Mode of interaction of TRIP13 AAA-ATPase with the Mad2-binding protein p31comet and with mitotic checkpoint complexes

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The AAA-ATPase thyroid hormone receptor interacting protein 13 (TRIP13), jointly with the Mad2-binding protein p31^{comet}, promotes the inactivation of the mitotic (spindle assembly) checkpoint by disassembling the mitotic checkpoint complex (MCC). This checkpoint system ensures the accuracy of chromosome segregation by delaying anaphase until correct bipolar attachment of chromatids to the mitotic spindle is achieved. MCC inhibits the anaphase-promoting complex/cyclosome (APC/C), a ubiquitin ligase that targets for degradation securin, an inhibitor of anaphase initiation. MCC is composed of the checkpoint proteins Mad2, BubR1, and Bub3, in association with the APC/C activator Cdc20. The assembly of MCC in active checkpoint is initiated by the conversion of Mad2 from an open (O-Mad2) to a closed (C-Mad2) conformation, which then binds tightly to Cdc20. Conversely, the disassembly of MCC that takes place when the checkpoint is turned off involves the conversion of C-Mad2 back to O-Mad2. Previously, we found that the latter process is mediated by TRIP13 together with p31^{comet}, but the mode of their interaction remained unknown. Here, we report that the oligomeric form of TRIP13 binds both p31^{comet} and MCC. Furthermore, p31^{comet} and checkpoint complexes mutually promote the binding of each other to oligomeric TRIP13. We propose that p31^{comet} bound to C-Mad2–containing checkpoint complex is the substrate for the ATPase and that the substrate-binding site of TRIP13 is composed of subsites specific for p31^{comet} and C-Mad2–containing complex. The simultaneous occupancy of both subsites is required for high-affinity binding to TRIP13.

mitosis | spindle checkpoint | cell cycle

Thyroid hormone receptor interacting protein 13 (TRIP13) is an AAA-ATPase that is required for the inactivation of the mitotic (spindle assembly) checkpoint (1, 2). This checkpoint system delays anaphase until correct bipolar attachment of sister chromatids to the mitotic spindle is achieved and thus ensures accuracy of chromosome segregation in mitosis (3–5). When the mitotic checkpoint system is on, it inhibits the action of the anaphase-promoting complex/cyclosome (APC/C), a ubiquitin ligase that targets for degradation specific cell cycle regulatory proteins, such as securin, an inhibitor of anaphase initiation (6). APC/C is inhibited by the mitotic checkpoint complex (MCC), which is composed of the checkpoint proteins Mad2, BubR1, and Bub3, in association with the APC/C activator Cdc20. The active checkpoint converts Mad2 from an open (O-Mad2) to a closed (C-Mad2) conformation, and the latter associates with Cdc20 in a very tight complex. It is thought that the C-Mad2–Cdc20 (MC) subcomplex associates with BubR1-Bub3 to form the MCC (4, 5).

In studying the mechanisms of the disassembly of MCC, we found that ATP hydrolysis is required for this process (7). ATP was also required for the action of p31^{comet}, a Mad2-binding protein involved in the exit from the mitotic checkpoint (8) and in MCC dissociation (9). Subsequently, we purified a factor that promotes ATP- and p31^{comet}-dependent release of Mad2 from MC and MCC and identified it as the TRIP13 ATPase (1). The role of TRIP13 in checkpoint inactivation was corroborated by in vivo results of other investigators indicating that TRIP13 knockdown delays metaphase–anaphase transition (2). We proposed that the energy of ATP hydrolysis is used by the TRIP13 ATPase to promote conformational transition of C-Mad2 to O-Mad2, thus leading to its release from MCC or MC (1). The action of TRIP13 to convert C-Mad2 to O-Mad2 was recently demonstrated by direct methods (10).

