

Analysis of Bub3 spindle checkpoint function in *Xenopus* egg extracts

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Summary

The spindle checkpoint delays the onset of anaphase if there are any defects in the interactions between spindle microtubules and kinetochores. This checkpoint has been reconstituted *in vitro* in *Xenopus* egg extracts, and here we use antibodies to *Xenopus* Bub3 (XBub3) to show that this protein is required for both the activation and the maintenance of a spindle checkpoint arrest in egg extracts. We detect two forms of XBub3 in egg extracts and find both to be complexed with the XBub1 and XBubR1 kinases. Only one form of XBub3 is apparent in *Xenopus* tissue culture (XTC) cells, and localisation studies reveal that,

unlike the Mad proteins, which are concentrated at the nuclear periphery, XBub3 is diffusely localised throughout the nucleus during interphase. During early prophase it is recruited to kinetochores, where it remains until chromosomes align at the metaphase plate. We discuss the mechanism by which our α -XBub3 antibodies interfere with the checkpoint and possible roles for XBub3 in the spindle checkpoint pathway.

Key words: Bub3, Spindle checkpoint, Kinetochores

Introduction

During cell division it is essential that cells attach all of their kinetochores to spindle microtubules before the onset of anaphase, otherwise daughter cells will gain or lose genetic material, resulting in aneuploidy (Nicklas, 1997). A cell cycle control termed the spindle checkpoint (also known as the mitotic or kinetochore-attachment checkpoint) acts to ensure this dependency (for reviews, see Musacchio and Hardwick, 2002; Shah and Cleveland, 2000). Kinetochores (Pluta et al., 1995; Rieder and Salmon, 1998) are key players in this checkpoint, and it has been shown that unattached kinetochores (Rieder et al., 1995) and/or kinetochores lacking tension (Li and Nicklas, 1995; Stern and Murray, 2001) can activate a checkpoint-dependent metaphase delay.

Budding yeast genetics originally identified the Mad and Bub proteins, and later the Mps1 kinase, as components of this checkpoint (Hoyt et al., 1991; Li and Murray, 1991; Weiss and Winey, 1996). These have since been shown to be conserved across species from yeast to man. Their precise functions in the checkpoint pathway are now the subject of intense study in many model organisms. More recently a number of kinetochore proteins, including the CENP-E microtubule motor protein (Chan et al., 1998; Yao et al., 2000), and ROD and Zw10 (Basto et al., 2000; Chan et al., 2000), have also been shown to play roles in the checkpoint.

The Bub3 protein binds constitutively to two other checkpoint proteins, the Bub1 kinase (Brady and Hardwick, 2000; Martinez-Exposito et al., 1999; Roberts et al., 1994; Taylor et al., 1998) and Mad3 (Fraschini et al., 2001; Hardwick et al., 2000; Millband and Hardwick, 2002). Mad3 is the yeast orthologue of vertebrate BubR1, which also binds to Bub3

(Chan et al., 1999; Chen, 2002; Yao et al., 2000). The precise function of Bub3 remains unclear but it has been suggested to target Bub1 and BubR1/Mad3 to kinetochores (Millband and Hardwick, 2002; Taylor et al., 2001). In *Saccharomyces cerevisiae*, there are two quite distinct Bub3-containing complexes. Upon checkpoint activation, Bub3 and Bub1 bind to Mad1 (Brady and Hardwick, 2000), and Bub3 is also found in a large complex associated with Mad3, Mad2 and the spindle checkpoint effector Cdc20 (Fraschini et al., 2001; Hardwick et al., 2000). In HeLa cells a similar complex containing BubR1, Bub3, Mad2 and Cdc20 has been detected (Fang, 2002; Sudakin et al., 2001; Tang et al., 2001). Thus it is possible that Bub3 has a number of distinct functions in the spindle checkpoint.

To prevent anaphase onset the Mad and Bub proteins inhibit the function of the checkpoint effector Cdc20/fizzy (Fang et al., 1998; Hwang et al., 1998; Kallio et al., 1998; Kim et al., 1998; Wassmann and Benezra, 1998). Cdc20 targets specific substrates to an E3 ubiquitin ligase known as the cyclosome/anaphase promoting complex (APC) (Visintin et al., 1997), which marks them for destruction by the proteasome (for reviews, see Page and Hieter, 1999; Zachariae and Nasmyth, 1999). A key APC substrate that needs to be destroyed for anaphase onset is securin Pds1/Cut2 (Funabiki et al., 1996; Yamamoto et al., 1996; Zou et al., 1999), which complexes with and inhibits the separase Esp1 (Ciosk et al., 1998). Once Esp1 is released it cleaves a cohesin, Scc1, in budding yeast (Uhlmann et al., 1999) and sister-chromatid separation and anaphase ensue.

The spindle checkpoint pathway has been reconstituted in *Xenopus* egg extracts and shown to be MAP kinase dependent (Minshull et al., 1994). It has since been shown to require the

XMad1 (Chen et al., 1998), XMad2 (Chen et al., 1996), XBub1 (Sharp-Baker and Chen, 2001), XBubR1 (Chen, 2002), XMps1 (Abrieu et al., 2001), Aurora B (Kallio et al., 2002) and CENP-E proteins (Abrieu et al., 2000). Here we have produced a number of antibodies specific for the XBub3 protein. With these antibodies we show that XBub3 function is required both for spindle checkpoint activation and for maintenance of a checkpoint arrest in *Xenopus* egg extracts. We find that XBub3 is complexed with both XBub1 and XBubR1 kinases, and we localise XBub3 throughout the cell cycle in *Xenopus* tissue culture (XTC) cells. During interphase XBub3 has a diffuse nuclear localisation, and it is recruited to kinetochores during early prophase. Once chromosomes align at the metaphase plate, XBub3 staining is lost. In egg extracts, our antibodies do not interfere with kinetochore localisation of XBub3 or of the other checkpoint proteins tested. We discuss their possible mechanisms of interference with the spindle checkpoint and different roles that XBub3 might play.

Materials and Methods

cDNA cloning of *Xenopus* Bub3

A cDNA encoding a partial *Xenopus* Bub3 (XBub3) fragment was isolated from a λ gt10 *Xenopus* oocyte cDNA library (Clontech) by PCR amplification. We used degenerate oligonucleotide primers directed against the sequences MVTGSWD (amino acids 112-117 of human Bub3) containing an *Eco*RI restriction site and NKKRLCQFHRY (amino acids 265-275 of human Bub3) containing a *Bam*HI restriction site. The resulting cDNA fragment was subcloned by digesting with *Eco*RI and *Bam*HI and ligated into Bluescript (KS⁻, Stratagene). DNA sequencing, using an Applied Biosystems Rhodamine sequencing kit and an ABI prism 377 sequencer, and a BLAST search of a nucleotide database confirmed that the partial cDNA fragment corresponded to amino acids 162-210 of a recently deposited *Xenopus* Bub3 sequence (Goto et al., 1999). The cDNA encoding full-length *Xenopus* Bub3 was then isolated from a *Xenopus* oocyte cDNA library (Clontech) by PCR amplification. We used oligonucleotide primers directed against the sequences MNTQTDM (amino acids 1-7 in XBub3) with the restriction site for the enzyme *Eco*RI placed at the 5' end, and DAETKPK (amino acids 324-330 in XBub3) with a restriction site for *Sal*I placed at the 5' end because XBub3 has an internal *Bam*HI site. DNA sequencing confirmed that the sequence of the full-length XBub3 DNA (990bp) was identical to a deposited XBub3 sequence [GenBank accession number AF119790 (Schwab et al., 2001)]. As such it has a single base change (GTT to GTC encoding valine at residue 210) when compared to that of Goto et al. (Goto and Kinoshita, 1999).

