Activation of the Budding Yeast Spindle Assembly Checkpoint without Mitotic Spindle Disruption

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ural site of action. This activity is specific to Int-6 because Tax does not alter the PCD structure, as shown by examination of PML localization in the presence of Tax. Further analyses will determine not only the importance of the interaction in the onset of adult T cell leukemia in patients infected with HTLV-I but also a possible role of Int-6 in human nonviral cancers.

**REFERENCES AND NOTES**


6. We used the MATCH-MAKER two-hybrid system (Clontech, Palo Alto, CA). The Tax coding sequence was fused to the GUS DNA binding domain in the pGB-T9 yeast expression vector, giving pGB-Tax. The human cDNA library we used has been described (5). Briefly, cDNAs from EBN-transformed human peripheral lymphocytes were fused to the GAL4 transcriptional activation domain into the ACTH phage vector. pGB-Tax was introduced into the HFr7 yeast strain, and the resulting cells were transformed by the fusion cDNA library. The HFr7 strain possesses the His synthease gene (HIS3) and the lacZ gene under the control of GAL4 binding sites. From an initial screen of 250,000 transformants, 700 were found to grow on a minimal medium lacking His. Of these, 94 were also positive when assayed for β-galactosidase expression. Plasmid DNAs from the double positive clones were extracted and sequenced.


9. Forty hours after transfection by the calcium phosphate precipitation method, cells were fixed for 15 min in 4% paraformaldehyde, followed by 20 min in 0.1 M glycine, and permeabilized for 5 min in 0.5% Triton X-100. Cells were then incubated for 1 hour with the monoclonal antibody M2 (Kodak) directed against the FLAG epitope or Tax antigen. After washing, cells were further incubated for 45 min with appropriate secondary antibodies conjugated with fluorescein or rhodamine. Finally, nuclei were stained with Hoescht 33258.


11. C. Desbiez, P. R. Roussel, F. Bantignes, P. Jainot, data not shown.


15. K. Weiss et al., ibid., p. 345.

16. Immunofluorescence experiments were performed as described for Fig. 3. Cells were examined with a confocal laser scan microscope (Zeiss). Fluorescein was excited at 488 nm, and rhodamine, at 543 nm. The two channels were recorded independently. Pseudocolor images were generated and superimposed with Photoshop software.


21. HeLa cells (0.7 × 10⁵) were transfected by the calcium phosphate precipitation method with 1 μg of the pGUSCAT vector construct, 10 ng of pG4M or pG4S-Tax (8), and 100 ng of pSG-FNv or pSG-FNv-70. pSG-FNv is a pSG5 derivative encoding the FLAG epitope fused to the SV40 nuclear localization signal and to the VP16 activation domain (amino acids 403 to 479). The clone 70 Int-6 cDNA was inserted into this parental plasmid to give pSG-FNv-70. Duplicate transfections were done for each construct. After transfection, concentrations of the CAT protein were measured by enzyme-linked immunosorbant assay (Boehringer Mannheim).


23. Complementary DNA from the Int-6 clone 88 was inserted in-frame with the FLAG epitope into pSG5, giving pSGF-Int-6. COS7 cells (0.7 × 10⁶) were transfected with 1 μg of pSGF-Int-6 or 1 μg of pSG-Tax, or both. Cells were lysed in radiomunoprecipitation assay buffer. The immuno precipitates were separated on a 10% polyacrylamide–SDS gel and blotted onto nitrocellulose. The enhanced chemiluminescent detection system (ECL, Amer sham) was used to visualize bound antibodies.

24. We generated a polyclonal rabbit antiserum to a peptide corresponding to the COOH-terminal 20 amino acids of Int-6 coupled to ovalbumin. To purify this serum, we produced a FLAG-Int-6 fusion protein in bacteria and coupled it to a FLAG-M2 antibody affinity gel (Kodak). Covalent linkage between the protein and the M2 antibody was performed by treatment with glutaraldehyde. The antiserum to Int-6 was incubated with this matrix, and specific antibodies were eluted with 100 mM glycine (pH 2.5).