The question arose concerning what is the role of p31^{comet} in the action of the TRIP13 ATPase. Because it had been suggested by a proteomic data-mining study that TRIP13 interacts with p31^{comet} (11) and because p31^{comet} specifically binds to the closed conformation of Mad2 (12), it seemed reasonable to assume that p31^{comet} serves as an adaptor protein that targets the TRIP13 AAA-ATPase to C-Mad2–containing checkpoint complexes. However, in our previous experiments on immunodepletion of TRIP13 or p31^{comet} from checkpoint extracts, using antibodies directed against either protein, we could not detect coimmunodepletion of either protein with its presumed partner (1). The present investigation was initiated to solve this problem and to gain insight into the role of p31^{comet} in TRIP13 action. We find that p31^{comet} and checkpoint complexes mutually stimulate the binding of each other to the oligomeric form of the TRIP13 ATPase. We propose that p31^{comet} bound to C-Mad2–containing checkpoint complex is the substrate for the ATPase and that the substrate-binding site of TRIP13 is composed of subsites specific for p31^{comet} and the C-Mad2 moiety of the checkpoint complex. The simultaneous binding of p31^{comet} and

Significance

The mitotic checkpoint system is important to ensure accurate segregation of chromosomes in mitosis. It acts by the formation of a mitotic checkpoint complex (MCC), which inhibits the ubiquitin ligase anaphase-promoting complex/cyclosome (APC/C). When the checkpoint is turned off, MCC is disassembled by the joint action of an ATP-utilizing enzyme, thyroid receptor interacting protein 13 (TRIP13), and a Mad2-binding protein, p31comet. It is not well understood how p31comet targets TRIP13 to disassemble MCC. We show here that p31comet and MCC mutually promote the binding of each other to oligomeric TRIP13 and propose a model for the mode of the action of TRIP13 in MCC disassembly. Thus, the results reveal an important molecular mechanism in the inactivation of the mitotic checkpoint.

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C-Mad2 to these subsites is required for their high-affinity interaction with TRIP13.

Results

ATP Analogs Stimulate the Interaction of TRIP13 with p31 and with Mad2 in Extracts from Checkpoint-Arrested Cells. Previously, we could not detect coimmunoprecipitation of p31^{comet} with TRIP13 in extracts from checkpoint-arrested cells (1). Indeed, when checkpoint extracts were immunoprecipitated with anti-TRIP13 and immunoprecipitates were probed for p31^{comet}, no significant binding of p31^{comet} to TRIP13 was detected (Fig. 1, lane 1). We found, however, that, when checkpoint extracts were incubated with the ATP analog ATP-γ-S before immunoprecipitation, a significant portion of endogenous p31^{comet} was bound to TRIP13 (Fig. 1, lane 2). Much lower enhancement of TRIP13- p31^{comet} interaction was observed with another nonhydrolyzable ATP analog, AMP-PNP (Fig. 1, lane 3) whereas preincubation with ATP had no influence on this process (Fig. S1*A*, lane 3). Incubation of extracts with ATP-γ-S does not change the affinity of anti-TRIP13 to TRIP13 itself, as indicated by the finding that equal amounts of TRiP13 were immunoprecipitated in samples incubated with or without ATP-γ-S (Fig. S1*A*, *Lower*). Because it has been observed that ATP-γ-S promoted the conversion of different AAA-ATPases to oligomeric forms (13), we assumed that the ATP analog may cause oligomerization of TRIP13, which is necessary for its interaction with p31^{comet}. This assumption was examined in subsequent experiments.

Because p31^{comet} binds to the closed conformer of Mad2 (C-Mad2), which is present in MCC (14), and because checkpoint extracts contain considerable amounts of MCC and MC (7, 9), we asked whether preincubation of checkpoint extracts with ATP-γ-S also promotes the interaction of TRIP13 with Mad2. As shown in Fig. S1*B*, this notion indeed was found to be the case. Here again, the stimulatory effect of ATP-γ-S was much greater than that of AMP-PNP.

Checkpoint Protein Complexes Enhance the Binding of p31 to TRIP13. The above experiments, which showed coordinate binding of p31^{comet} and Mad2 to TRIP13, may be interpreted as indicating that p31^{comet} is bound directly to the oligomeric form of TRIP13 and that Mad2 may associate with TRIP13 indirectly, via its binding to p31^{comet}. It is also possible, however, that oligomeric TRIP13 directly binds the substrates that are disassembled by this ATPase, the mitotic checkpoint complex (MCC), and the MC subcomplex, which contain C-Mad2 and to which p31^{comet} binds. In the latter case, it would be expected that the binding of p31^{comet} to TRIP13 would be enabled by MCC or MC. To examine this possibility, MCC and MC were removed from checkpoint extracts by sequential immunodepletion with anti-BubR1 and anti-Mad2. As shown in Fig. 2*A*, this treatment removed from extracts most BubR1, almost all Mad2, and a large part of Cdc20. We then examined the effects of this immunodepletion on the binding of p31^{comet} to TRIP13. Because removal of mitotic checkpoint complexes also removed a part of endogenous p31^{comet} that was associated with these complexes, we also examined the effects of immunodepletion on the association with TRIP13 of exogenously supplied, recombinant his6-p31^{comet}. In the experiment shown in Fig. 2*B*, the effect of anti-Mad2 and anti-BubR1 immunodepletion on p31^{comet}–TRIP13 interaction was examined, with or without treatment with ATP-γ-S. It may be seen that the binding of both endogenous p31^{comet} and of exogenously supplied his6-p31^{comet} was markedly reduced by the removal of endogenous mitotic checkpoint complexes. However, p31^{comet}–TRIP13 interaction was not completely abolished in immunodepleted extracts, suggesting that some p31^{comet} can bind to TRIP13 in the absence of mitotic checkpoint complexes.