Expression of GST-XBub3 and production of XBub3 antibodies

The full-length XBub3 DNA was subcloned into pGEX-6P-2 (Pharmacia) for the expression of GST-tagged XBub3 in the *E. coli* strain BL21.DE3 (Stratagene). GST-XBub3 was induced overnight at 16°C and purified from *E. coli* lysates on glutathione-agarose resin (Sigma). The protein was eluted using 20 mM glutathione and dialysed into TBS (25 mM Tris, pH 8.0, 150 mM NaCl). Antibodies were raised in rabbits and sheep against full-length GST-XBub3 protein (Diagnostics Scotland) and affinity purified against GST-XBub3 protein coupled to Affigel 10 resin (BioRad). Antibodies were also raised in rabbits and sheep against a peptide corresponding to the C-terminal amino acids 316-330 (C-AIYIRQVTD AETKPK) of XBub3. The peptide was coupled with glutaraldehyde to keyhole limpet hemocyanin for immunisation in rabbits and sheep and to Affigel-10 resin (BioRad) for affinity purification of XBub3 peptide antibodies (Harlow and Lane, 1988).

Immunoprecipitation of XBub1 and XBubR1 and λ protein phosphatase treatment

Polyclonal XBub1 and XBubR1 antibodies were a gift from Rey-Huei Chen (Department of Molecular Biology and Genetics, Cornell University, Ithaca, NY). To immunoprecipitate XBub1 or XBubR1 20 μ l of Affi-prep protein A support beads (BioRad, Hercules, CA) were incubated with 3 μ g of XBub1 antibody for 1 hour at room temperature in TBS. The beads were washed into Extract Buffer (XB; 10 mM HEPES, pH 7.8, 50 mM sucrose, 100 mM potassium chloride, 10 mM magnesium chloride, 1 mM calcium chloride and 5 mM EGTA) containing 10 μ g/ml each of leupeptin, pepstatin and chymostatin (LPC) as protease inhibitors, and 50 mM sodium fluoride, 1 mM sodium vanadate and 80 mM sodium- β -glycerophosphate as phosphatase inhibitors. Excess buffer was removed and the washed beads were then incubated with 80 μ l of CSF-arrested *Xenopus* egg extract for 1 hour at 4°C. The beads were then washed three times with XB, containing protease and phosphatase inhibitors, twice in XB and twice with λ protein phosphatase buffer containing 10 mM MnCl₂. The XBub1 beads were then split into two aliquots, which were incubated in the presence and absence of 20 units of λ protein phosphatase (New England Biolabs Inc., Beverly, MA) for 30 minutes at 30°C. The bound proteins were eluted in SDS-PAGE sample buffer, resolved by SDS PAGE and detected by immunoblotting using rabbit XBub1 antibody or sheep XBub3 peptide antibody.

Immunoprecipitation of *Xenopus* Bub3 protein from denatured egg extracts

40 μ l of CSF-arrested *Xenopus* egg extract was denatured in 1 ml of TCA buffer (20 mM Tris pH 8.0, 10% TCA, 50 mM ammonium acetate, 2 mM EDTA). The denatured proteins were pelleted at 14,000 g for 10 minutes at 4°C in a microfuge. The pellets were then resuspended in 100 μ l of resuspension buffer (100 mM Tris, pH 11, 3% SDS, 3 mM DTT) by heating to 65°C for 5 minutes and 95°C for 10 minutes. 1 ml of immunoprecipitation buffer suspension (10 mM Tris pH 8.0, 150 mM NaCl, 1% Triton X-100) was then added to the extract protein suspension. 3 μ g of affinity-purified rabbit XBub3 peptide antibodies were coupled to 20 μ l of Affi-prep protein A support beads (Bio-Rad, Hercules, CA) in TBS (25 mM Tris, 150 mM sodium chloride, pH 8.0), and the washed beads were incubated with the above protein suspension for 90 minutes at 4°C. The beads were washed three times in immunoprecipitation buffer and the bound proteins eluted in SDS PAGE sample buffer. The proteins were resolved by SDS PAGE and detected using sheep XBub3 peptide antibodies.

Xenopus egg extracts and kinase assays

Fresh CSF-arrested *Xenopus* egg extracts were prepared from unfertilised *Xenopus* eggs as previously described (Murray, 1991). For analysis of spindle checkpoint activation the CSF egg extracts were pre-incubated with XBub3 antibodies to a final concentration of 50 μ g/ml for 1 hour at 4°C. The extracts were released from metaphase arrest by addition of 0.8 mM CaCl₂ in the presence or absence of 10 μ g/ml nocodazole and 10,000 sperm nuclei/ μ l extract. For analysis of spindle checkpoint maintenance the checkpoint was activated by addition of 10 μ g/ml nocodazole and 10,000 sperm nuclei/ μ l extract for 20 minutes at room temperature, antibodies added and the extracts incubated for a further 30 minutes at room temperature. 0 time points were taken and then CaCl₂ added to 0.8 mM. The reactions were incubated at room temperature for 60 minutes and 1 μ l samples were removed from the extracts during this time for histone H1 kinase activity analysis. The products of the histone H1 kinase reaction were resolved by 15% SDS-PAGE and the dried gels exposed to hyper β max X-ray film (Amersham). Nuclear morphology in egg extracts was analysed by fixing aliquots of extract in an equal volume of fix/stain solution (10 mM HEPES, 200 mM sucrose, 7.4% formaldehyde

containing 10 µg/ml Hoechst 33258) and the DNA visualised by fluorescence microscopy. Images were captured using a Photometrics Sensys charge-coupled device (CCD) camera linked to a using a Zeiss Axioskop fluorescence microscope using Smartcapture imaging software (Vysis).

Gel filtration analysis

Aliquots of CSF egg extract were incubated in the presence of 10,000 sperm/µl egg extract, diluted fivefold in XB containing 10 µg/ml each of leupeptin, pepstatin and chymostatin and 50 mM sodium fluoride, 1 mM sodium vanadate, 80 mM sodium-β-glycerophosphate, 1mM DTT and then filtered through a 0.22 µm filter unit (Millipore). The diluted samples were resolved on Superose-6 gel filtration column (Pharmacia) equilibrated in XB and 1 mM DTT at a flow rate of 0.4 ml/minute, and 500 µl fractions were collected. The samples were precipitated in 15% TCA, washed in acetone and resuspended in SDS PAGE sample loading buffer.

Immunofluorescent staining of XTC cells

Asynchronous and nocodazole-treated (10 µg/ml for 4 hours) *Xenopus* tissue culture cells were grown on coverslips for 24 hours, rinsed in MMR buffer (100 mM NaCl, 2 mM KCl, 1 mM MgSO₄, 2 mM CaCl₂, 10 mM EDTA, 5 mM HEPES, pH 7.8) and incubated in 25% Hanks balanced salt solution (Gibco-BRL, Paisley, Scotland) for 10 minutes at room temperature. The cells were fixed in MMR containing 3% paraformaldehyde, rinsed in MMR and then blocked in TBS containing 5% milk. Primary antibodies were added to a final concentration of 1 µg/ml in TBS/5% milk and detected using fluorescent Alexa-labelled secondary antibodies (Molecular Probes). Nuclear morphology was analysed by placing the coverslips on slides containing mounting medium containing DAPI (Vector Labs).

Immunofluorescent staining of chromosomes assembled in *Xenopus* egg extracts

Replicated metaphase sperm chromatin was produced in *Xenopus* egg

extracts using the method previously described (Chen et al., 1998). Briefly, 20 µl of CSF egg extract was released into interphase by addition of 0.8 mM CaCl₂ in the presence of 1500 sperm/µl extract. The extracts were incubated at room temperature for 90 minutes to replicate the DNA and then induced to enter mitosis by addition of 10 µl of CSF egg extract and incubated at room temperature for a further 90 minutes. Affinity-purified XBub3 antibodies were added to 0.1 µg/ml during this incubation. The extracts were then incubated in the presence or absence of 10 µg/ml nocodazole for 10 minutes at room temperature and followed by dilution with 10 volumes of XB containing 0.5% Triton X-100. The diluted extracts were then layered over 5ml of 30% glycerol made in XB plus 0.5% Triton-X100 with a coverslip placed at the bottom of the tube. The chromosomes were collected by centrifugation at 8000 rpm in a Beckman JS13.1 rotor and the coverslips processed for immunofluorescence as described for staining of XTC cells.

