p31\textsuperscript{comet} promotes disassembly of the mitotic checkpoint complex in an ATP-dependent process

Adar Teichner\textsuperscript{1}, Esther Eytan\textsuperscript{1}, Danielle Sitry-Shevah, Shirly Minioiwitz-Shemtov, Elena Dumin, Jonathan Gromis, and Avram Hershko\textsuperscript{2}

Unit of Biochemistry, The Rappaport Faculty of Medicine, Technion-Israel Institute of Technology, Haifa 31096, Israel

Contributed by Avram Hershko, January 6, 2011 (sent for review December 12, 2010)

Accurate segregation of chromosomes in mitosis is ensured by a surveillance mechanism called the mitotic (or spindle assembly) checkpoint. It prevents sister chromatid separation until all chromosomes are correctly attached to the mitotic spindle through their kinetochores. The checkpoint acts by inhibiting the anaphase-promoting complex/cyclosome (APC/C), a ubiquitin ligase that targets for degradation securin, an inhibitor of anaphase initiation. The activity of APC/C is inhibited by a mitotic checkpoint complex (MCC), composed of the APC/C activator Cdc20 bound to the checkpoint proteins MAD2, BubR1, and Bub3. When all kinetochores acquire bipolar attachment the checkpoint is inactivated, but the mechanisms of checkpoint inactivation are not understood. We have previously observed that hydrolyzable ATP is required for exit from checkpoint-arrested state. In this investigation we examined the possibility that ATP hydrolysis in exit from checkpoint is linked to the action of the Mad2-binding protein p31\textsuperscript{comet} in this process. It is known that p31\textsuperscript{comet} prevents the formation of a Mad2 dimer that it thought to be important for turning on the mitotic checkpoint. This explains how p31\textsuperscript{comet} blocks the activation of the checkpoint but not how it promotes its inactivation. Using extracts from checkpoint-arrested cells and MCC isolated from such extracts, we now show that p31\textsuperscript{comet} blocks the activation of checkpoint, but not its deactivation. The activation of APC/C is caused by the hydrolysis of MCC and that this process requires βγ-hydrolyzable ATP. Although p31\textsuperscript{comet} binds to Mad2, it promotes the dissociation of Cdc20 from BubR1 in MCC.

The mitotic (or spindle assembly) checkpoint system is a surveillance mechanism that prevents the initiation of anaphase until all sister chromatids are correctly attached to the mitotic spindle through their kinetochores. It thus acts as a safeguard mechanism to ensure the accuracy of chromosome segregation in mitosis (reviewed in refs. 1–3). The target of the mitotic checkpoint is the anaphase-promoting complex/cyclosome (APC/C), a ubiquitin–protein ligase that acts on cell cycle regulatory proteins such as mitotic cyclins and securin, an inhibitor of anaphase initiation (reviewed in refs. 4, 5). When the checkpoint system is turned on it inhibits APC/C, securin cannot be degraded, and thus anaphase cannot be initiated. Components of the mitotic checkpoint system include members of Mad (mitotic arrest deficient) and Bub (budding uninhibited by benzimidazole) proteins, which are conserved among eukaryotes. However, the mechanisms by which the activity of APC/C is regulated by the mitotic checkpoint system are not well understood. Genetic work in yeast first indicated that the interaction of the checkpoint protein Mad2 with the APC/C activator Cdc20 is essential for the action of the checkpoint system (6, 7). More recent structural studies have shown that Mad2 exists in two conformations, open (O-Mad2) and closed (C-Mad2) conformers, of which only the latter binds to Cdc20 (8, 9). Most of Mad2 in the interphase is in the open conformation. When the mitotic checkpoint is turned on, O-Mad2 dimerizes with C-Mad2 that is associated with Mad1 on the kinetochore. It has been shown that the interaction of O-Mad2 with C-Mad2 is essential for turning on the mitotic checkpoint system, and it has been proposed that this process converts cytosolic O-Mad2 to C-Mad2, which in turn binds to Cdc20 (reviewed in 10, 11). However, the binding of Mad2 to Cdc20 does not seem to inhibit Cdc20 simply by the sequestration of the activator Cdc20. Rather, the activity of APC/C is inhibited during checkpoint by inhibitory complexes, such as one composed of Cdc20, Mad2, BubR1, and Bub3, called the mitotic checkpoint complex (MCC) (12). It has been suggested that C-Mad2–Cdc20 may combine with BubR1–Bub3 to form the MCC (10, 11), but the pathways of MCC assembly have not been defined. Also unknown are the mechanisms involved in the disassembly of MCC when all kinetochores acquire bipolar attachment and the checkpoint is inactivated.