26. We thank S. Eledge for providing the cDNA library; B. Cullen for the antiserum to Tax; A. Dejean for the PML rabbit polyclonal serum; R. van Driel for the PML monoclonal antibody; P. Chambon for the PML expression vector; C. Souchier for help with confocal microscopy; F. Chatelat for assistance in preparing the figures; and J. Maryansky for critical reading of the manuscript. This work was supported by the Agence Nationale de Recherches sur le Sida et les Associa tion pour la Recherche contre le Cancer (F.B.).

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**Activation of the Budding Yeast Spindle Pole Body Assembly Checkpoint Without Mitotic Spindle Disruption**

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The spindle assembly checkpoint keeps cells with defective spindles from initiating chromosome segregation. The protein kinase Mps1 phosphorylates the yeast protein Mad1p when this checkpoint is activated, and the overexpression of Mps1p induces modification of Mad1p and arrests wild-type yeast cells in mitosis with morphologically normal spindles. Spindle assembly checkpoint mutants overexpressing Mps1p pass through mitosis without delay and can produce viable progeny, which demonstrates that the arrest of wild-type cells results from inappropriate activation of the checkpoint in cells whose spindle is fully functional. Ectopic activation of cell-cycle checkpoints might be used to exploit the differences in checkpoint status between normal and tumor cells and thus improve the selectivity of chemotherapy.

The spindle assembly checkpoint keeps cells with spindle defects from segregating their chromosomes. The defects the checkpoint detects include the absence of spindle microtubules (1, 2), monopolar spindles (3), and the misalignment of a single chromosome on the spindle (4). Mutations in the budding yeast MAD (mitotic arrest defective) (2) or BUB (budding uninhibited by benomylazole) (1) genes inactivate the checkpoint and allow cells with defective spindles to proceed through mitosis. Another checkpoint component, the essential protein kinase Mps1p (5, 6), has two roles in the cell cycle; it is required for spindle pole body duplication in G₀ (7) and for the spindle assembly checkpoint in mitosis (3). We found that Mps1p directly phosphorylated Mad1p and that overexpression of Mps1p constitutively activated the spindle assembly checkpoint in wild-type cells.

Spindle depolymerization causes hyperphosphorylation of Mad1p that leads to a decrease in the protein’s/gel mobility during SDS–polyacrylamide gel electrophoresis (PAGE) (8). To determine whether spindle pole body defects also induced this modification, we examined the phosphorylation of Mad1p at 37°C in cdc31 mutants, mps2 and mps1, mutants, which are all defective in spindle pole body duplication (7, 9). The cdc31 and mps2 mutants, which induce mitotic arrest,
also cause increased phosphorylation of Mad1p (Fig. 1A). In contrast, no modification of Mad1p was detected in mps1 cells, which failed to arrest at restrictive temperatures even when the cells were treated with nocodazole to induce microtubule depolymerization (3). These results link the hyperphosphorylation of Mad1p (8) with the role of Mps1p in the spindle assembly checkpoint (3) and suggest that modification of Mad1p is an essential step in the activation of the spindle assembly checkpoint.

To test whether Mps1p directly phosphorylates Mad1p, we used in vitro kinase assays with purified components. Active Mps1p was purified from yeast as a glutathione S-transferase (GST)–Mps1p fusion protein, and the NH₂-terminal third of Mad1p was expressed and purified from Escherichia coli. Mps1p directly phosphorylated Mad1p (Fig. 1B), and this in vitro phosphorylation led to reduced mobility of Mad1p, as seen in vivo upon checkpoint activation (10). It remains to be determined whether this modification is required for the activation of the checkpoint.

To determine whether increasing the activity of Mps1p would lead to Mad1p phosphorylation and mitotic arrest in cells whose spindle was normal, we overexpressed the MPS1 gene by fusing it to the galactose-inducible GAL1-10 promoter. We exposed cells transformed with this construct to galactose and monitored their spindle morphology by both light microscopy and electron microscopy (EM). Overexpression of Mps1p led to a cell-cycle arrest with a G2 DNA content. These cells contained short bipolar spindles whose length and overall appearance when observed by EM were no different from those of normal mitotic cells that had not initiated anaphase B (Fig. 2) (11). Transfer of the mitotically arrested cells from galactose to glucose allowed them to complete mitosis and produce viable progeny, which showed that overexpression of Mps1p did not induce permanent spindle damage.

