP-170 is a phosphoprotein, we investigated whether CLIP-170 was phosphorylated in a rapamycin-sensitive manner *in vivo*. Brief treatment of HEK293 cells with rapamycin caused little detectable change in CLIP-170 gel mobility, as judged by one-dimensional SDS–PAGE (Figure 3A). Because FRAP-dependent phosphorylation is counteracted by PP2A (Dennis *et al.*, 1999; Schmelzle and Hall, 2000; Gingras *et al.*, 2001), we treated HEK293 cells with calyculin A, a PP2A inhibitor, to enhance possible FRAP-dependent phosphorylation. Brief exposure of cells to calyculin A indeed caused a significant retardation of CLIP-170 gel mobility and rapamycin partially inhibited this mobility decrease (Figure 3A). In order to resolve such phosphorylated species, long electrophoresis is required. These gel mobility changes were due to phosphorylation because treatment of HEK293 cell lysates with calf intestine phosphatase (CIP) (insensitive to calyculin A) caused all CLIP-170 species to migrate in a fast mobility form (Figure 3B). Interestingly, CLIP-170 proteins from cells before exposure to calyculin A migrated slightly more slowly than CIP-treated CLIP-170, the fully dephosphorylated CLIP-170 (Figure 3B and C, lanes 1, 5 and 9 versus lanes 3 and 7), indicating that CLIP-170 is normally

Fig. 1. Interaction between FRAP and CLIP-170. (A) Interaction of endogenous FRAP and CLIP-170. HEK293 cell extracts were incubated with a FRAP-specific rabbit polyclonal serum (Imm) or a preimmune serum (Pre). The bound materials were analyzed by western blotting with FRAP (upper) and CLIP-170 (lower) antibodies. T, total input. (B) Interaction of recombinant FRAP and CLIP-170. Left: FLAG-FRAP was immunoprecipitated (IP) from HEK293 cells transiently expressing MYC-CLIP-170 in the presence or absence of FLAG-FRAP. Right: MYC-CLIP-170 was precipitated from lysates of HEK293 cells expressing FLAG-FRAP with or without MYC-CLIP-170. T, total input. (C) FRAP binds to CLIP-170 *in vitro*. Bacterial recombinant His₆-FRAP(aa 501–930) was incubated with bacterial GST–CLIP-170(aa 1–348) or GST. The bound materials were analyzed by western blotting with an anti-His₆ antibody. T, total input.

hypophosphorylated and calyculin A treatment causes CLIP-170 hyperphosphorylation. Additionally, these results suggest that two types of phosphorylation of CLIP-170 exist: rapamycin-sensitive and rapamycin-insensitive phosphorylation.

To confirm that such CLIP-170 phosphorylation did occur *in vivo*, we labeled HEK293 cells with [³²P]orthophosphate in the presence or absence of rapamycin. Consistent with the gel mobility study, CLIP-170 was normally phosphorylated (likely in the hypophosphorylated form). Its overall phosphorylation did not significantly change in the presence of rapamycin (Figure 3D). Two-dimensional (2D) tryptic phosphopeptide mapping of [³²P]CLIP-170 revealed five predominant ³²P-labeled phosphopeptides (phosphopeptides 1–5, Figure 3E). Phosphopeptide 1 contained the most ³²P counts. Phosphopeptides 1, 2 and 3 remained constant in the absence or presence of rapamycin (Figure 3E). In contrast, phosphopeptides 4 and 5 were significantly reduced by rapamycin. These results are in

Fig. 2. Mapping the FRAP-CLIP-170 interaction domains. **(A)** The N-terminus of CLIP-170 is sufficient to interact with FRAP. Wild type and mutant MYC-CLIP-170 proteins were co-expressed with FLAG-FRAP in HEK293T cells. T, total input; IP, immunoprecipitated materials. **(B)** The N-terminus of Bik1 interacts with Tor1. Gal4 AD-fused wild type and mutant Bik1 proteins were co-expressed with Gal4 BD-fused Tor1 in a yeast two-hybrid strain. Their interaction was detected by the ability of the yeast strain to grow on a Trp, Leu and His dropout plate containing 5 mM 3-AT. **(C)** The second HEAT domain of FRAP is responsible for binding to CLIP-170. Wild type and mutant FLAG-FRAP were co-expressed with MYC-CLIP-170 in HEK293T cells. FLAG-FRAP were immunoprecipitated with an anti-FLAG mAb. C, MYC-CLIP-170 was co-transfected with a plasmid vector.