We have been using extracts from nocodazole-arrested HeLa cells to study the mechanisms by which the mitotic checkpoint system regulates the activity of APC/C. These “checkpoint extracts” faithfully reproduce downstream events in this system (13). They have been used to characterize mitotic checkpoint inhibitors of APC/C such as MCC (12, 13) and an additional inhibitor, MCF2 (mitotic checkpoint factor 2) (14). Checkpoint extracts could also be used to characterize the molecular mechanisms of exit from the arrested state. When checkpoint extracts were incubated in the presence of ATP, APC/C was converted to an active form following a lag period. Experiments in which “activated” and “checkpoint-arrested” extracts were mixed indicated that the activation of APC/C is due to the elimination of labile inhibitor(s) (13). Indeed, the activation of APC/C was accompanied by the disassembly of MCC and the disappearance of APC/C-bound MCF2 (14). More recently, we found that ATP is required both for the activation of APC/C and for the disassembly of MCC in exit from mitotic checkpoint arrest. The cleavage of ATP at the βγ position was required for these processes, as indicated by the finding that replacing ATP with the nonhydrolyzable analogue adenosine-5′-(β,γ-imido)-triphosphate (AMP–PNP) prevented both the activation of APC/C and the disassembly of MCC (15). This observation ruled out the possibility that ATP is required only for ubiquitylation, as suggested by other investigators (16), because ubiquitylation involves the scission of the α-β bond of ATP (17) and thus can utilize AMP–PNP. The mode of action of ATP in exit from checkpoint remained unknown.

In the present investigation, we have examined the possibility that ATP hydrolysis in exit from the checkpoint-arrested state is linked to the action of p31\textsuperscript{comet} in this process. This protein, also called CMT2, was originally discovered as a Mad2-binding protein involved in the regulation of progression through late mitosis (18). The binding of p31\textsuperscript{comet} to Mad2 is maximal during the exit of cells from mitotic checkpoint, suggesting that it may play a role in checkpoint silencing (18, 19). This notion was corroborated by the finding that depletion of p31\textsuperscript{comet} delayed escape of cells from...

Author contributions: A.T., E.E., S.M.-S., J.G., and A.H. designed research; A.T., E.E., S.M.-S., J.G., and A.H. performed research; A.T., E.E., S.M.-S., J.G., and A.H. analyzed data; D.S.-S. and E.D. contributed new reagents/analytic tools; and A.H. wrote the paper.

The authors declare no conflict of interest.

1A.T. and E.E. contributed equally to this work.

2To whom correspondence should be addressed. E-mail: hershko@tx.technion.ac.il.
mitotic checkpoint arrest (18, 19). p31\textsuperscript{comet} was also shown to stimulate the release of APC/C from checkpoint arrest in extracts incubated with the E2 enzyme UbcH10 (16). It was furthermore shown that p31\textsuperscript{comet} specifically binds to the closed conformation of Mad2 (19). Binding of p31\textsuperscript{comet} to C-Mad2 precludes the binding of the latter to O-Mad2 but does not interfere with C-Mad2-Cdc20 interaction (19). The structure of p31\textsuperscript{comet} is remarkably similar to that of Mad2 (20, 21). It has been suggested that by acting as a competitive inhibitor of the assembly of the C-Mad2: O-Mad2 dimer, p31\textsuperscript{comet} blocks the activation of Mad2 (20, 21). These findings explain how p31\textsuperscript{comet} may inhibit the activation of the mitotic checkpoint, but it remained unclear how it promotes the inactivation of the checkpoint and exit from checkpoint arrest. We now show that p31\textsuperscript{comet} promotes the disassembly of MCC and that this process requires the participation of βγ-hydrolyzable ATP.

Results

p31\textsuperscript{comet} Accelerates the Release of APC/C From Checkpoint Inhibition and Stimulates the Disassembly of MCC in Extracts From Nocodazole-Arrested Cells. It has been reported that p31\textsuperscript{comet} promotes exit of cells from mitotic checkpoint arrest (18, 19). Release of APC/C from checkpoint inhibition can be recapitulated in vitro by incubation of extracts from nocodazole-arrested cells with ATP (13). Under these conditions, APC/C is converted to an active form in lag kinetics, a process accompanied by the disassembly of MCC and that this process requires the participation of βγ-hydrolyzable ATP.

