Preparation of Monoclonal Antibodies Against the Spindle Checkpoint Kinase Bub1

ROWENA L. LOCK,1,2 TIM H. SZETO,3 ALAN ENTWISTLE,1 OLE V. GJOERUP,4 and PARMJIT S. JAT1,3

ABSTRACT

The Bub1 kinase is a critical component of the spindle checkpoint involved in monitoring the separation of sister chromatids at mitosis. The viral oncoprotein Simian virus 40 large T antigen (LT) can bind and perturb the spindle checkpoint function of Bub1. We have developed three highly specific monoclonal antibodies against the Bub1 protein and have demonstrated that they can all detect Bub1 via Western blotting and immunofluorescence, in addition to their ability to immunoprecipitate Bub1.

INTRODUCTION

E VERY TIME A CELL DIVIDES, IT IS CRUCIAL THAT EACH DAUGHTER cell receives one and only one copy of each chromosome. Failure to ensure the fidelity of sister chromatid segregation in each mitosis would result in aneuploidy (a chromosome number that is not an exact multiple of the haploid number). Aneuploidy can lead to either cell death or the accumulation of cancer-causing mutations through gains or losses of DNA (oncogenes or tumor suppressors). The spindle checkpoint is a surveillance mechanism that blocks chromosome segregation until all sister chromatid pairs in the cell have successfully formed a bipolar attachment to spindle microtubules. The spindle checkpoint pathway comprises many components, including Bub1, Bub2, and Bub3 (budding uninhibited by benomyl) proteins; Mad1, Mad2, and Mad3 (mitotic arrest deficient) proteins; and the Mps1 protein. The genes encoding these spindle checkpoint proteins were first identified by genetic screens in yeast. Vertebrate homologs of several of these proteins are associated with the kinetochore, a specialized DNA-protein complex located at the centromere of each sister chromatid.

Bub1 is a serine-threonine kinase that is important for recruitment of spindle checkpoint proteins to kinetochores of sister chromatids that are not properly attached to or are misaligned with the mitotic spindle. Murine Bub1 (mBub1) is a 1058-residue protein with a predicted molecular weight of 119.5 kDa. mBub1 has a GLEBS-like (GLE2p-binding sequence) motif at residues 229–266 that is sufficient for Bub3 binding, as well as a carboxy-terminal kinase domain (Fig. 1). The human Bub1 (hBub1) is a protein of 1085 amino acids, with a predicted molecular weight of 122 kDa and has a similar domain structure to mBub1.

Mutations in the Bub1 gene can result in loss of spindle checkpoint function and are related to the chromosome instability (CIN) phenotype in human neoplasias. We have identified an interaction between the viral protein SV40 large T antigen (LT) and Bub1, and we suspect that aneuploidy induced by LT may be caused by modification of Bub1 activity. Interestingly, the interaction of Bub1 with LT is closely correlated with LT antigen induced transformation. Inhibition of Bub1 activity has been shown to result in genomic instability and anchorage independent growth of normal human fibroblasts. To dissect the mechanism by which LT compromises Bub1 function, we have generated three novel and highly specific monoclonal antibodies against Bub1. We successfully produced antibodies specific to the amino- and carboxy-terminal parts of the Bub1 protein, all of which are applicable to Western blotting, immunoprecipitation, and immunofluorescence studies of the LT-Bub1 interaction. Preliminary results using these antibodies suggest that LT does not affect Bub1 activity by perturbing its subcellular localization under normal growth conditions.

1Ludwig Institute for Cancer Research, Royal Free and University College School of Medicine, London, United Kingdom.
2Department of Biochemistry and Molecular Biology, University College London, London, United Kingdom.
3Department of Neurodegenerative Disease, Institute of Neurology, University College London, London, United Kingdom.
4Department of Cancer Biology, Dana-Farber Cancer Institute and Harvard Medical School, Boston, Massachusetts.
MATERIALS AND METHODS

Cell lines

Cell lines were maintained at 37°C in Dulbecco’s modified Eagle medium (DMEM, Invitrogen, Carlsbad, CA) for NIH 3T3 and U20S cells, or RPMI 1640 medium with GlutaMAX I and 25 mM HEPES (Invitrogen) for hybridoma cells. Medium was supplemented with 10% heat-inactivated donor calf serum (NIH 3T3) or fetal calf serum (U20S and hybridoma).

Expression and purification of mBub1 fusion proteins

mBub1 cDNA (a gift from the McKeon laboratory that originally cloned it) was PCR amplified in two sections, incorporating restriction sites and a 6 x His-tag, and cloned into the pET-23a bacterial expression vector (Novagen). The mBub1 fusion proteins were expressed as described in the Novagen, (Madison, WI) pET system manual.