agreement with the gel mobility result (Figure 3A) and indicate that CLIP-170 is phosphorylated at multiple sites *in vivo*, only some of which are rapamycin-sensitive. In addition, the gel

mobility decrease by the hypophosphorylated CLIP-170 in comparison to the fully dephosphorylated CLIP-170 is due to the rapamycin-insensitive phosphorylation (Figure 3C). To verify

Fig. 3. Analysis of CLIP-170 phosphorylation. (A) CLIP-170 becomes hyperphosphorylated in the presence of calyculin A. HEK293 cells were treated with and without rapamycin and calyculin A for 30 min. CLIP-170 was resolved on a long 6% SDS polyacrylamide gel and detected by western blotting. (B) CIP-treatment of hyperphosphorylated CLIP-170. Lysates of HEK293 cells treated with calyculin A in the presence and absence of rapamycin were incubated with CIP with or without Na₄P₂O₇. The samples were analyzed by western blotting with a long gel. (C) CLIP-170 is normally hypophosphorylated *in vivo*. Lysates of HEK293 cells, were incubated with CIP with or without Na₄P₂O₇. CLIP-170 was analyzed by western blotting with a long gel. (D) Metabolic labeling of CLIP-170 with [³²P]orthophosphate. CLIP-170 was isolated from HEK293 cells, metabolically labeled with [³²P]orthophosphate for 3 h and treated without or with rapamycin for 30 min before cell lysis. ³²P-labeled CLIP-170 was separated on an SDS polyacrylamide gel, transferred to a nitrocellulose filter and detected by autoradiography. (E) Tryptic phosphopeptide analysis of *in vivo* phosphorylated CLIP-170. Samples from (C) were analyzed by 2D tryptic phosphopeptide mapping. Highly reproducible tryptic phosphopeptides were labeled as 1–5. Arrows point to rapamycin-sensitive phosphopeptides. (F) FRAP phosphorylates CLIP-170 *in vitro*. Wild type and kinase-dead (kd) FLAG-FRAP were incubated with or without CLIP-170 in the presence of [³²P]ATP. The phosphorylated proteins were separated on an SDS polyacrylamide gel, transferred to nitrocellulose filter and detected by autoradiography. (G) FRAP phosphorylates CLIP-170 at the rapamycin-sensitive sites. The 2D tryptic phosphopeptides of *in vitro* and *in vivo* phosphorylated CLIP-170 were merged.

whether FRAP directly phosphorylates CLIP-170, we immunopurified FRAP and CLIP-170, and assayed for the ability of FRAP to phosphorylate CLIP-170 in the presence of [³²P]ATP. The wild type FRAP, but not a kinase-dead FRAP [FRAP(kd)] significantly phosphorylated CLIP-170 (Figure 3F). More importantly, FRAP phosphorylated CLIP-170 at the rapamycin-sensitive sites (Figure 3G). Taken together, these results suggest that FRAP is one of the CLIP-170 kinases.

To study the role of phosphorylation, we examined the ability of CLIP-170 to bind to MTs in the absence or presence of rapamycin. In this experiment, HeLa cells were treated with or without rapamycin. Cell extracts were then prepared and incubated with *in vitro* polymerized bovine MTs. These samples were layered over a sucrose cushion and centrifuged. The MT-bound CLIP-170 was precipitated with MTs and assayed by western blotting with a CLIP-170-specific antibody. CLIP-170

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from HeLa cells was capable of binding to MTs *in vitro*. This binding, however, was significantly inhibited after the cells were exposed to rapamycin (Figure 4A). In contrast, FK506 did not have any effect (data not shown). Hence, interference with FRAP function by rapamycin inhibits the ability of CLIP-170 to bind to MTs. We further investigated the effects of rapamycin-sensitive and -insensitive phosphorylation on CLIP-170 binding to MTs. We treated HeLa cells with calyculin A in the absence or presence of rapamycin, and assayed for CLIP-170 to bind to taxol-stabilized bovine tubulin. Although a long gel was good for resolving differentially phosphorylated CLIP-170 species, it usually resulted in smearing of the hyperphosphorylated forms (Figure 3A and B). For better quantitative comparison, we decided to use a short gel to analyze the amount of CLIP-170 associated with MTs under different conditions. Such a short gel did not separate different CLIP-170 species. We found that CLIP-170 was capable of binding to MTs in the presence of calyculin A, but not in the presence of both calyculin A and rapamycin (Figure 4B). These observations suggest that rapamycin-insensitive phosphorylation is inhibitory to CLIP-170 MT-binding and rapamycin-sensitive phosphorylation allows CLIP-170 to bind to MTs in the presence of rapamycin-insensitive phosphorylation.