![Figure 1](https://www.pnas.org/cgi/doi/10.1073/pnas.1100023108)

Fig. 1. p31\textsuperscript{comet} stimulates the release of APC/C from checkpoint inhibition and accelerates the disassembly of MCC. (A) Effect of p31\textsuperscript{comet} on APC/C activation in checkpoint extracts. Extracts were incubated with ATP as described in Methods, in the absence (“Control”) or presence of bacterially expressed p31\textsuperscript{comet}, as indicated. Samples taken at the times indicated were immunoprecipitated with anti-Cdc27 beads and APC/C activity in the ligation of ubiquitin to S-\textsuperscript{\textgamma}cyclin was assayed. (B) Effect of p31\textsuperscript{comet} on MCC in checkpoint extracts incubated with ATP. Extracts were incubated with p31\textsuperscript{comet} or without it (“Control”), as described in A. Samples withdrawn at the indicated times were subjected to sequential immunoprecipitations with anti-Cdc27 and anti-BubR1 antibodies (see Methods). The amounts of MCC components associated with anti-BubR1 immunoprecipitates were estimated with quantitative immunoblotting. (C) Binding of \textsuperscript{35}S-labeled p31\textsuperscript{comet} to components in anti-BubR1 immunoprecipitates. Extracts were incubated with ATP as described in A, (“Control”), and samples were subjected to immunoprecipitation with anti-BubR1 as described in B. The binding of \textsuperscript{35}S-p31\textsuperscript{comet} to anti-BubR1 immunoprecipitates was estimated as described in Methods. (D) Relationship of bound \textsuperscript{35}S-p31\textsuperscript{comet} to MCC components in anti-BubR1 immunoprecipitates. Binding of \textsuperscript{35}S-p31\textsuperscript{comet} to immunoprecipitates was estimated as in C, and the amounts of Mad2, Cdc20, and BubR1 in the same immunoprecipitates were measured as in B. Ratios were normalized to 1.0 at time zero of incubation.
incubation (Fig. 1B, Left). We have furthermore observed that the release of Cdc20 from anti-BubR1 immunoprecipitates was also markedly stimulated by p31comet (Fig. 1B, Right), although the rate of the release of Cdc20 was slower than that of Mad2. This finding was surprising, because p31comet was reported to bind to Mad2 (18–21), and yet it also promoted the dissociation of Cdc20 from BubR1. Details of interactions between subunits of MCC are not known, but it is known that Cdc20 binds to both Mad2 and to BubR1 (22, 23). To examine the possibility that p31comet may also bind to some other component in MCC, such as to BubR1 or to Cdc20, we compared the binding of 35S-labeled p31comet to different species of MCC of varying compositions. The composition of MCC-related complexes is altered during incubation of checkpoint extracts with ATP; because the release of Mad2 is faster than that of Cdc20 (15) (Fig. 1B). In the experiment shown in Fig. 1C, checkpoint extracts were incubated with ATP; samples taken in the course of the incubation were precipitated with anti-BubR1 antibody, and the binding of 35S-p31comet to immunoprecipitated material was estimated. 35S-p31comet had no significant influence on MCC dissociation during binding assay, because this assay was carried out at 4 °C and the concentration of 35S-labeled p31comet was approximately 50-fold lower than that of the bacterially expressed protein effective in MCC dissociation. Significant binding of 35S-p31comet to anti-BubR1 immunoprecipitates derived from checkpoint extracts was observed, and the amount of bound material decreased rapidly upon incubation of extracts with ATP (Fig. 1C). This rapid decrease in the capacity of anti-BubR1 immunoprecipitates to bind 35S-p31comet correlated well with the rapid decrease in their content of Mad2, as opposed to the slower decay of Cdc20 or BubR1. Indeed, examination of the ratio of 35S-p31comet bound to BubR1 immunoprecipitates with the amount of MCC components (estimated by quantitative immunoblottting) in the same immunoprecipitates showed that this ratio in relation to Mad2 was stable but declined markedly with Cdc20 and BubR1 in the course of the incubation (Fig. 1D). These observations suggested that 35S-labeled p31comet is bound to Mad2 in MCC, as expected, and not to Cdc20 or to BubR1 that remain in these immunoprecipitates at longer times of incubation. This conclusion further suggested that the binding of p31comet to Mad2 in MCC (presumably in the form of C-Mad2) triggers an alteration of the capacity of anti-BubR1 immunoprecipitates with the amount of MCC component of C-Mad2 (14).