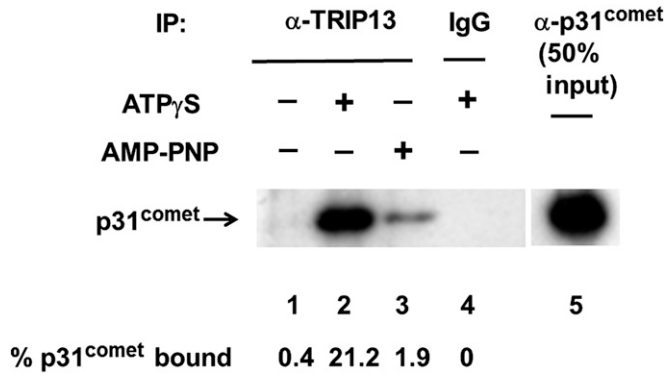

Fig. 1. Effect of ATP analogs on the binding of p31^{comet} to TRIP13 in checkpoint extracts. Mixtures contained in a volume of 30 μL: extract from checkpoint-arrested HeLa cells (400 μg of protein), 25 mM Tris·HCl (pH 7.6), 3 mM $MgCl_2$, 1 mM DTT, 1 mg/mL BSA, and 7% (vol/vol) glycerol. Samples were either not incubated (lane 1), or incubated at 23 °C for 60 min with the ATP analogs indicated (5 mM). Subsequently, samples were subjected to immunoprecipitation with anti-TRIP13 and to immunoblotting for p31^{comet}, as described Materials and Methods. The input of endogenous p31^{comet} was determined by immunoprecipitation of extract with anti-p31. Results are expressed as the percentage of p31 bound to TRIP13.

The notion that mitotic checkpoint complexes enhance the binding of p31^{comet} to TRIP13 was further examined by testing the effects of the supplementation of recombinant mitotic checkpoint complexes on p31^{comet}–TRIP13 interaction. In the experiment shown in Fig. 2*C*, extracts that had been subjected to immunodepletion with anti-Mad2 and anti-BubR1 were incubated with his6-p31 in the presence of ATP-γ-S, and the effects of the addition of recombinant Mad2– or MCC complexes on p31^{comet}–TRIP13 interaction were examined. There was some binding of his6-p31 to TRIP13 in the absence of checkpoint complexes (Fig. 2*C*, lane 1), but the supplementation of recombinant MC or MCC complexes greatly enhanced this interaction. These results suggest that most of the interaction of p31^{comet} with TRIP13 require the presence of mitotic checkpoint complexes.

p31^{comet} Stimulates the Binding of Mitotic Checkpoint Complexes to Oligomeric TRIP13. Another possibility to be considered was that strong interaction with oligomeric TRIP13 may take place only when both binding partners are present at high levels. According to this model, it could be expected that not only the binding of p31^{comet} to TRIP13 is stimulated by mitotic checkpoint complexes, as described above, but also the binding of mitotic checkpoint complexes to the ATPase would be stimulated by p31^{comet}. This notion could be tested in untreated mitotic checkpoint extracts because p31^{comet} is present in such extracts in limiting amounts, as indicated previously by the marked stimulation of checkpoint inactivation by exogenous p31^{comet} (9). In the experiment shown in Fig. 3*A*, checkpoint extracts were incubated with increasing amounts of his6-p31^{comet}, in the presence of ATP-γ-S, the samples were precipitated with anti-TRIP13, and the association of components of endogenous mitotic checkpoint complexes with TRIP13 was estimated by immunoblotting. It may be seen that increasing amounts of added his6-p31^{comet} strongly stimulated the binding of endogenous MCC components (Mad2, Cdc20, and BubR1) to endogenous TRIP13 (Fig. 3*A*).