Amino terminal (NT, residues 1–561) His-tagged mBub1 protein was purified under native conditions, dialyzed into PBS at 4°C, and concentrated 10-fold using Centricon-30 columns. Carboxy terminal (CT, residues 562–1058) His-tagged mBub1 protein was purified under denaturing conditions, dialyzed into 2 M urea at 4°C, and concentrated 10-fold using Centricon YM-10 columns. Purification procedures were essentially as described in the Qiagen QIAexpressionist manual, except that eluted His-tagged proteins were collected via centrifugation.

Immunizations, generation of hybridomas, and ELISA screening

Immunizations of mice with the His-tagged fusion proteins, generation of hybridomas via somatic cell fusions, single cell cloning, ELISA screening, and isotyping of the resulting hybridomas were carried out by Dr. Terry Jowett (University College London Monoclonal Antibody Facility) using standard protocols.

DNA transfection of mammalian cells

Cells were transfected using FuGENE 6 Transfection reagent (Roche, Nutley, NJ) according to manufacturer’s instructions.

Preparation of whole cell lysates

Subconfluent cells were washed with cold PBS and lysed in the presence of Protease Inhibitor Cocktail (Sigma-Aldrich, St. Louis, MO) for 30 min on ice in either RIPA or NP40 lysis buffer. RIPA lysates were passed three times through a 21-gauge needle to shear the DNA. Cell extracts were centrifuged for 30 min at 10,000 rpm at 4°C and the supernatant stored at -20°C.

Western blot analysis

Protein lysates (along with Bio-Rad Broad Range Prestained Marker, San Diego, CA) were separated on 8% SDS-PAGE gels, transferred to nitrocellulose membrane and immunoblotted using standard protocols. Proteins were visualized using horseradish peroxidase (HRP) detection reagents, according to manufacturer’s instructions (ECL, Amersham Biosciences, Piscataway, NJ), and exposed to autoradiographic film.

Immunoprecipitation

Proteins were immunoprecipitated from whole cell lysates with hybridoma tissue culture supernatants using standard protocols. Protein A-sepharose beads (Amersham) were used to collect the immune complexes.

FIG. 1. The murine Bub1 (mBub1) protein contains a GLEBS-like motif that is involved in binding the spindle checkpoint protein Bub3, and a carboxy-terminal serine-threonine kinase domain. The histidine-tagged mBub1 recombinant proteins used for generation of monoclonal antibodies are also shown.
**Immunofluorescence**

Cells were grown on glass coverslips and fixed with ice-cold methanol for 10 min at 20°C. All antibody incubations and washes were carried out in immunofluorescence (IF) buffer (PBS with 1% BSA and 0.5% CHAPS) at room temperature. Cells were blocked with tissue culture medium (1:2 dilution) for 10 min, washed, incubated with primary antibody (1:8 dilution of hybridoma supernatant) for 30 min, and washed again. Cells were then incubated with secondary antibody for 30 min in the dark (1:100 dilution of FITC- and TRITC-conjugated IgG subtype specific secondary antibodies [Jackson Immunoresearch Laboratories, West Grove, PA] with 1 μM propidium iodide added), and washed. Coverslips were mounted on microscope slides using Dako (Carpenteria, CA) Fluorescent Mounting Medium, and images of the cells were obtained using a Zeiss LSM 510 confocal laser-scanning microscope.

**RESULTS**

**Purification of recombinant mBub1 proteins**

To facilitate studies of the proposed distinct activities of the amino and carboxy terminal parts of the Bub1 protein in the spindle checkpoint and chromosome congression, respectively, we generated mouse monoclonal antibodies that were specific for each of these regions. The mouse Bub1 (mBub1) cDNA was cloned into the expression vector pET-23a in two halves (amino terminal [NT, residues 1–561] and carboxy-terminal [CT, residues 562–1058]). A histidine tag was incorporated into the carboxy terminus of each protein to aid in the purification process (Fig. 1).

IPTG induction of *Escherichia coli* transformed with the recombinant pET vectors resulted in overproduction of a ~90 kDa protein (NT mBub1-His tagged protein) or a ~70 kDa protein (CT mBub1-His tagged protein) (Fig. 2). The His-tagged mBub1 proteins were then affinity purified on Ni-NTA His-bind resin. The NT mBub1 antigen was purified in a native form, while the CT mBub1 antigen was purified under denaturing conditions to give sufficient yield for immunization of mice and ELISA screening (Fig. 2). Pooled elution fractions of each mBub1 protein were dialyzed into PBS (NT mBub1) or 2 M urea (CT mBub1), concentrated to 2 mg/mL, and used to immunize mice.