Results

Isolation and characterisation of *Xenopus* Bub3

A partial XBub3 cDNA was isolated from a λgt10 *Xenopus* oocyte cDNA library by degenerate PCR using primers directed against the sequences MVTGSWD (amino acids 112-117 of human Bub3) and NKKRLCQFHRY (amino acids 265-275 of human Bub3). DNA sequencing and a search of the database confirmed that the cDNA fragment encoded amino acids 162-210 of a recently deposited XBub3 sequence (Goto and Kinoshita, 1999). A full-length cDNA was then isolated by PCR amplification of a *Xenopus* oocyte cDNA library using oligonucleotide primers directed against the known full-length XBub3 sequence (Goto and Kinoshita, 1999). Our DNA sequencing confirmed that the PCR fragment isolated was identical to that recently deposited (Schwab et al., 2001).

Fig. 1 shows an alignment between the Bub3 proteins of *Xenopus*, human, *Drosophila* and budding yeast. The bulk of the Bub3 protein is highly conserved across species. This is

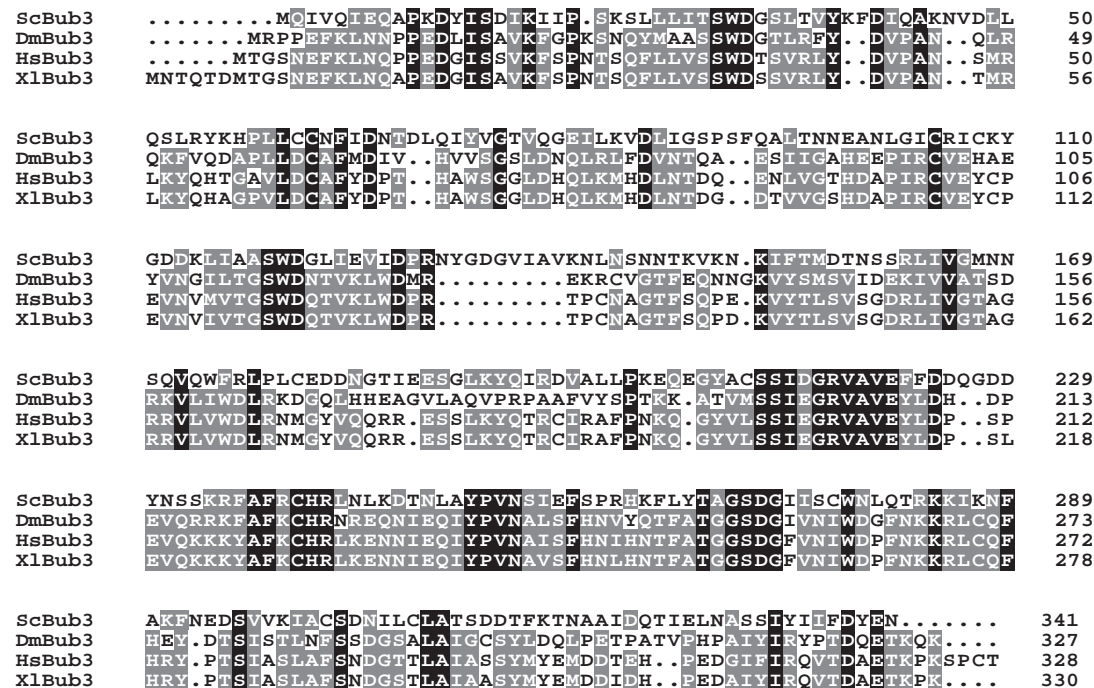


Fig. 1. Alignment of Bub3 homologues. A Clustal alignment of Bub3 proteins from *Saccharomyces cerevisiae* (M64707), *Drosophila melanogaster* (AF106679), *Homo sapiens* (AF047473) and *Xenopus laevis* (AB018419/AF119790). Identical residues are shown in black and similar residues are highlighted in grey. The region against which the peptide antibodies were raised is underlined.

likely to be because of its 3D structure, which consists almost entirely of seven WD repeats that fold together to form a β -propeller-like structure (David Wilson, UC Davis, personal communication).

As the *Xenopus* Bub3 protein remained uncharacterised, we raised antibodies against full-length bacterially expressed GST-XBub3 and a peptide corresponding to the C-terminal residues 316-330 of XBub3 in both rabbits and sheep. The affinity-purified antibodies recognise bacterially expressed GST-XBub3 by western blotting (data not shown), confirming that the antibodies were capable of recognising XBub3 protein and detected a single band in *Xenopus* tissue culture cell (XTC) lysates running slightly larger than the predicted molecular weight of XBub3 (37kDa) (Fig. 2A). The antibodies recognised a doublet of bands in both CSF and interphase egg extracts (Fig. 2A). Although our antibodies can immunoprecipitate XBub3 protein expressed in the rabbit reticulocyte lysate system and a small amount of XBub3 from egg extracts (data not shown), none of the full-length or peptide

antibodies can efficiently immunodeplete XBub3 from egg extracts unless the extract is first denatured. After TCA precipitation and renaturation, both bands of the doublet could be immunoprecipitated by the rabbit XBub3 peptide antibody (Fig. 2B).

Xenopus Bub1 protein has recently been isolated and shown to undergo a mobility decrease on SDS-PAGE consistent with the active form of XBub1 being a phosphoprotein in *Xenopus* egg extracts (Schwab et al., 2001; Sharp-Baker and Chen, 2001). We immunoprecipitated XBub1 and XBubR1 using affinity-purified antibodies and we probed the immunoprecipitates with our XBub3 antibodies. Both bands of the XBub3 protein doublet were present in XBub1 and XBubR1 immunoprecipitates (Fig. 2C,E), demonstrating that both forms of XBub3 associate with each of these kinases. We found no evidence for a complex containing both XBub1 and XBubR1 (data not shown) (see also Chen, 2002).

To determine whether the two forms of XBub3 found in egg extracts were due to modification by phosphorylation, we