We also tested the influence of p31^{comet} on the binding of recombinant mitotic checkpoint complexes to TRIP13 (Fig. 3*B*). For this purpose, checkpoint extracts were subjected to triple immunodepletion, consisting of treatment with anti-p31^{comet}, followed by immunodepletions with anti-Mad2 and anti-BubR1. The resulting preparation contained TRIP13. We used "native" TRIP13 from HeLa cells rather than recombinant TRIP13 for all experiments because recombinant TRIP13 was prone to aggregation and

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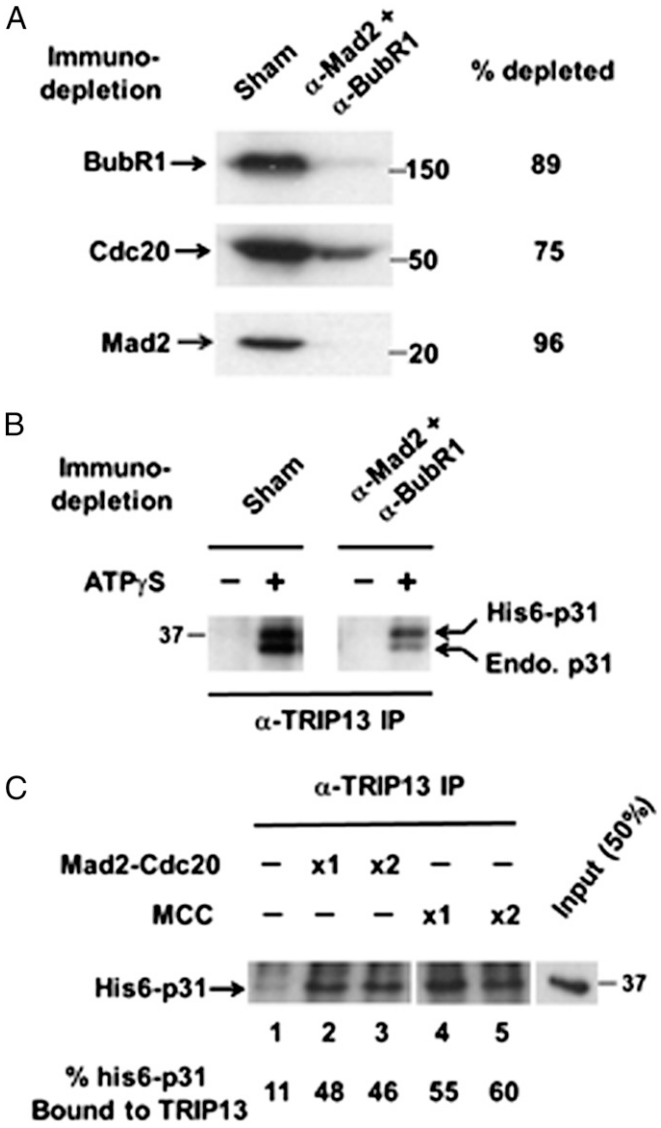

Fig. 2. (*A*) Removal of mitotic checkpoint proteins from extracts by immunodepletion. Immunodepletion of extracts by anti-Mad2 and anti-BubR1 was carried out as described in Material and Methods. Sham treatment was under similar conditions, with nonimmune rabbit IgG. Samples (30 μg of protein) of supernatants were immunoblotted for the indicated proteins. (*B*) Influence of the removal of endogenous mitotic checkpoint proteins on the binding of p31^{comet} to TRIP13. Samples (90 μg of protein) of immunodepleted or sham-treated extracts were incubated in the absence or presence of ATPγS, under conditions similar to those described in Fig. 1, except that bacterially expressed purified his6-p31^{comet} (30 nM) was added to all samples and the concentration of BSA was increased to 4 mg/mL Subsequently, samples were subjected to immunoprecipitation with anti-TRIP13, followed by immunoblotting for p31^{comet}. The positions of his6-p31^{comet} and of endogenous p31^{comet} ("Endo") are indicated. The number on the left indicates the electrophoretic migration position of the 37-kDa marker protein. (*C*) Recombinant mitotic checkpoint complexes stimulate the binding of p31 to TRIP13. Checkpoint extracts were subjected to immunodepletion with anti-Mad2 and anti-BubR1 and were incubated with his6- p31^{comet}, as described in Fig. 2*B*. Where indicated, recombinant Mad2–Cdc20 or recombinant MCC were added at 30 nM ("x1") or 60 nM ("x2") concentration. Samples were subjected to immunoprecipitation and immunoblotting as in Fig. 2*B*. Results are expressed as the percentage of his6-p31^{comet} bound to TRIP13.