We next investigated the MT-binding behavior of CLIP-170 *in vivo*. A significant population of CLIP-170 is localized to the growing ends of MTs, as indicated by the patches at these ends of MT fibers (Figure 4C; Perez *et al.*, 1999; Hoogenraad *et al.*, 2000). Rapamycin treatment eliminated the CLIP-170 patches (Figure 4C). In addition, rapamycin treatment altered MT structures from well-defined fibers to discontinuous patterns (Figure 4C). This latter phenomenon was also seen with the yeast *Saccharomyces cerevisiae*, in which rapamycin treatment led to MT disorganization (Choi *et al.*, 2000), indicating that the control of MT organization by TOR proteins is conserved in eukaryotes. Interestingly, the *bik1* mutation in yeast has a similar effect on MT structures (Berlin *et al.*, 1990; Choi *et al.*, 2000), raising the possibility that inhibition of FRAP/TOR signaling to Bik1/CLIP-170 by rapamycin is the cause of MT instability *in vivo*.

In this study, we show that CLIP-170 is phosphorylated at multiple sites *in vivo*, some of which are rapamycin-sensitive. FRAP phosphorylates CLIP-170 at the rapamycin-sensitive sites *in vitro*, suggesting that it is responsible for phosphorylation at these sites in the cells. The two types of CLIP-170 phosphorylation

Fig. 4. FRAP regulates the ability of CLIP-170 to bind to microtubules. (A) Rapamycin inhibits the binding of CLIP-170 to MTs. Extracts of cells treated with or without rapamycin were incubated with bovine MTs. MT and associated materials were separated on a short gel. MT-bound CLIP-170 was examined by western blotting and bovine tubulin by Coomassie Blue staining. S, supernatant; P, pellet. (B) Rapamycin-sensitive and -insensitive phosphorylation on the MT-binding behavior of CLIP-170. HeLa cells were cultured in the presence or absence of rapamycin before treatment with calyculin A for 15 min. Cell extracts were incubated with bovine MTs. MT-bound materials were separated on a short gel. CLIP-170 was detected by western blotting and bovine tubulin was examined by Coomassie Blue staining. S, supernatant; P, pellet. (C) The effect of rapamycin on CLIP-170 distribution and MT. HeLa cells were cultured in the presence or absence of rapamycin. The cells were stained with CLIP-170- (green) and MT-specific (total MTs, red) antibodies. The highlighted areas were enlarged to show staining details. The arrows show CLIP-170 patches on the growing ends of MTs. (D) A model indicating a possible regulatory mechanism by which CLIP-170 is controlled by phosphorylation.

appear to have opposing effects on the ability of CLIP-170 to bind to MTs. CLIP-170 from cells treated with rapamycin or rapamycin plus calyculin A has reduced affinity for MTs, suggesting that rapamycin-sensitive phosphorylation is stimulatory and rapamycin-insensitive phosphorylation is inhibitory. Detergent is known to be critical to extract FRAP from cells and tissues (Brown *et al.*, 1994; Sabatini *et al.*, 1994; Withers *et al.*, 1997). In two recent studies on CLIP-170 *in vitro* phosphorylation (Rickard and Kreis, 1991; Hoogenraad *et al.*, 2000), cell-free extracts were prepared in a buffer without detergent (these extracts should be depleted of FRAP). As a result, when treated with the phosphatase inhibitor okadaic acid, CLIP-170 became hyperphosphorylated and failed to bind to MTs (Rickard and Kreis, 1991; Hoogenraad *et al.*, 2000). Since there was no FRAP in the cell extracts, CLIP-170 was likely to be hyperphosphorylated only at the rapamycin-insensitive sites, which would inhibit the binding of CLIP-170 to MTs. These results also suggest that rapamycin-sensitive phosphorylation is required for CLIP-170 to bind to MTs in the presence of the rapamycin-insensitive phosphorylation (Figure 4D). Additionally, a bacterially produced N-terminal fragment of CLIP-170 is known to bind to MTs (Scheel *et al.*, 1999), suggesting that CLIP-170 is capable of binding to MTs in the unphosphorylated form. While this form of CLIP-170 was not detected in HeLa cells, dephosphorylation could be used as an alternative means to control the binding of CLIP-170 to MTs under certain cellular conditions (Figure 4D). Taken together, these observations suggest a possible mechanism by which the MT-binding behavior of CLIP-170 is regulated by multiple kinases, and possibly phosphatase(s) *in vivo* (Figure 4D).