Overexpression of Mps1p could arrest the cell cycle by three different mechanisms: (i) activation of the spindle assembly checkpoint without impairment of spindle function, (ii) activation of the checkpoint by induction of spindle defects, or (iii) activation of a pathway that does not involve the checkpoint. To distinguish among these possibilities, we overexpressed Mps1p in wild-type cells and in mad1, mad2, mad3, bub1, bub2, and bub3 mutants and analyzed cell-cycle progression by pedigree analysis of individual cells growing on solid medium or by monitoring cell morphology and the amount of a mitotic cyclin, Cbid2p, in cell populations in liquid culture. All of the mutants failed to arrest, which demonstrated that Mps1p overexpression arrests wild-type cells by activating the MAD- and BUB-dependent checkpoint (Fig. 3).

The mad3, bub1, bub2, and bub3 mutants that overexpressed Mps1p produced viable progeny. In contrast, mad1 and mad2 cells that passed through mitosis with high concentrations of Mps1p produced dead progeny (Fig. 3). This lethality may be due to interference with spindle pole body duplication: Mps1p overexpression that was temporarily restricted so that it did not overlap with the period of spindle pole body duplication still induced a mitotic arrest in wild-type cells but did not induce lethality in mad1 or mad2 cells. (12). The viability of the dividing mad and bub cells shows that Mps1p overexpression activates the spindle assembly checkpoint without producing defects that interfere with chromosome segre-
Fig. 3. Failure of overexpression of MPS1 to arrest mad and bud strains, even though Mad1p was hyperphosphorylated. The graphs depict microcolony assays (A) and budding index in liquid cultures (B) of the indicated strains. Certain mutants died as a result of the mitotic divisions (strain viability in (A): wild type (WT), 94%; mad1Δ, 9%; mad2Δ-1, 17%; mad3Δ, 94%; bub1Δ, 72%; bub2Δ-1, 92%; and bub3Δ, 88%). (C) Immunoblot of Mad1p show that none of the known Mad or Bub proteins are necessary for Mad1p modification when MPS1 is overexpressed (29).

REFERENCES AND NOTES

2. R. Li and A. W. Murray, ibid., p. 519.

22. Cells were grown in rich growth medium (yeast extract, peptone, and dextrose (YFD) for 3 hours at the indicated temperatures (Fig. 1). Nocodazole was used at 20 μg/ml in combination with benomyl at 30 μg/ml. Both drugs were necessary to maintain the arrest in wild-type cells at 37°C. Cell lysates were prepared, and immunoblotting was done as described (8). Strains are wild type (KH1445, mps1Δ-1 (W2421-2b), mps2Δ-1 (W5178-3c), and cdc31-2 (ELW65-9c). The cdc31-2 strain showed delayed mitosis when grown at 24°C. Mps3p was purified as a GST-fusion protein from yeast cells (6), and the GST was removed by cleavage with thrombin (18). Mad1–GST fusion proteins were purified and dialyzed as described (8). Kinase reaction conditions were as described (6) with the addition of approximately 18 nM GST-Mad1p, GST, or mycin basic protein (MBP). All substrates were present in Coomassie blue-stainable quantities. The Mad1–GST fusion contained residues 27 through 310 of Mad1p. A COOH-terminal fragment of Mad1p (residues 593 through 749) was also phosphorylated in this assay (12).

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24. Mps1p overexpression was achieved by placing the MPS1 gene behind the GAL10 promoter on a 2-μm plasmid (Fig. 2A). A single mc317 epitope was inserted with custom oligonucleotides into a Not I site that had been engineered by inserting linkers into a Hinc II site that follows the second codon of the MPS1 open reading frame. To create pElW025 [p2 μ, LEU2, GAL10-Nmyc(MPS1)], we inserted the Nmyc-terminally mc317-tagged MPS1 gene in an Afl III to Swa I fragment into pHC22 digested with Nco I and Smal I. The pElW025 was then transformed into yeast strain BJ/168, and galactose-driven overexpression of Mps1p was confirmed by immunoblotting with the 9E10 antibody (19) (Fig. 2A, inset). Cells (ELW175) grown in medium containing 2% raffinose or exposed to 3% galactose for 6 hours were prepared for EM (29) and viewed on a Phillips CM10 microscope (Mahwah, NJ). Spindle lengths were measured from spindle pole body to spindle pole body directly on the negatives from the EM and corrected for magnification (Fig. 2B). Several spindles traversed serial sections and were required spindle length correction for section thickness (70 to 80 nm) by triangulation. KH135 cells (which contain plasmid 1M120, a GAL1-Nmyc...
Regulation of Cardiac Na⁺,Ca²⁺ Exchange and K⁺ATP Potassium Channels by Pip₂