loss of solubility and had very low specific activity (1). Immunodepleted extract was incubated with recombinant MC or MCC preparations, in the presence or absence of his6-p31^{comet} or ATP-γ-S, and was subjected to immunoprecipitation with anti-Cdc20, which binds mitotic checkpoint complexes through their Cdc20 moiety. The binding of TRIP13 to immunoprecipitated MC or MCC was determined by immunoblotting for TRIP13. The association of TRIP13 with MC (Fig. 3*B*, lanes 1–3) or with MCC (lanes 4–6) was virtually completely dependent upon the supplementation of both p31 and ATP-γ-S. These results indicate that not only the binding of TRIP13 to p31^{comet} is stimulated by mitotic checkpoint complexes, but also its association with mitotic checkpoint complexes is dependent upon the presence of p31^{comet} (Fig. 3*B*, lanes 2 and 5). These data support a model according to which the interaction of p31^{comet} with C-Mad2–containing checkpoint complexes results in their simultaneous high-affinity binding to TRIP13 (Discussion).

Oligomeric TRIP13 Binds p31^{comet} and Mad2–Cdc20 Complex. In all above-described binding experiments, done by immunoprecipitation procedures, it was assumed that the obligatory role of ATPγS in binding was due to its action to convert TRIP13 to an oligomeric form. We next examined, by size exclusion chromatography, whether this notion is indeed so. In the experiment shown in Fig. 4, partially purified TRIP13 from HeLa cells was incubated with ATP analogs and then was separated on a Superdex-200 column, and fractions were examined by immunoblotting for TRIP13. Without ATP analogs, TRIP13 eluted at a size corresponding to its monomeric form (~45 kDa), as described earlier (1). After incubation with ATP-γ-S, most was converted to a high-molecular-size oligomeric form, as expected. The apparent size of the oligomeric form was around 500 kDa, which is higher than that expected for a hexamer (~270 kDa). This finding may be due to the formation of a double-hexameric structure, as observed for some other AAA-ATPases (15, 16) or to imprecise estimation by size exclusion chromatography. After incubation with AMP-PNP, there was much less conversion of TRIP13 to the high-molecular-size oligomer, and mostly conversion to intermediate-sized forms was observed (Fig. 4). This finding may account for the much lower efficiency of AMP-PNP in promotion of the binding of p31^{comet} and Mad2 to TRIP13 (Fig. 1 and Fig. S1*B*).

We next asked whether p31^{comet} and the Mad2–Cdc20 subcomplex indeed bind to the high-molecular-size form of TRIP13, as was suggested by the immunoprecipitation experiments. Indeed, we found that, after incubation of all components, the Mad2–Cdc20 subcomplex eluted from the Superdex column at a size corresponding to oligomeric TRIP13, as indicated by immunoblotting of column fractions for Mad2 (Fig. S2*A*). Similarly, p31^{comet} comigrated with the same high-molecular-size form of TRIP13 after incubation with Mad2–Cdc20, but not in its absence (Fig. S2*B*). These results support the notion that the obligatory role of ATP-γ-S in the binding experiments is to stabilize the oligomeric form of TRIP13 that binds substrate at a high affinity (Discussion).

Discussion

Previously, we observed that the disassembly of mitotic checkpoint complexes, which is required for the inactivation of the mitotic checkpoint, requires the joint action of the AAA-ATPase TRIP13 and of the Mad2-binding protein p31comet (1). It seemed reasonable to assume that p31^{comet} acts as an adaptor protein that targets the ATPase to its substrates, the Mad2-containing mitotic checkpoint complexes. This notion was in agreement with previous information from proteomic studies on interaction between p31^{comet} and TRIP13 (11). However, in coimmunoprecipitation experiments, we could not detect significant binding of p31^{comet} to TRIP13 in extracts from mitotic checkpoint-arrested cells (1). We now find that p31^{comet}–TRIP13 interaction does exist and can be observed in checkpoint extracts in the presence of the ATP analog ATPγS (Fig. 1). Nucleotide-dependent oligomerization has been observed with other AAA-ATPases (13), but TRIP13 is unique in this family of proteins in