**Hybridoma screens**

To ensure selection of monoclonal antibodies with several different experimental applications, hybridomas were initially screened via ELISA, followed by screening via Western blot and immunoprecipitation. ELISA screens identified 40 pools specific for the NT mBub1 antigen and 22 for the CT mBub1 antigen. These pools were then analyzed by Western blots of whole cell lysates from NIH 3T3 cells expressing LT and bacterial lysates from induced cultures expressing either the N-terminal or C-terminal Bub1 fusion proteins. A rabbit polyclonal antiserum raised against the N-terminal region of hBub1 was used as a positive control to

**FIG. 2.** Purification of recombinant amino-terminal (NT) and carboxy-terminal (CT) mBub1 proteins tagged with 6 histidine residues. For each protein, Coomassie-stained SDS-PAGE of total protein extracted before (−) and after (+) IPTG induction of recombinant protein expression are shown, along with the purified protein used for immunization of mice.
identify authentic Bub1.(13) NIH 3T3 cells expressing LT were chosen for this screen because of our intention to use the antibodies to investigate the LT-Bub1 interaction in rodent cell systems.(13)

Figure 3 shows the results of the Western blot screen of four CT mBub1 hybridoma supernatant pools (3, 12, 19 and 20) that were strongly positive for detecting the bacterial CT mBub1 (lane 2). As expected, this band was not seen in the negative control (media only) blot or in the blot using a rabbit polyclonal antiserum raised against the N-terminal 303 residues of the human Bub1 (hBub1) protein. This NT hBub1 polyclonal antiserum detected many strong bands in the NIH 3T3 cell lysate (Fig. 3, lane 1), including a band of ~120 kDa (the predicted molecular weight of mBub1) and a very strong ~150 kDa band. None of the four CT mBub1 hybridomas detected the 120 kDa band in lane 1, but pool 19 detected a strong 150 kDa band, also seen by the NT hBub1 polyclonal antiserum.

All hybridoma supernatant pools that were positive (albeit weakly) in the ELISA assay were also screened for their ability to immunoprecipitate the Bub1 protein, as detected by Western blot using the NT hBub1 polyclonal antiserum (which cross-reacts with the mBub1 protein). Figure 4 shows the immunoprecipitation screen for the CT mBub1 hybridomas. Four of the hybridoma supernatant pools immunoprecipitated a protein that was detected by the NT hBub1 polyclonal antiserum, but was not detected when using media only as a negative control for the immunoprecipitation. These four pools (3, 12, 19, and 20) were also the pools that were strongly positive for detecting the bacterially expressed CT mBub1 by Western blot. Again, it was noted that the protein migrated slower on SDS-PAGE than the expected molecular weight for Bub1 of 120 kDa. A rabbit polyclonal antiserum raised against the C-terminal 395 residues of hBub1, used as a positive control, also pulled down a protein of ~150 kDa that was recognized strongly by the NT hBub1 polyclonal antiserum (data not shown).

Similar Western blot and immunoprecipitation screens were carried out for the hybridoma pools from the NT mBub1 fusion that were positive in the ELISA assay (data not shown). One pool (pool 8) was identified that was able to detect the bacterially expressed 90 kDa NT mBub1 antigen in the Western blot screen, and a ~150 kDa band from the NIH 3T3 lysates in the immunoprecipitation screen.

The hybridoma pools that were positively identified by these screens were single cell cloned and isotypified, and the resulting clones were tested for specificity to both mouse and human Bub1 protein via Western blot (data not shown). NT mBub1 hybridoma pool 8 gave rise to an IgG1 subclass single cell clone designated 2B8, able to detect a band of ~150 kDa in both mouse and human cell lysates. CT mBub1 hybridoma pools 12 and 19 gave rise to single cell clones designated 13G9 (IgG2a subclass) and 17E9 (IgG2b subclass), respectively. These CT mBub1 hybridoma supernatants were able to detect a strong band of ~150 kDa in mouse, but not in human cell lysates. None of the mBub1 monoclonals generated detected a band of the predicted molecular weight for mBub1 of 120 kDa via Western blot. However, they strongly and consistently detected the appropriate bacterially expressed mBub1 fusion protein.

Confirmation that the antibodies detect Bub1

Our next aim was to determine whether the mBub1 monoclonal antibodies were specific to the mouse Bub1 protein. To
do this we exploited the finding that all three monoclonal antibodies strongly detect only the mouse protein. We therefore transiently transfected human (U20S) cells with an HA-tagged mouse Bub1 expression vector, prepared whole cell lysates, and attempted to detect the HA-mBub1 protein via Western blot analysis using the mBub1 monoclonals. 2B8, 13G9, and 17E9 antibodies all detected an protein that co-migrated with the protein detected using an anti-HA-tag antibody and the polyclonal NT-hBub1 antiserum (Fig. 5, lane 3). This indicates that the mBub1 monoclonal antibodies are specific to the Bub1 protein.