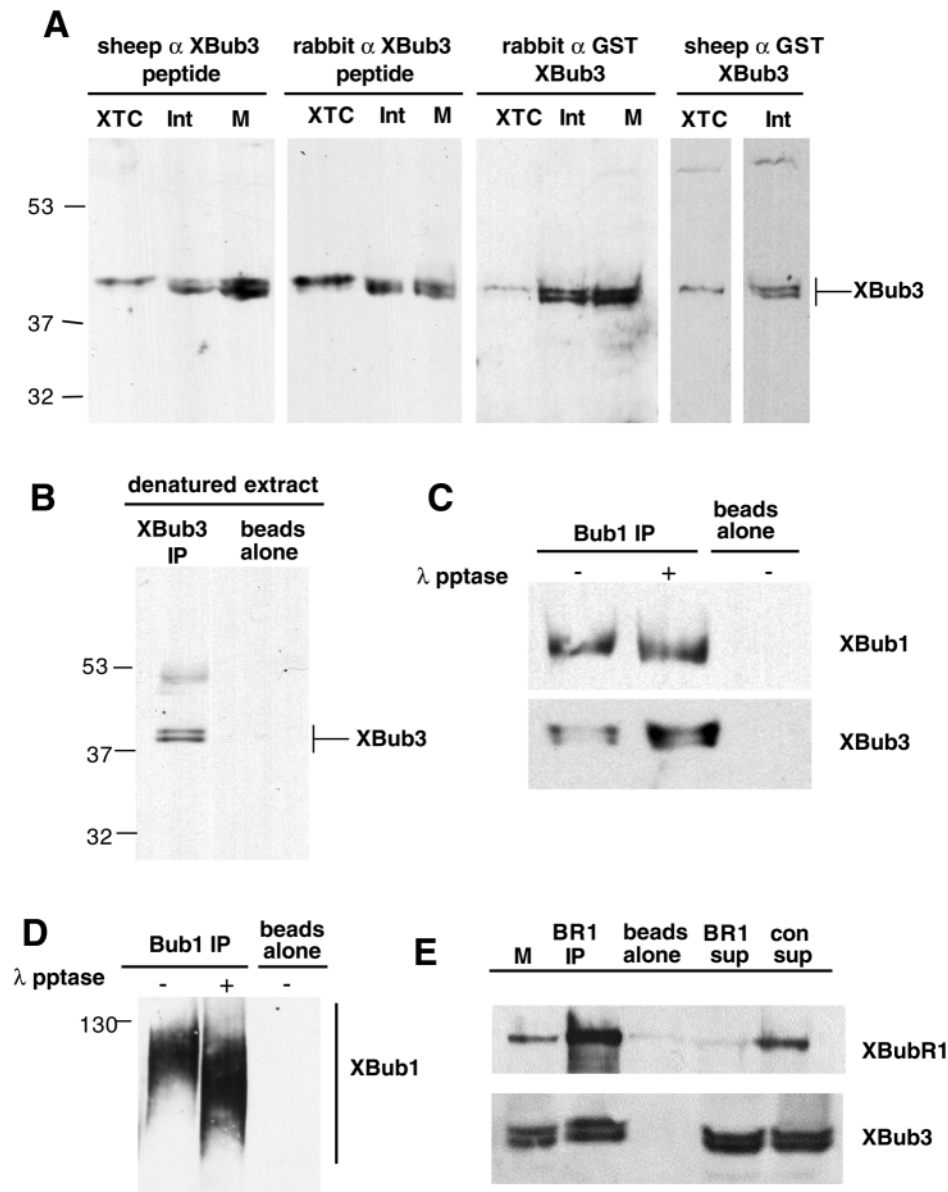


Fig. 2. Detection of XBub3 by immunoblotting and its co-immunoprecipitation with XBub1/XBubR1. (A) Immunoblot analysis of XBub3 in XTC cell lysates (XTC), interphase egg extracts (Int) or CSF egg extracts (M), using sheep and rabbit XBub3 peptide and full-length XBub3 antibodies. A doublet of bands in egg extracts and a single band in XTC cell lysates were specifically detected. (B) Immunoblot analysis of proteins immunoprecipitated from a denatured (TCA precipitated and re-natured) CSF egg extract using XBub3 antibody beads (XBub3 IP) or control beads without antibody. Both bands of the XBub3 were immunoprecipitated, although neither can be efficiently immunoprecipitated from a native extract (data not shown). (C) XBub1 and XBub3 co-immunoprecipitate. CSF egg extracts were incubated with beads alone or XBub1 beads and the immunoprecipitates were then treated with (+) or without (-) λ protein phosphatase (λ pptase). Bound proteins were eluted in sample buffer, separated by SDS-PAGE and the Bub proteins were then detected by immunoblotting using rabbit anti-XBub1 antibody or sheep anti-XBub3 peptide antibody. (D) As in C, except that the XBub1 was further resolved by running a large 7.5% SDS polyacrylamide gel for 12 hours. (E) XBubR1 and XBub3 co-immunoprecipitate. CSF egg extracts (M) were immunoprecipitated with BUBR1 antibodies (BR1 IP) or beads alone. Samples of BUBR1-depleted supernatant (BR1 sup) or beads alone supernatant (con sup) are shown. Bub proteins were detected by immunoblotting using rabbit anti-XBubR1 antibody or sheep anti-XBub3 peptide antibody.

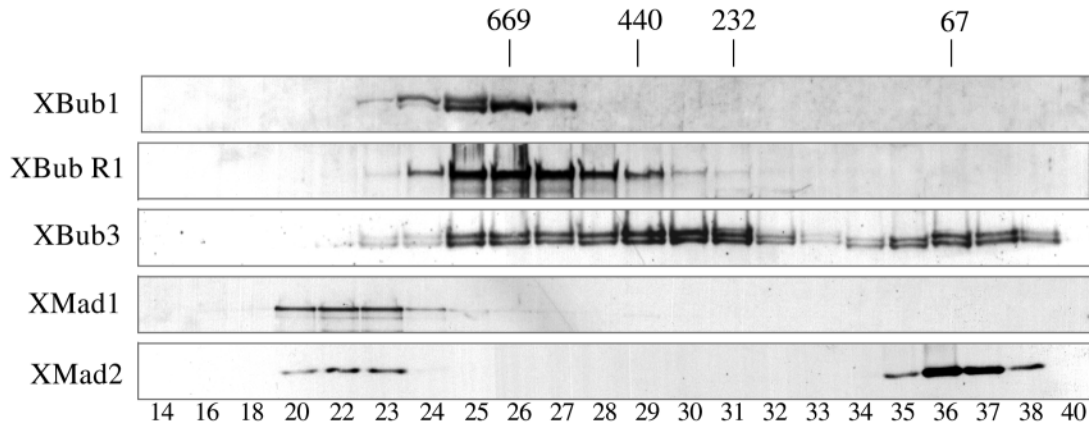


Fig. 3. Gel filtration analysis of XBub3 protein complexes in egg extracts. A CSF egg extract was fractionated by Superose-6 gel filtration chromatography (see Materials and Methods). Protein-containing fractions were analysed by western blotting with antibodies to XBub1, XBubR1, XBub3, XMad1 and XMad2 proteins. No protein was detected by Ponceau staining or western blotting prior to fraction 14 and after fraction 40. The elution positions of molecular weight standards are indicated.

treated the XBub1 immunoprecipitates with λ protein phosphatase. Such treatment led to an increase in the gel mobility of XBub1, consistent with dephosphorylation of XBub1 (Fig. 2C,D) but the doublet of XBub3 remained. These results demonstrate that both forms of the XBub3 protein are capable of binding XBub1 and XBubR1 in egg extracts and that the XBub3 doublet is unlikely to be the result of phosphorylation. Thus, while only one major form of XBub3 is present in XTC cells, there appear to be two different forms in *Xenopus* egg extracts.

We next analysed how the two forms of XBub3 fractionate upon gel filtration chromatography of CSF egg extracts containing sperm nuclei. We observed that both bands of the doublet of XBub3 behave identically upon gel filtration analysis, forming two major peaks consisting of free Bub3 (Fig. 3, fractions 35–38) and Bub3 complexes (fractions 25–31). Although a substantial proportion of the XBub3 protein co-elutes with Bub1 and BubR1 in complexes of around 669 kDa, a major proportion of XBub3 is found in complexes of 440–232 and does not co-elute with either of these proteins (fractions 29–31). A small amount of the Mad1 protein overlaps with the elution profile of the XBub3, but we observed no differences in the distribution or the amounts of any of these checkpoint proteins upon checkpoint activation or in the absence of sperm chromatin.

Xenopus Bub3 is required to establish and maintain spindle checkpoint arrest in *Xenopus* egg extracts

Xenopus eggs are arrested in metaphase of their second meiotic division by cytostatic factor (CSF) when they are laid. Fertilisation produces a transient peak of calcium, which induces exit from this meiotic arrest and the initiation of mitotic divisions. The spindle checkpoint can be reconstituted in *Xenopus* egg extracts by releasing the extracts from metaphase arrest with CaCl_2 in the presence of 10,000 sperm/ μl extract and 10 $\mu\text{g}/\text{ml}$ nocodazole (Minshull et al., 1994). Activation of the spindle checkpoint in these egg extracts produces a mitotic arrest that cannot be overridden by the addition of calcium and is characterised by the presence of

high histone H1 kinase activity and highly condensed chromosomes. We wished to investigate the role of XBub3 in the spindle checkpoint. To do this we tested whether our XBub3 antibodies had any effect on activation of the spindle checkpoint in egg extracts. CSF-arrested extracts were pre-incubated with affinity-purified full-length rabbit anti-XBub3 antibodies or mock affinity-purified rabbit pre-immune serum and then released from metaphase by calcium addition in the presence of 10 $\mu\text{g}/\text{ml}$ nocodazole and 10,000 sperm/ μl of extract (Fig. 4B). It is important to note that pre-incubation of CSF-arrested egg extracts with our rabbit full-length XBub3 antibody had no effect on the CSF arrest of the extract: histone H1 kinase levels were still high at the zero time point, before calcium addition (Fig. 4B). Thus XBub3 is not required to maintain a CSF arrest. This fits well with the findings that neither XBub1 (Sharp-Baker and Chen, 2001; Tunquist et al., 2002) nor XBubR1 (Chen, 2002) are required to maintain a CSF arrest.

In the presence of a pre-immune antibody the spindle checkpoint was activated, as shown by the presence of condensed chromatin and maintenance of high levels of histone H1 kinase activity up to 60 minutes after calcium addition (Fig. 4A,B). However, upon addition of calcium, H1 kinase levels failed to be maintained in the extracts containing XBub3 antibodies. We conclude that these antibodies are 'function-blocking' and that XBub3 is required to establish a spindle checkpoint arrest. Interphase nuclei were formed in the presence of nocodazole and XBub3 antibody within 20 minutes, and these grew to normal proportions by 60 minutes (Fig. 4Aa,c), indicating that inhibition of XBub3 function does not cause major disruption of nuclear structure.