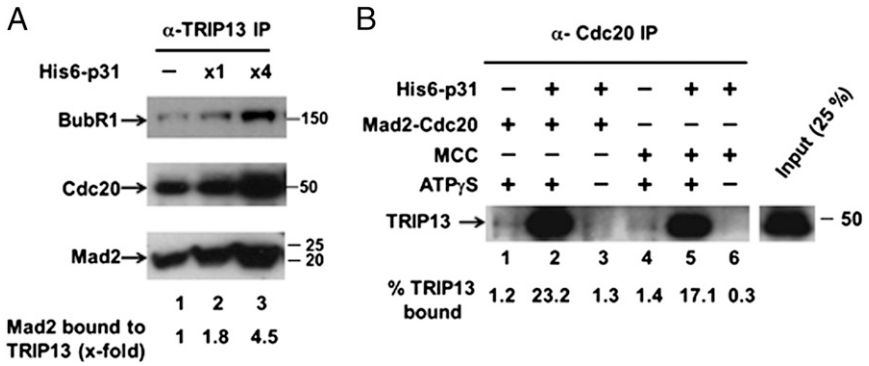

Fig. 3. p31^{comet} stimulates the binding of mitotic checkpoint complexes to TRIP13. (*A*) p31^{comet} stimulates the binding of endogenous mitotic checkpoint proteins to TRIP13. Extract from checkpoint-arrested HeLa cells (400 μg of protein) was incubated as described in Fig. 1, in the presence of 5 mM ATPγS and with increasing amounts of recombinant purified his6-p31^{comet}. The "x1" denotes 30 nM his6-p31^{comet}. Samples were immunoprecipitated with anti-TRIP13 and then were immunoblotted for the indicated endogenous mitotic checkpoint proteins. Numbers on the right side indicate the migration position of marker proteins (kDa). The increase of Mad2 bound to TRIP13, relative to that bound in the absence of added his6-p31^{comet}, is indicated at the bottom. (*B*) p31^{comet} stimulates the binding of TRIP13 to recombinant mitotic checkpoint complexes. Extracts from mitotic checkpoint-arrested cells were subjected to immunodepletion with anti-Mad2 and anti-BubR1 and then were further immunodepleted with anti-p31^{comet} (*Materials and Methods*). Samples of immunodepleted extracts (100 μg of protein) were incubated under conditions described in Fig. 1. Where indicated, 120 nM his6-p31^{comet}, 60 nM recombinant Mad2–Cdc20, or recombinant MCC and 5 mM ATPγS were supplemented. After incubation at 23 °C for 60 min, samples were subjected to immunoprecipitation with anti-Cdc20, followed by immunoblotting for TRIP13. Results are expressed as the percentage of TRIP13 bound to Cdc20-containing mitotic checkpoint complexes.

that, in the native state, it exists mostly in the monomeric form (1). The oligomeric, active form of most AAA-ATPases is a hexameric ring (13, 17, 18) although, in some cases, double-hexameric structures have been described (15, 16). It seems reasonable to assume that ATPγS binds to TRIP13 but cannot be hydrolyzed and thus stabilizes the oligomeric form of TRIP13 (which is transient in the normal reaction cycle) that binds substrate at a high affinity.

The use of ATPγS for binding studies allowed us to define the characteristics of the binding of p31^{comet} and of mitotic checkpoint complexes to TRIP13. We found that mitotic checkpoint complexes, such as MCC or the Mad2–Cdc20 subcomplex, strongly enhanced the binding of p31^{comet} to oligomeric TRIP13 (Fig. 2 and Fig. S2*B*). Furthermore, the binding of mitotic checkpoint complexes to TRIP13 was dependent on the presence of p31 (Fig. 3). A plausible explanation for this mutual interdependence for binding to TRIP13 is that the substrate for this ATPase is a complex between p31^{comet} and a mitotic checkpoint complex. Thus, p31^{comet} may first bind to mitotic checkpoint complex through their C-Mad2 moiety and then associate with TRIP13. Alternatively or in addition, p31^{comet} may bind first to TRIP13 at low affinity, and then its further binding to a checkpoint complex may increase its binding affinity to the ATPase. In support of the latter possibility is our observation that, in the absence of mitotic checkpoint complexes, there was binding of a low amount of p31^{comet} to TRIP13 (Fig. 2 *B* and *C*). We furthermore propose