The N-termini of FRAP and TOR contain 20 HEAT repeats (Andrade and Bork, 1995). HEAT repeat-containing proteins, including the A subunit of protein phosphatase 2A (PR65/A), Huntingtin and FRAP/TOR, play important roles in several human diseases. PR65/A HEAT repeats interact with PP2A catalytic subunits and tumor antigens of several small DNA viruses, and are frequently mutated in human cancers, resulting in a disruption of the binding of the catalytic subunits (Ruediger *et al.*, 2001). Here we show that the FRAP HEAT repeats are involved in recruiting substrate (CLIP-170) for the catalytic domain (Figure 2C). Interestingly, CLIP-170 is over-expressed in Hodgkin's disease and anaplastic large cell lymphoma (Bilbe, 1992; Delabie *et al.*, 1992, 1993). An ester derivative of rapamycin called CCI-779 is a potent inhibitor of many human cancers and is currently under advanced clinical trials. Hence, inhibition of CLIP-170 phosphorylation could play a significant role in the inhibition of human cancers by rapamycin.

METHODS

CLIP-170 phosphorylation. For calyculin A treatment, HeLa or HEK293 cells were treated with 50 nM calyculin A for 15 min. Some cells were pretreated with 1 mM rapamycin for 2 h. For CIP treatment, lysates of HEK293 cells were incubated with CIP buffer alone, 20 units CIP (Roche) or 20 units CIP plus 10 mM Na₄P₂O₇ for 10 min at 30°C. For *in vitro* phosphorylation, FLAG-FRAP or kinase-dead Flag-FRAP(kd) and MYC-CLIP-170 were immunoprecipitated from HEK293T cells with an anti-FLAG antibody. After extensive washing, the immunoprecipitates were

incubated with the kinase buffer (50 mM HEPES-KOH pH 7.4, 10 mM MnCl₂, 1 mM DTT, 100 μCi [γ-³²P]ATP, 3000 Ci/mM and 0.02 mM ATP) at 30°C. For *in vivo* labeling, HEK293 cells (3 × 10⁶) were transfected with plasmids expressing MYC-CLIP-170 using LipofecAMINE (Invitrogen). After 34 h, the cells were incubated with phosphate-serum-free DMEM containing 2.5 mCi/ml of [³²P]orthophosphate for 3 h. The cells were stimulated with 10% dialyzed fetal bovine serum with or without 50 nM rapamycin for 30 min. MYC-CLIP-170 was immunoprecipitated with the 9E10 mAb. The IP samples were separated by SDS-PAGE, transferred onto a nitrocellulose filter and detected by autoradiography. The regions of filter corresponding to phosphorylated CLIP-170 were excised, digested by trypsin (0.02 mg/ml in 50 mM ammonium bicarbonate) and analyzed by 2D phosphopeptide mapping (1D by electrophoresis at pH 1.9 and 2D by thin-layer chromatography in a buffer with *n*-butanol:pyridine:acetic acid:water ratio of 75/50/15/60).

***In vitro* MT-binding assay.** HeLa cells were pretreated in the absence or presence of rapamycin (1 μM) for 2 h, and then treated with 50 mM calyculin A for 15 min (Sigma). The cells were swelled for 5 min in EM (2 mM EGTA, 1 mM MgCl₂) and were lysed in PEM Buffer (PEM Wash Buffer plus 1 mM DTT, protease inhibitors; Roche) by dounce-homogenization. The cell lysates were cleared by centrifugation first at 20 000 g for 10 min and then at 50 000 g at 30°C for 1 h. For the co-sedimentation assay, 10 mg total protein was incubated in the presence or absence of Taxol-stabilized tubulin (Molecular Probes) in PEM Buffer containing 1 mM GTP for 15 min at 37°C in a reaction volume of 50 ml, and then layered over 100 ml of 10% sucrose in PEM Buffer and centrifuged at 30 000 g at 20°C for 30 min. The pellets were washed once in PEM Buffer.

Supplementary data. Supplementary data are available at *EMBO reports* Online.

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