We next tested whether our full-length XBub3 antibodies were capable of overriding the maintenance of a spindle checkpoint arrest if the checkpoint is activated prior to antibody addition. The spindle checkpoint was activated by the addition of nocodazole and 10,000 sperm/ μl extract for 20 minutes at room temperature. Full-length rabbit XBub3 antibodies were then added to the extract for 30 minutes at room temperature. Following this incubation calcium was added to release the egg extracts from CSF arrest. Under these

conditions, addition of our XBub3 antibody disrupted the maintenance of the previously established spindle checkpoint, observed as a decrease in histone H1 kinase activity (Fig. 4C) and the formation of interphase nuclei (data not shown) after

calcium addition. When taken together these results show that *Xenopus* Bub3 function is required for the activation and for the maintenance of a spindle checkpoint arrest in egg extracts.

XBub3 binds to kinetochores prior to XMad2 during early prophase

Bub3p has been localised to kinetochores in a number of species when the spindle checkpoint is active (Basu et al., 1998; Martinez-Exposito et al., 1999; Taylor et al., 1998). XMad2 localises to kinetochores in XTC cells treated with the microtubule-depolymerising drug, nocodazole, and during late prophase/prometaphase of the normal cell cycle (Chen et al., 1996).

Although Mad1 is required to recruit Mad2 to kinetochores (Chen et al., 1996) and Bub3 required for Bub1 kinetochore recruitment (Taylor et al., 1998), analysis of the timing of Mad1-Mad2 and Bub1-Bub3 kinetochore recruitment during the cell cycle and upon spindle checkpoint activation has not been carefully examined. To do this we first analysed XBub3 localisation in XTC cells in which the checkpoint had been activated using the microtubule-depolymerising drug, nocodazole. XBub3 was detected using our affinity-purified sheep anti-XBub3 peptide antibodies. The chromosomes of cells arrested in metaphase by nocodazole showed highly condensed chromosomes with strong punctate staining for XBub3, and this staining overlapped exactly with the kinetochore staining of XMad2 protein, indicating that XBub3 protein localises to kinetochores when the spindle checkpoint is activated in XTC cells (Fig. 5). We next examined the localisation of XBub3 protein in an asynchronous population of XTC cells. XBub3 protein localised exclusively to the nucleus during interphase, becoming bound to kinetochores in early prophase and persisting there until all the chromosomes had aligned at metaphase (Fig. 6a-e). At metaphase (Fig. 6e) a single chromosome stained brightly for XBub3 and XMad2, presumably because this chromosome had not yet, or had only just, aligned on the metaphase plate. XBub3 and XMad2 were not detectable at the kinetochores of chromosomes at the metaphase to anaphase transition or during anaphase, with cells simply showing general cytoplasmic staining at this time. Thus the bulk of both checkpoint proteins appear to leave the kinetochore at metaphase in XTC cells. By telophase, XBub3 was once again concentrated in the nucleus (Fig. 6g).

The XBub3 and XMad2 staining were equally intense at kinetochores in nocodazole-treated cells (Fig. 5). However, within the asynchronous culture of XTC cells, we observed intense XBub3 staining during early prophase at a time when a high proportion of the XMad2 protein was still diffusely nuclear and associated with the nuclear membrane. We only observed clear XBub3 and XMad2 colocalisation during late prophase and prometaphase (Fig. 6c,d). Thus we have

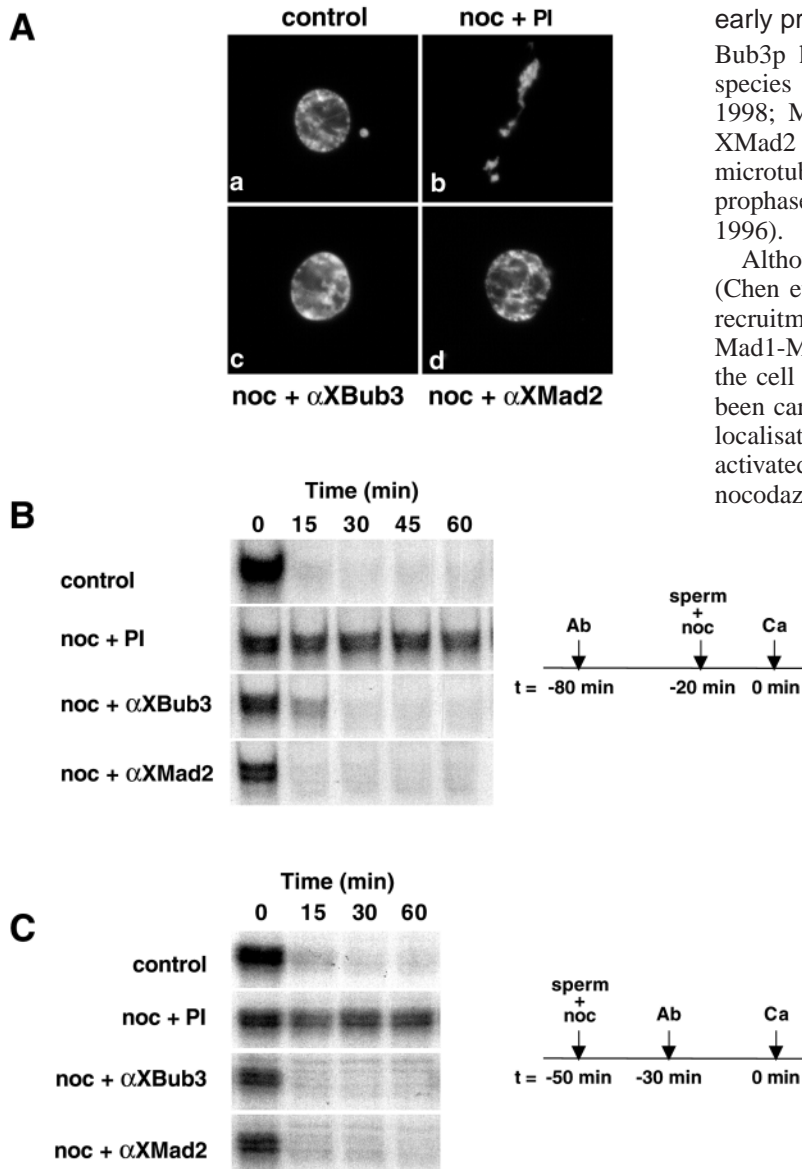


Fig. 4. XBub3 antibodies override spindle checkpoint activation and maintenance in egg extracts. (A) Checkpoint activation. Aliquots of CSF-arrested *Xenopus* egg extract were pre-incubated with the following additions and then released from metaphase arrest with calcium chloride. Buffer control, nocodazole plus pre-immune antibody, nocodazole plus rabbit XBub3 antibody, nocodazole plus XMad2 antibody. Chromosomes were visualised by the fluorescence of the DNA-binding dye Hoechst 33258 60 minutes after calcium addition. (B) Checkpoint activation. Samples were removed from the above egg extracts at the timepoints indicated for analysis of histone H1 kinase activity. The zero time point is histone H1 kinase activity prior to calcium addition. (C) Checkpoint maintenance. The spindle checkpoint was activated in aliquots of egg extract and the egg extracts further incubated at room temperature in the presence of a buffer control, nocodazole plus pre-immune antibody, nocodazole plus rabbit XBub3 antibody or nocodazole plus XMad2 antibody. Calcium was added to release the extracts from metaphase arrest and samples removed for analysis of H1 kinase activity at the timepoints indicated.