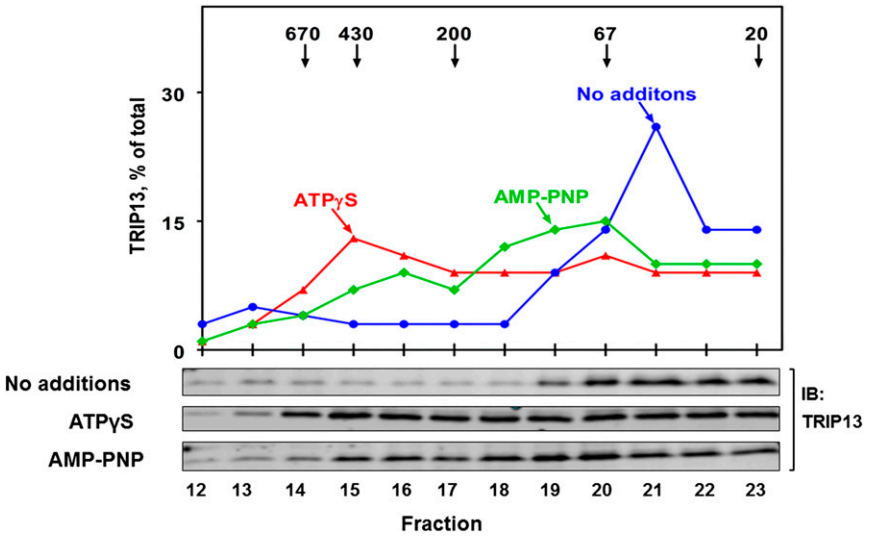

Fig. 4. Effects of ATPγS and AMP-PNP on the oligomerization of TRIP13. Partially purified TRIP13 from HeLa cells (Materials and Methods) was treated in a mixture that contained in a volume of 50 μL: 25 mM Tris·HCl (pH 7.6), 2–5 mM $MgCl_2$, 1 mM DTT, 5 mg/mL BSA, and 700 nM TRIP13. Where indicated, 2 mM ATPγS or 5 mM AMP-PNP were supplemented. After incubation at 23 °C for 60 min, samples were applied to a Superdex 200 Increase 5/150 GL column (GE Healthcare), equilibrated with 50 mM Tris·HCl (pH 7.2), 100 mM NaCl, 10% (vol/vol) glycerol, 1 mg/mL DTT, and 1 mM $MgCl_2$. When TRIP13 was treated with ATPγS or AMP-PNP, column buffers also contained 0.1 mM ATPγS or 0.2 mM AMP-PNP, respectively. Fractions of 100 μL were collected at a flow rate of 0.2 mL/min. Samples of 10 μL of column fractions were immunoblotted for TRIP13. Arrows, elution position of molecular mass marker proteins (kDa).

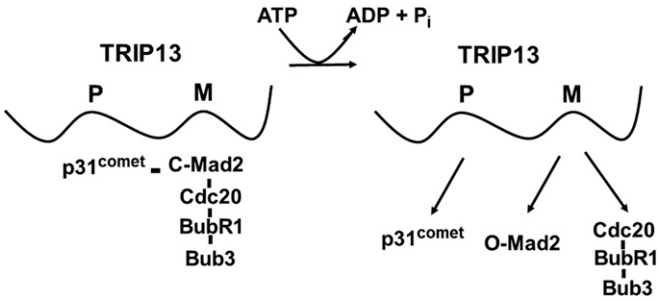

Fig. 5. Proposed interactions of TRIP13 with p31^{comet} and MCC (Discussion). It is proposed that the substrate-binding site of TRIP13 consists of two subsites, P and M, for the binding of p31^{comet} and the C-Mad2 moiety of MCC, respectively. p31^{comet} bound to MCC is tightly associated with TRIP13 by interactions with the two subsites. ATP hydrolysis promotes conformational transition of C-Mad2 to O-Mad2. Thereby, Mad2 loses affinity to both Cdc20 and p31^{comet}, leading to release of substrates.

that the substrate binding site of TRIP13 is composed of two subsites, one for p31^{comet} and another for the C-Mad2 moiety of the mitotic checkpoint complex, and that these two subsites are in close proximity (Fig. 5, P and M subsites). Both subsites have to be occupied for high-affinity binding of the p31^{comet}-mitotic checkpoint complex substrate to oligomeric TRIP13. It should be noted that, in our proposed model, p31^{comet} still serves in an adaptor role for the binding of a specific protein (C-Mad2), although not in the usual sense in which the adaptor is a subunit of the AAA-ATPase.