Identification of Products of the Dissociation MCC by p31comet and ATP. The results described above suggested that the combined action of p31comet and ATP caused the release of Cdc20 and Mad2 from MCC but did not reveal whether these components were released individually or as a subcomplex. In addition, there was a concern that in experiments carried out with immunoprecipitated MCC, the structure of this complex may have been altered by the binding to antibody. Because of these reasons, we carried out incubations of soluble, partially purified MCC with p31comet and ATP and analyzed dissociation products by size exclusion chromatography. The results are shown in Fig. 3A, and their quantitation in Fig. 3B. Following incubation without additions, all three components (BubR1, Cdc20, and Mad2) eluted at the region of the 440-kDa marker protein, near the void volume of the Superdex-200 column. This is in agreement with previous estimate of the apparent molecular mass of MCC at around 400 kDa (12). Following incubation with p31comet and ATP, there was a marked change in the elution position of Mad2 and Cdc20; A large part of both Mad2 and Cdc20 disappeared from the high molecular size region and shifted to the lower size region. There was not much change in the elution position of BubR1 following this incubation, possibly due to lack of resolution of the Superdex-200 column at the high molecular size region. These results with soluble preparations of MCC confirmed the validity of our previous data obtained with anti-BubR1 immunoprecipitates. We furthermore noticed that at the lower size region, the elution profiles of released Cdc20 and Mad2 were remarkably similar (Fig. 3B, Right). This suggested that a Cdc20–Mad2 subcomplex may be released from MCC in this process. The peak of the pu
Fig. 2. MCC is dissociated by the joint action of p31\textsuperscript{comet} and ATP. (A) p31\textsuperscript{comet} abrogates the inhibition of purified APC/C by MCC. Reaction mixture was similar to that described in ref. 13 for the assay of APC/C activity, except that purified mitotic APC/C and recombinant Cdc20 were supplemented as in ref. 24. Where indicated, 250 nM p31\textsuperscript{comet} was added. MCC and MCF2 were purified from salt eluate of APC/C immunoprecipitate by chromatography on MonoQ as described (14). Residual MCC was removed from the preparation of MCF2 by immunodepletion with anti-BubR1 (14). The amounts of MCC and MCF2 used were adjusted to produce an inhibition of approximately 40–50% of APC/C activity. The ligation of P\textsuperscript{2}I\textsuperscript{-}cyclin to ubiquitin was determined following an incubation of 60 min at 30°C. Results were expressed relative to APC/C activity in the control incubation (without inhibitor and without p31\textsuperscript{comet}). (B) p31\textsuperscript{comet} and \(\gamma\)P\textsuperscript{3}ATP synergistically promote the release of Mad2 and Cdc20 from anti-BubR1 immunoprecipitates. The release of MCC components from anti-BubR1 immunoprecipitates to supernatants was determined as described in Methods. Where indicated, ATP or AMP-PNP were added at 5 mM, and p31\textsuperscript{comet} at 250 nM. “Input” was the amount of MCC components associated with anti-BubR1 immunoprecipitate prior to release. (C) Time course of the release of Mad2 and Cdc20 from anti-BubR1 immunoprecipitates incubated with p31\textsuperscript{comet} and ATP. Reaction conditions were similar to those in B. Samples of released material were analyzed by quantitative immunoblotting. (D) Nucleotide specificity of MCC dissociation. BubR1 immunoprecipitates were incubated with 2 mM of the indicated nucleotides, in the absence (“Control”) or presence of 250 nM p31\textsuperscript{comet}. GMP-PNP, guanosine-5’-[\(\gamma\)P\textsuperscript{3}]triphosphate. The release of MCC components was determined as described in Methods. “No nucl.,” without nucleotide added. Shown is the release of Mad2; similar results were obtained with the release of Cdc20.