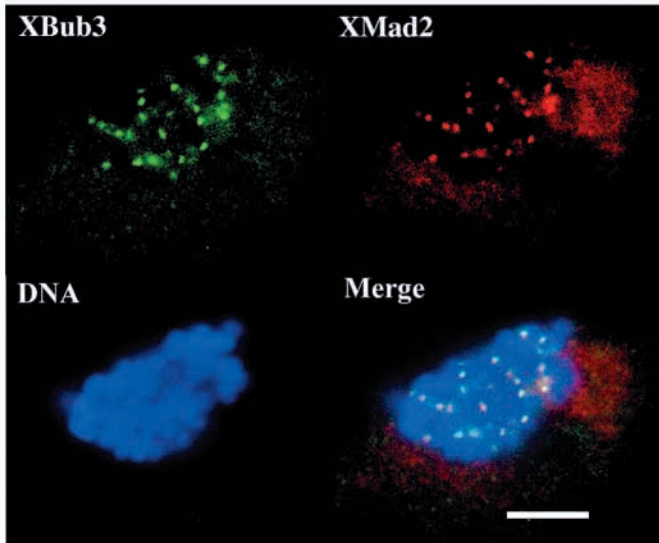


Fig. 5. Colocalisation of XBub3 and XMad2 at kinetochores in nocodazole-treated XTC cells. XTC cells grown on coverslips were treated with 10 $\mu\text{g/ml}$ nocodazole for 4 hours and then fixed and stained using rabbit XMad2 antibody (XMad2) and a sheep XBub3 peptide antibody (XBub3). The antibodies were detected using fluorescence-labelled secondary antibodies (XBub3 is green, XMad2 is red). Chromosomes were detected by mounting the coverslips in medium containing DAPI (DNA). The merge of all three fluorescent images is also shown. Bar, 10 μm .

consistently observed XBub3 at kinetochores before XMad2 kinetochore staining (Fig. 6, panel b) and before XMad1 kinetochore staining (data not shown).

XBub3 antibodies do not override the spindle checkpoint by preventing kinetochore recruitment of Bub3 or Mad2 proteins

Both XMad1 and XMad2 have been shown to localise to replicated chromosomes when the spindle checkpoint is activated in *Xenopus* egg extracts (Chen et al., 1998). Addition of XMad1 antibodies to these extracts prevents kinetochore binding of both XMad1 and XMad2 proteins and prevented the establishment of the spindle checkpoint. Addition of XMad2 antibodies did not affect the kinetochore localisation of either protein, yet interfered with checkpoint function (Chen et al., 1998).

As XBub3 appears to be recruited to kinetochores before XMad2, we wished to determine whether our XBub3 antibodies would inhibit the establishment of the spindle checkpoint in egg extracts by preventing XBub3 and/or XMad1/Mad2 binding to kinetochores. To do this we examined the kinetochore localisation of XBub3 on chromosomes assembled in egg extracts in the presence of our XBub3 antibodies. Metaphase chromosomes and spindles were first allowed to assemble in egg extracts, and then nocodazole was added to induce microtubule depolymerisation. When chromosomes were isolated (see Materials and Methods) during the metaphase arrest we found no XBub3 detectable at kinetochores. Chromosomes isolated after nocodazole treatment showed bright, punctate XBub3 staining, which was

confirmed as kinetochore staining by colocalisation with XMad2 (Fig. 7, top 2 rows). Pre-incubation of the egg extracts with our full-length or peptide XBub3 antibodies did not disrupt the kinetochore localisation of XBub3 or XMad2 as the kinetochore staining obtained is identical to that seen in the absence of pre-incubation with XBub3 antibodies (Fig. 7, bottom two rows). We also observed no disruption of XBub1 kinetochore staining on chromosomes isolated from egg extracts containing XBub3 antibodies (data not shown). These results show that although our XBub3 antibodies disrupt the establishment and maintenance of a spindle checkpoint arrest (Fig. 4) they do not do this by preventing XBub3, XMad2 or XBub1 binding to kinetochores in the egg extracts.

Discussion

The *Xenopus Bub3* gene was originally isolated by researchers looking at mRNA which accumulates in only the animal pole of early (eight-cell stage) embryo blastomeres, although the reasons for the differential mRNA localisation remain unclear (Goto and Kinoshita, 1999). Here we have characterised *Xenopus* Bub3 protein and report that there are two forms of XBub3 detectable in egg extracts, both of which bind to XBub1 and XBubR1 kinases (Fig. 2), and one form in XTC cells. As is the case in other organisms, we found no evidence for XBub3 modification by phosphorylation (Fig. 2) (Sharp-Baker and Chen, 2001). The precise nature of the two forms of XBub3 observed in egg extracts remains unclear, and as both interact with XBub1 and XBubR1 (Fig. 2) and fractionate identically by gel filtration chromatography (Fig. 3) there is no evidence that they have distinct functions.

In *Xenopus* egg extracts, spindle checkpoint activation and maintenance both require the functions of XMad1 and XMad2 (Chen et al., 1998; Chen et al., 1996), the kinesin-related microtubule motor protein CENP-E (Abrieu et al., 2000), the protein kinases XBub1 (Sharp-Baker and Chen, 2001), XBubR1 (Chen, 2002), XMps1 (Abrieu et al., 2001), Aurora B (Kallio et al., 2002) and a *Xenopus* MAP kinase (Cross and Smythe, 1998; Minshull et al., 1994; Takenaka et al., 1997). Here we have shown that *Xenopus* Bub3 is also required for spindle checkpoint activation and for maintenance of a spindle checkpoint signal in egg extracts (Fig. 4). This is the first demonstration that Bub3 is required to maintain a spindle checkpoint arrest in any system.

Our work (Fig. 4) also demonstrates that XBub3 function is not required for the maintenance of a meiotic metaphase arrest by CSF. This agrees with experiments showing that XBub1 and XBubR1 depletion have no effect on the maintenance of a CSF arrest (Chen, 2002; Sharp-Baker and Chen, 2001; Tunquist et al., 2002). Note, it has recently been shown that XBub1 is required to establish a CSF arrest (Tunquist et al., 2002), but a similar role for XBub3 has yet to be analysed.

XBub3 complexes

Bub3 is a small WD-repeat protein, and the budding yeast homologue has been crystallised and shown to consist of seven WD-repeat domains that fold together to form a β -propeller structure (David Wilson, UC Davis, personal communication). Many WD-repeat-containing proteins are known to form multi-protein complexes (for a review, see Smith et al., 1999). Bub3p

forms complexes with Bub1p and Mad3p in both *S. cerevisiae* and *Schizosaccharomyces pombe* (Hardwick et al., 2000; Millband and Hardwick, 2002; Roberts et al., 1994) and the kinases Bub1 and BubR1 in vertebrates, the second of which is homologous to yeast Mad3 (Chan et al., 1999; Chan et al., 1998; Martinez-Exposito et al., 1999; Taylor et al., 1998). In

budding yeast, two distinct Bub3-containing multi-protein complexes are formed during metaphase and upon spindle checkpoint activation. The first of these complexes is a Bub3-Bub1-Mad1 complex containing phosphorylated Mad1, the formation of which has been suggested to cause the release of Mad2 from Mad1 (Brady and Hardwick, 2000; Sironi et al., 2001). The second complex contains Bub3-Mad3-Mad2-Cdc20 (Fraschini et al., 2001; Hardwick et al., 2000). In vertebrates a similar complex has been detected between Bub3-BubR1, Cdc20 and Mad2 proteins. However, it is currently unclear as to whether Bub3-BubR1 and Mad2 are part of one APC inhibitory complex or function as distinct complexes both of which bind Cdc20 and inhibit APC activation (Fang, 2002; Sudakin et al., 2001; Tang et al., 2001).

Our gel filtration and co-immunoprecipitation analyses show that the Mad1-Mad2, Bub1-Bub3 and BubR1-Bub3 complexes are present in metaphase-arrested egg extracts (Fig. 3). We do not observe any major change in the elution profiles of these proteins upon checkpoint activation (data not shown). Thus, if spindle checkpoint activation in egg extracts requires the formation of a Mad1-Bub1-Bub3 complex or a Bub3-BubR1-Mad2 complex then the amount of protein found in such complexes corresponds to a small proportion of the total protein present in the egg extract. Alternatively, such complexes may be kinetochore bound and therefore not detectable by our gel filtration analysis. Consistent with the former explanation, less than 1% of Mad2 forms a complex with Cdc20 when the checkpoint is active in *Xenopus* egg extracts (Chung and Chen, 2002), and gel filtration analysis in budding yeast shows no change in the elution profiles of Bub1-Bub3, Mad1-Mad2 and Mad3 between cycling yeast cell cultures and spindle-checkpoint-arrested cultures (Fraschini et al., 2001).