Our observations on the properties of the binding of the substrate to TRIP13 in the presence of ATPγS may also shed light on the mode of the action of the enzyme with ATP. In this case, both ATP binding and hydrolysis take place, and both processes are transient. We have previously proposed that the energy of ATP hydrolysis is used for the conversion of C-Mad2 to O-Mad2 conformation, thus terminating the checkpoint signal (1). This suggestion has recently been verified by direct methods by other investigators (10). Because p31^{comet} binds only to C-Mad2 and not to O-Mad2 (12), at the end of the ATPase cycle, it should be dissociated and released from TRIP13, along with other low-affinity products, as schematically shown in Fig. 5.

Materials and Methods

Antibodies. For immunoprecipitation and immunodepletion, we used rabbit polyclonal antibodies raised against the following antigens: α-TRIP13, α-Mad2, α-p31^{comet}, and α-Cdc20, the corresponding his6-tagged full-length human proteins; and α-BubR1, his6-tagged 38- to 486-amino acid fragment of BubR1. All antibodies were affinity purified on their respective antigens. For immunoblotting, the following rabbit polyclonal antibodies were used: α-TRIP13, the antibody described above; and α-p31^{comet}, antibody raised against 17-amino acid C-terminal peptide of p31. In addition, the following commercial antibodies were used for immunoblotting: BubR1 (612503; BD Transduction Laboratories), Cdc20 (sc-13162; Santa Cruz Biotechnology), and Mad2 (K0167; MBL Laboratories).

Immunodepletion, Immunoprecipitation, and Immunoblotting. Extracts from nocodazole-arrested HeLa cells were prepared as described (19). Immunodepletion of extracts was carried out as described previously (1). Briefly, antibodies were bound to Affi-Prep Protein A beads (Bio-Rad), were mixed with extracts at a ratio of 5:1 (vol/vol), and were rotated at 4 °C for 2 h. For removal of mitotic checkpoint complexes, extracts were subjected to sequential immunodepletions with anti-Mad2 and anti-BubR1. Sham depletions were carried out by subjecting extracts to a similar procedure, with Protein A beads bound to nonimmune rabbit IgG. Immunoprecipitation and immunoblotting procedures were as carried out previously (7). When rabbit antibodies were used for immunoblotting, we used, for secondary antibody, EasyBlot anti-Rabbit IgG (HRP) (GTX 231666; GeneTex), to minimize interference by immunoprecipitated rabbit IgG. Immunoblots were quantified with Odyssey (Li-Cor) or with ImageQuant RT ECL instrument (GE Healthcare).

Preparations. His6-p31^{comet} was expressed in bacteria and purified as described (9). Recombinant Mad2–Cdc20 complex was formed in SF9 insect cells by coinfection with baculoviruses expressing his6-Cdc20 and Flag-Mad2. This complex was purified on Ni-NTA-agarose (Qiagen) followed by anti-DYKDDDD (Flag) affinity resin (GeneScript), according to instructions of the manufacturers. Recombinant MCC was formed by coexpression in insect cells of streptavidin-binding peptide (SBP)-BubR1, his6-Cdc20, and Flag-Mad2 and was purified on Strep Tactin Agarose, High performance (GE Healthcare), as instructed by the manufacturer. Bub3 was not coexpressed for recombinant MCC formation because it has no influence on MCC structure or function (14).

TRIP13 from HeLa cell extracts was partially purified by a modification of a previously described anion exchange chromatographic procedure (1). Briefly, 8 mL of extract (~160 mg of protein) was applied to 8 mL of Source 15Q (GE Healthcare; packed in Tricorn 10/100 column), equilibrated with 50 mM Tris·HCl (pH 7.4) and 1 mM DTT. Elution was with a gradient of NaCl in the same buffer, and fractions of 8 mL were collected. TRIP13 was eluted at 120–140 mM NaCl. The pooled fractions were concentrated by ultrafiltration and were stored at −70 °C in a buffer consisting of 50 mM Tris·HCl (pH 7.6), 30% (vol/vol) glycerol, and 1 mM DTT.

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