Discussion

The results presented in this paper show that p31\textsuperscript{comet} promotes the dissociation of the mitotic checkpoint complex in a process that requires the participation of hydrolyzable ATP. It has been previously observed that p31\textsuperscript{comet} plays a role in exit from mitotic checkpoint arrest (18, 19), but the underlying mechanisms of action remained poorly understood in spite of good progress in the elucidation of the structure and interactions of this protein. The structure of p31\textsuperscript{comet} is remarkably similar to that of Mad2, and it binds to C-Mad2 in a tight complex (20, 21). It has been proposed that by such “structural mimicry”, p31\textsuperscript{comet} prevents the formation of the C-Mad2:O-Mad2 dimer (20, 21), which is thought to be important for the binding of C-Mad2 to Cdc20 and eventually for the assembly of MCC (10, 11). Such a sequence of events may explain how p31\textsuperscript{comet} might block the assembly of MCC and the activation of the mitotic checkpoint, but it remained unclear how it turns off the checkpoint, a process that requires the disassembly of MCC. It is possible that a state of dynamic equilibrium exists in cells between assembly and disassembly of MCC, so that when assembly is inhibited by the quenching of the checkpoint signal, disassembly of MCC will take place. However, we find that p31\textsuperscript{comet}, in the presence of ATP, promotes the disassembly of MCC under conditions that do not allow assembly to take place. Soluble extracts from nocodazole-arrested cells do not contain chromosomes, which are removed by centrifugation, so they lack the primary checkpoint signal that originates at unattached kinetochores. Still, p31\textsuperscript{comet} strongly stimulates MCC disassembly in such extracts incubated with ATP (Fig. 1). More conclusively, p31\textsuperscript{comet} in the presence of ATP also
Cdc20 or Mad2 (12) adsorbed to 10 immunoprecipitation with 5 blotting with the indicated monoclonal antibodies. samples of supernatants (S) and precipitates (P) were subjected to immuno-with a similar amount of nonimmune rabbit IgG (Pierce), as indicated. Equal is not explained by blocking Mad2 activation, it appears reason-

ATP and material released to the supernatant was collected as described

tion mixtures contained in a volume of 100 μl: 40 mM Tris-HCl (pH 7.6), 5 mM MgCl₂, 1 mM DTT and 80 μl MCC. Where indicated, 5 mM ATP and 250 nM p31comet were added. Following incubation at 23 °C for 60 minutes, samples were separated on a 2.4-ml Superdex 20 PC3.2/30 column (GE Healthcare) equilibrated with Buffer B. Fractions of 100 μl were collected at a flow rate of 50 μl/min. Samples of column fractions of 10 μl were analyzed by immu-

nit was produced by in vitro transcription-translation with TnT T7 Quick kit (Promega) and [35S]methionine (Amerham).

Extracts were incubated with 10 μM Tris-HCl (pH 7.6), 5 mM MgCl₂, 1 mM DTT, 1 mM ATP, 10 mM phosphocreatine and 100 μg/ml creatine phosphokinase. Where indicated, 250 nM recombinant p31comet was added. Following incubation at 23 °C for the time periods indicated in the figures, samples were subjected to sequential immunoprecipitations with anti-Cdc27 fol-

ized ATP . It is possible that the binding of

mente by a protein kinase that is tightly associated with MCC be involved. Clearly, much more investigation is required to elucidate the mechanism by which ATP cooperates with p31comet to promote MCC disassembly.

While the results reported in this paper allow insight into an important step in mitotic checkpoint inactivation, additional steps in MCC disassembly remain to be identified. We found that p31comet-promoted disassembly of MCC releases a subcomplex of Mad2 bound to Cdc20 (Fig. 3 C and D). This is presumably fol-

ated by another process that liberates free Mad2 and Cdc20 from this subcomplex. In addition, we observed that in extracts incubated with ATP, Mad2 is released from MCC faster than Cdc20 (15) (Fig. 1B), suggesting the existence of a parallel path-

way that liberates Mad2. Another unsolved mystery in checkpoint inactivation is the role of ubiquitylation in this process (16), which is required for the dissociation of MCC from APC/C but not for the disassembly of MCC (15). Much remains therefore to be learned about the complex mechanisms by which the activity of APC/C is regulated by the mitotic checkpoint system.

Methods

Extracts from nocodazole-arrested cells were prepared as described pre-

viously (13). His6-p31comet (full-length human protein) was expressed in bac-

teria. Much of the expressed protein was not soluble; the soluble part was purified by affinity chromatography on Ni-NTA agarose (Qiagen) followed by gel filtration on Superdex 75 100/300 GL (GE Healthcare). [35S]-labeled p31comet was produced by in vitro transcription-translation with TnT T7 Quick kit (Promega) and [35S]methionine (Amerham).