Our gel filtration analysis also revealed that although a substantial proportion of the XBub3 protein co-elutes with Bub1 and BubR1 in complexes of around 670 kDa, a major proportion of XBub3 is found in complexes of 440-232 where it does not co-elute with the XBub kinases. The precise composition of these complexes remains to be determined, but we find no co-enrichment of *Xenopus* Cdc20 protein, which is present in most of the fractions between 670 and 67 kDa (L.C. and K.G.H., unpublished) (Lorca et al., 1998).

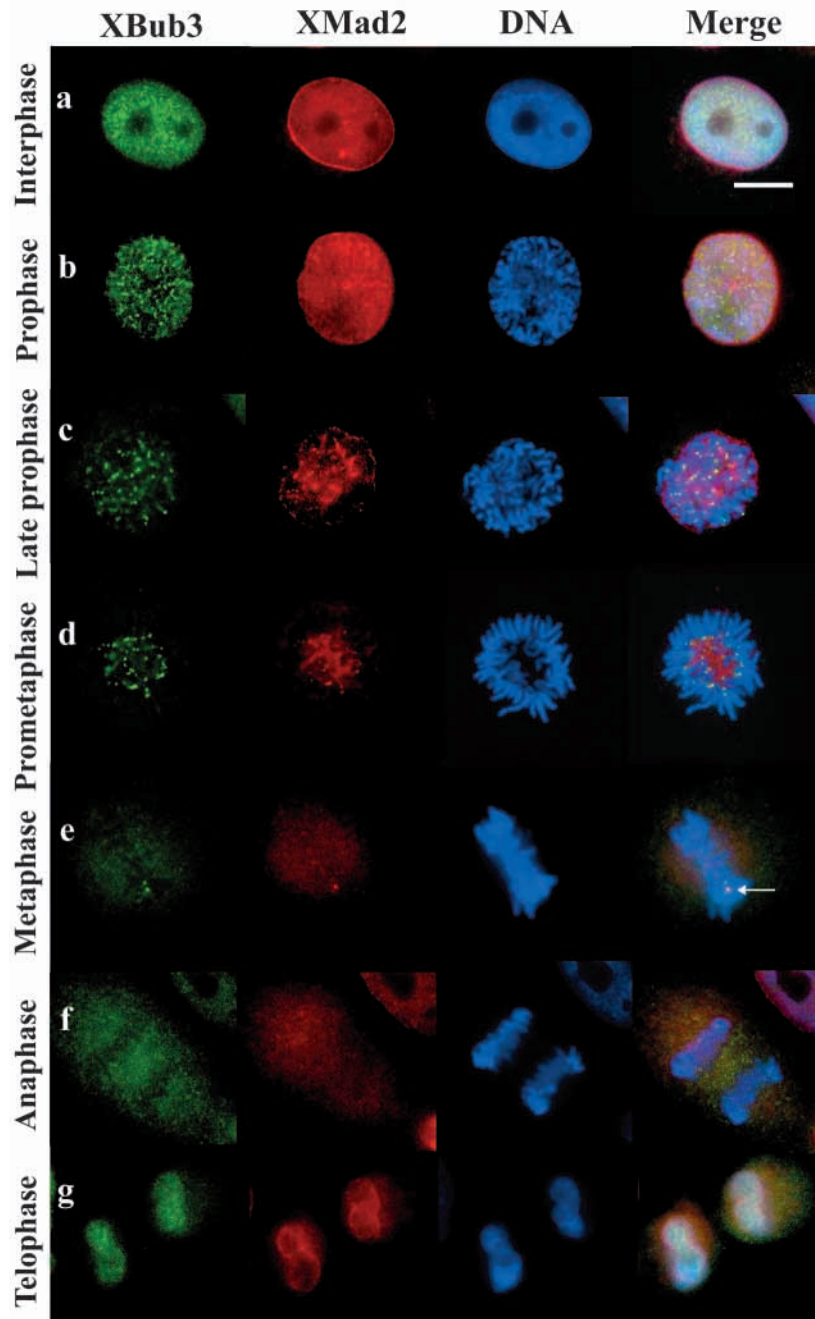


Fig. 6. Localisation of XBub3 in asynchronous XTC cells. Asynchronously growing XTC cells were fixed and stained using rabbit polyclonal XMad2 antibody (XMad2) and a sheep XBub3 peptide antibody (XBub3). The antibodies were detected using fluorescent Alexa-labelled secondary antibodies. Chromosomes were detected by mounting the coverslips in medium containing DAPI. The merge of all three fluorescent images is also shown. Representative pictures of cell cycle stages are shown as judged by DAPI staining. A lagging chromosome is marked (row e) with an arrowhead. Bar, 10 μ m.

XBub3 localisation

Drosophila, mouse and human Bub3 homologues are known to bind to kinetochores during prophase and prometaphase of the cell cycle and are associated with lagging chromosomes that have not yet formed correct bipolar attachment to the mitotic spindle (Basu et al., 1999; Martinez-

Exposito et al., 1999; Taylor et al., 1998). Here we show that XBub3 localises to kinetochores in XTC cells during prophase and prometaphase and is undetectable on kinetochores that have aligned on the spindle at metaphase (Fig. 5). In HeLa cells, Bub1 and BubR1 have been shown to decrease 3.7- and 3.9-fold respectively upon kinetochore attachment, whereas Mad2 decreases 152-fold (Zhou et al., 2002). We estimate that the decrease in XBub3 binding upon kinetochore attachment is at least fivefold from analysis of kinetochore pixel intensities during our immunofluorescence assay (data not shown). This is consistent with the decrease in Bub3 binding reported at mouse kinetochores at metaphase upon kinetochore attachment of three- to fivefold (Martinez-Exposito et al., 1999). Although the authors in that study still detected small amounts of Bub3 at metaphase we were unable to detect any Bub3 or Mad2 protein associated with kinetochores in the high general cytoplasmic staining of XBub3 present in metaphase cells (Fig. 6).

In tissue culture cells, Mad1 and Mad2 proteins are localised to kinetochores during prophase and prometaphase and to unattached kinetochores during metaphase (Chen et al., 1998;

Chen et al., 1996). Mad1 is required to recruit Mad2 kinetochore localisation in this system (Chen et al., 1998). Interestingly, we have consistently observed XBub3 binding to kinetochores very early in prophase, before we observe XMad2 (Fig. 6) or XMad1 (L.C. and K.G.H., unpublished). Human Bub1 is known to localise to kinetochores before BubR1 (Jablonski et al., 1998). Such observations suggest that the Bub1 and Bub3 proteins could be required for the recruitment of the Mad and BubR1 proteins to kinetochores. We attempted to test this in egg extracts using our XBub3 antibodies, but they did not interfere with the kinetochore binding of any checkpoint component tested, including XBub3 itself (Fig. 7). Unfortunately, it has not been possible to efficiently immunodeplete XBub3 from egg extracts with any of our antibodies. Our antibodies do recognise native XBub3 as shown by our immunofluorescence data (Fig. 7), but they are only able to immunoprecipitate a small proportion of XBub3 from native egg extracts (L.C. and K.G.H., unpublished). However, since these antibodies override the spindle checkpoint when added to egg extracts, this limited binding is clearly capable of disrupting XBub3 spindle