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Cardiac Na⁺,Ca²⁺ exchange activity can be enhanced by several acidic lipids (1, 2) that may occur in domains in cell membranes (3). In cardiac membrane patches treated with ATP, acidic lipids are generated on the cytoplasmic side of the membrane in parallel with a stimulation of Na⁺,Ca²⁺ exchange current (2, 4). The underlying mechanism might be (i) an ATP-dependent transport of phosphatidylserine (PS) from the extracellular to the cytoplasmic side by an amino phospholipid "flipase" (5), (ii) the phosphorylation of diacylglycerol (DAG) to form phosphatidic acid (FA) (6), or (iii) the phosphorylation of PI to form PIP₂ and PIP₃ (7). We used specific phospholipases and phospholipid vesicles to modify the lipid composition of giant cardiac membrane patches (8) and determined that the major mechanism is the generation of PIP₂ from PI.

Outward Na⁺,Ca²⁺ exchange current was increased by addition of Mg-ATP to the cytoplasmic side of inside-out giant cardiac membrane patches (Fig. 1A) (9). The current was first activated by application of 90 mM Na⁺ to the cytoplasmic side of the patch with 2 mM extracellular (pipette) Ca²⁺. With the free cytoplasmic Ca²⁺ concentration used (0.5 μM) the current inactivated (decreased) by about 80% over 15 s. Subsequent application of Mg-ATP (2 mM) for 40 s increased the current sifxold, and after ATP was removed the current remained stimulated for 100 s, after which it was turned off by removal of Na⁺.

The record in Fig. 1A is a control experiment from a randomized series of patches, one-half of which were treated for 4 min with a phospholipase C that specifically hydrolyzes PI (PL-PLC) (10). The PI-PLC treatment (0.6 U/ml) did not significantly decrease the current before application of ATP (11) (Fig. 1B), and PI-PLC had no effect after the current had been stimulated by ATP (12). However, the treatment decreased the ATP effect by 96% (P < 0.001). PIP₂ (50 μM) strongly activated the exchange current, although ATP did not (Fig. 1B). Pure PI vesicles (0.3 mM) were applied for 60 s to other treated patches that failed to respond to ATP (Fig. 1C). PI had no effect by itself, but it restored the capacity of ATP to stimulate the exchange current.

The effect of ATP was reversed by a recombinant PIP₂-specific phospholipase C, PLC-β₁, that is fully activated by 0.5 μM free Ca²⁺ under standard assay conditions (Fig. 2A) (13). This PLC-β₁ was histidine-tagged, expressed in Sf9 cells, purified by Ni²⁺-chelate affinity chromatography, and dialyzed against the solution used in the experiments. Reversal of the ATP effect after ATP removal was very slow (Fig. 2A). However, upon application of PLC-β₁ (0.2 mg ml⁻¹ with a maximal specific activity of 100 μmol min⁻¹ mg⁻¹), the current declined to its original value within 40 s (in three similar experiments). PLC-β₁ had no effect when it was applied to patches in which the exchange current had been stimulated by PS rather than ATP (12).

High concentrations of cytoplasmic free Ca²⁺ induced a faster reverse of the ATP effect, probably mediated by an endogenous Ca²⁺-dependent PLC (Fig. 2B). After ATP was applied and removed, 20 μM free Ca²⁺ was added. At first, the exchange current was slightly stimulated because cytoplasmic Ca²⁺ activates the exchange by an intrinsic regulatory mechanism (14). Thereafter, the exchange current declined rapidly over 30 s, and it declined to below its original level when free Ca²⁺ was reduced back to 0.5 μM (15). To determine the Ca²⁺ dependence of endogenous cardiac membrane-associated PLC, a crude membrane fraction was prepared from cultured myocytes, and PLC activity was measured as inositol trisphosphate (IP₃) released from exogenous vesicles containing [³H]PIP₂ (16). The PLC activity of the cardiac membranes was slightly activated with 0.5 μM free Ca²⁺ and was maximally activated with 20 μM free Ca²⁺ (Fig. 2C), which correlates with the ability of 20

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