Extracts were incubated with 10 μM Tris-HCl (pH 7.6), 5 mM MgCl₂, 1 mM DTT, 1 mM ATP, 10 mM phosphocreatine and 100 μg/ml creatine phosphokinase. Where indicated, 250 nM recombinant p31comet was added. Following incubation at 23 °C for the time periods indicated in the figures, samples were subjected to sequential immunoprecipitations with anti-Cdc27 fol-

owed by anti-BubR1 antibodies, as described previously (15), except that the amounts of these antibodies bound to Affi-prep Protein A beads were 1.0 and 0.25 μg/μl of packed beads, respectively. Following both immunopre-

cipitations, beads were washed three times with Buffer A consisting of 50 mM Tris-HCl (pH 7.2), 1 mg/ml BSA, 20% (v/v) glycerol and 0.5 mM DTT, and were resuspended in 2-4 volumes of the same buffer. The amounts of MCC components in anti-BubR1 immunoprecipitates were estimated by immunoblotting, using mouse monoclonal antibodies as described (15). Anti-BubR1 immunoprecipitates used for assay of release of MCC compo-

nents (see below) were prepared as above from checkpoint extracts without incubation, were stored at −70 °C in small samples and were thawed only once.

The release of MCC components from anti-BubR1 immunoprecipitates was determined by resuspending 3 μl (packed volume) anti-BubR1 beads in 30 μl of a buffer consisting of 40 mM Tris-HCl (pH 7.6), 1 mg/ml BSA, 5 mM MgCl₂, 1 mM DTT and 10% (v/v) glycerol. Where indicated, 250 nM p31comet or 5 mM ATP were added. Following incubation at 23 °C with shaking at 1,400 rpm for 1 h, samples were subjected to brief centrifugation and supernatants were passed through 0.45 μm Ultra-free centrifugal filters (Millipore), to ensure complete removal of beads from supernatants. Samples of 10 μl of supernatants were analyzed for MCC components by immuno-

blotting. The release of MCC components was expressed as the percentage of the amounts associated with anti-BubR1 beads prior to incubation.

Soluble MCC was partially purified from salt eluates of APC/C immunopre-

cipitates of checkpoint extracts (14) by gel filtration chromatography. Salt eluate originating from 16 ml of extract was concentrated to a volume of 2.5 ml by ultrafiltration and was applied to a 125-ml column of Superose
6 XX16 equilibrated with 50 mM Tris-HCl (pH 7.2), 100 mM NaCl, 0.1 mg/ml BSA and 1 mM DTT (Buffer B). Fractions of 2.5 ml were collected at a flow rate of 1 ml/min and were concentrated 10-fold by ultrafiltration. The peak of MCC was located by immunoblotting for the MCC components BubR1, Cdc20, and Mad2 and by inhibition of APC/C activity (14). MCC eluted at an apparent molecular size of 450–500 kDa. The central peak fractions of MCC (usually fractions 28–30) were collected.

APC/C activity was assayed by the ligation of [35S]-cyclin to ubiquitin, as described previously (14), with 1-μl samples (packed beads) of anti-Cdc27 immunoprecipitates. Unless otherwise stated, incubations were carried out at 23 °C for 60 min, with shaking at 1,000 rpm. To estimate the binding of [35S]-p31comet to anti-BubR1 immunoprecipitates, anti-BubR1 beads (3 μl) were suspended in 30 μl of Buffer A containing 2 μl of in vitro-translated [35S]-p31comet. The final concentration of [35S]-p31comet was approximately 5 nM, estimated by immunoblotting with rabbit polyclonal antibody raised against p31comet. Samples were incubated at 4 °C for 60 min with shaking at 1,200 rpm, beads were washed four times with 1-ml portions of Buffer A and subjected to SDS/PAGE. Radioactivity at the region of p31comet was estimated by phosphorimager analysis. Results were corrected for low nonspecific adsorption of [35S]-p31comet to beads, which was estimated in parallel samples with nonimmune IgG bound to protein A beads.

ACKNOWLEDGMENTS. This work was supported by grants from the Israel Science Foundation and the Diane and Guilford Glazer Distinguished Chair of the Israel Cancer Research Fund.