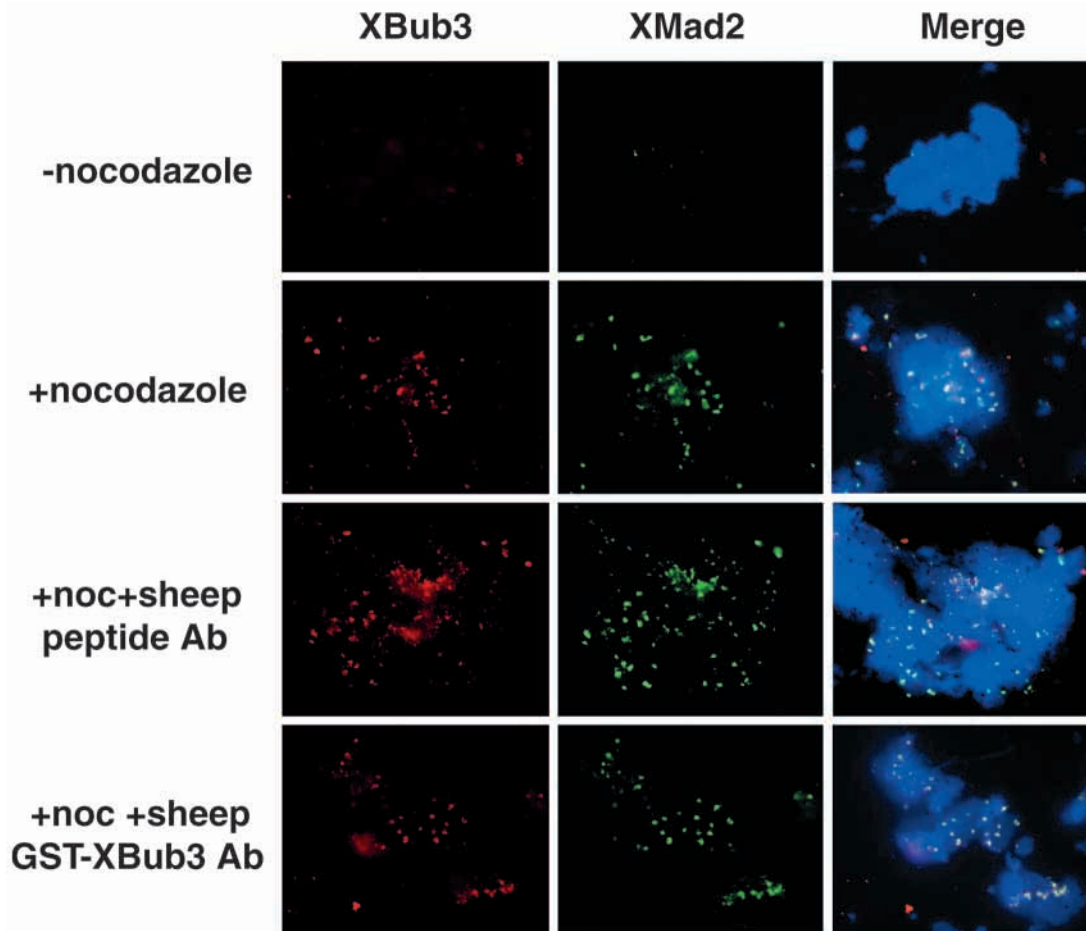


Fig. 7. Effect of XBub3 antibodies on XBub3 and XMad2 localisation in egg extracts. Metaphase chromosomes were assembled in egg extracts and treated with (+noc) or without (-noc) nocodazole. Sheep peptide or full-length XBub3 antibodies were added to the extracts 90 minutes before nocodazole addition. Chromosomes were isolated through a 30% glycerol cushion onto coverslips and stained with a sheep peptide XBub3 antibody followed by a fluorescent anti-sheep antibody (-noc, +noc) or fluorescent anti-sheep antibody alone (+noc +sheep XBub3 peptide Ab, +noc +sheep GST XBub3 Ab) to detect the sheep antibodies pre-incubated in the egg extract. Co-staining with XMad2 was achieved by incubating the coverslips with the rabbit XMad2 antibody. Chromosomes were detected by mounting the coverslips in medium containing DAPI (DNA).

checkpoint function. Sharp-Baker and Chen have recently reported that immunodepletion of XBub1 from egg extracts prevents the recruitment of XBub3, XMad1, XMad2, CENP-E and XBubR1 to kinetochores (Chen, 2002; Sharp-Baker and Chen, 2001). This is consistent with our analysis that Bub3 kinetochore binding occurs before Mad1-Mad2 recruitment during cell division and suggests that Bub3-Bub1 binding to kinetochores may occur at an early stage in spindle checkpoint activation, facilitating Mad1-Mad2 kinetochore recruitment (Sharp-Baker and Chen, 2001). In mammalian cells Bub1 associates with kinetochores before BubR1 and the kinesin-like protein, CENP-E (Jablonski et al., 1998). CENP-E binds to BubR1 (Chan et al., 1999; Yao et al., 2000), and immunodepletion of CENP-E prevents XMad1 and XMad2 kinetochore recruitment and checkpoint activation (Abrieu et al., 2000). Furthermore, immunodepletion of XMps1 also prevents the kinetochore association of Mad1 and Mad2 at kinetochores, perhaps by preventing CENP-E binding to kinetochores (Abrieu et al., 2001). Thus Mad1 and Mad2 appear to require a complex platform of checkpoint proteins at *Xenopus* kinetochores before they can be recruited.

Although XBub3 and XMad2 are both nuclear during interphase we never found XBub3 concentrated at the nuclear envelope, as observed for XMad2 (Fig. 5) (Chen et al., 1996) and XMad1 (Chen et al., 1998). It has recently been shown that human Mad1 and Mad2 are associated with nuclear pore complexes in interphase (Campbell et al., 2001). In this regard it is interesting to note that most organisms contain Bub3-related proteins (named Rae1 or Gle2), which have been shown to be involved in nuclear-cytoplasmic transport (Bharathi et al., 1997; Murphy et al., 1996). In addition, it has recently become clear that the region of Bub1 and Mad3 that interacts with Bub3 (Hardwick et al., 2000; Taylor et al., 1998) is closely related to the GLEBS (for GLE2p-binding sequence) motif of the nuclear pore complex protein hNUP98 (Wang et al., 2001). However, why the Mad proteins are associated with nuclear pores throughout interphase, when Bub3 and Bub1 are not, remains unclear. Perhaps it is important to keep the Mad and Bub proteins apart, in relatively restricted cellular locations, until the onset of mitosis.

Possible modes of action of XBub3 antibody

Our analysis of XBub3 function has relied upon the addition of function-interfering antibodies to egg extracts. Many efforts were also made to immunodeplete XBub3, but it proved impossible to do so efficiently. Others have had similar problems (Chen, 2002), and it is not clear why immunodepletion has proven so difficult to achieve, although the XBub3 protein is certainly quite abundant. We are confident that our antibody interference experiments, which are often used in analyses of *Xenopus* spindle checkpoint function (Chen et al., 1998; Abrieu et al., 2001; Kallio et al., 2002), have clearly demonstrated a role for XBub3 in spindle checkpoint activation and maintenance.

Our XBub3 antibodies prevent spindle checkpoint activation, without preventing the kinetochore association of XBub3, XMad2 (Fig. 7), XMad1 or XBub1 kinetochore association (L.C. and K.G.H., unpublished). In this regard they behave like α -Mad2 antibodies (Chen et al., 1998). It is possible that our XBub3 antibodies prevent kinetochore

association of XBubR1, CENP-E or other spindle checkpoint components, but this seems unlikely as immunodepletion of CENP-E prevents Mad1 and Mad2 kinetochore recruitment (Abrieu et al., 2000) and immunodepletion of XBubR1 reduces the kinetochore association of several checkpoint components (Chen, 2002). Our antibodies have no obvious effect on Mad/Bub protein recruitment to kinetochores (Fig. 7) and thus it seems most likely to us that they interfere with a 'downstream' function, after kinetochore recruitment of the checkpoint proteins has taken place. For example, they could act by preventing Bub3-BubR1 from forming inhibitory complexes with the APC activator, Cdc20/fizzy (Fang, 2002; Tang et al., 2001). We attempted to test this by immunoprecipitation of Cdc20 or XBubR1 from egg extracts in the presence and absence of our XBub3 antibody. As yet no convincing effects have been observed: we can detect no difference in the XBub3, XBubR1 or XMad2 levels associated with Cdc20 in the presence and absence of our function-blocking XBub3 antibodies (data not shown).

The effect of our antibodies may be more subtle. For example they could disrupt transmission of the checkpoint signal from unattached kinetochores throughout the cell, which is necessary to ensure that anaphase onset is inhibited globally. The molecular nature of this global signal and its mode of transmission remain a mystery. Real-time studies of hsMad2 (Howell et al., 2000) have demonstrated that Mad2 is only a transient component of kinetochores, with a $t_{1/2}$ of 24-28 seconds. In addition, Mad2-binding sites were observed to move from kinetochores to spindle poles via microtubules. It will be of great interest to see whether Bub3 and its associated kinases, Bub1 and BubR1, also turnover rapidly at kinetochores and whether some components of the spindle checkpoint pathway are more stably associated with kinetochores, providing a platform for the rapid turnover of Mad2 protein.

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