As expected, NT hBub1 polyclonal antiserum was able to detect the endogenous human Bub1 protein from lysates of untransfected U20S cells or cells transfected with empty vector.

FIG. 4. Immunoprecipitation (IP) screen of the 22 CT mBub1 hybridoma tissue culture supernatants that were positive in the ELISA screen. Fifty μL of each hybridoma culture supernatant were used for IP from whole cell lysates of NIH 3T3 cells expressing LT (500 μg). Ability to IP Bub1 was determined by blotting with a polyclonal antiserum raised against NT hBub1 (1:2000). The lanes with arrows (CT mBub1 supernatants 3, 12, 19, and 20) were the only monoclonals to pull down a protein in the region expected for Bub1, but the protein migrated at ~150 kDa rather than the predicted molecular weight for Bub1 of 120 kDa. A negative control (media only) is also shown.

FIG. 5. Western blot analysis of 50 μg human (U20S) whole cell lysate ectopically expressing HA-tagged murine Bub1 (lane 3) confirms that the mBub1 monoclonal antibodies 2B8, 13G9, and 17E9 (hybridoma supernatant used at 1:25 dilution) are specific to Bub1, a protein that appears to migrate at ~150 kDa on SDS-PAGE. Control lysate from untransfected U20S cells (lane 1), and cells transfected with empty vector (lane 2), along with Western blot using anti-HA antibody (1:1000) as a positive control confirm the specificity of the antibodies. This experiment also confirms that only NT mBub1 monoclonal 2B8 and the NT hBub1 polyclonal antiserum (1:2000) positive control are able to detect the endogenous human Bub1 protein in the U20S cells.
Further application of the mBub1 monoclonal antibodies: immunofluorescence studies

We next determined whether the mBub1 monoclonal antibodies could be used to examine the subcellular localization of Bub1. NT mBub1 monoclonal 2B8 detected a punctate staining pattern in early prophase of the cell cycle in NIH 3T3 cells expressing LT (Fig. 6A). Bub1 is localized at the kinetochore of the sister chromatids during mitosis in normal mammalian cells. Therefore, the punctate staining suggests that this antibody is applicable to immunofluorescence and also that Bub1 localization may not be affected by the presence of LT in these cells.

Figure 6B shows another NIH 3T3 cell (expressing LT) stained using the 2B8 mBub1 monoclonal, this time in the prometaphase of mitosis. During a short period in prometaphase, all mammalian chromosomes are aggregated into a single, wheel-shaped ring, known as a chromosome rosette, with most chromosomes oriented with their arms projecting outward and their centromeres (and therefore also their kinetochores) arranged at the center. The ring of dots seen in Figure 6B was observed in many mitotic cells and is consistent with localization of mBub1 with kinetochores in a chromosome rosette structure. Furthermore, murine Bub1 has previously been noted to localize at the kinetochores in the center of a prometaphase chromosome rosette. Although this observation does not provide absolute proof of kinetochore localization of Bub1 in these LT-expressing cells, it suggests that Bub1 localization (as seen using the mBub1 monoclonals) is very similar to that observed by others in the absence of LT.

The proteins identified by the three mBub1 monoclonal antibodies also co-localized (Fig. 6C). 2B8 and 13G9 were raised against different recombinant proteins (NT and CT mBub1, respectively), providing a very good indication of the specificity of the antibodies to mBub1 (Fig. 6C, upper panels). Co-localization was also observed between the two CT mBub1 monoclonals (Fig. 6C, 13G9 and 17E9, lower panels).

The mBub1 monoclonal antibodies were also used to examine the localization of Bub1 in NIH 3T3 cells expressing LT at all stages of the cell cycle (data not shown). Preliminary results suggested that the subcellular localization of Bub1 was not affected by the presence of LT, even though we have shown that spindle checkpoint function is compromised in mammalian cells expressing this viral protein.

DISCUSSION

We have successfully generated three monoclonal antibodies against the mouse Bub1 protein. 2B8 (IgG1 sub-class) is specific to the N-terminus of mBub1; 13G9 (IgG2a) and 17E9 (IgG2b) are independent monoclonals specific to the C-terminal region of mBub1. The specificity of all three antibodies for the Bub1 protein was verified via detection of ectopically expressed HA-tagged mBub1 protein. The CT mBub1 monoclonals were specific to the mouse Bub1 protein, which they recognize strongly and very specifically in Western blots, whereas the NT mBub1 monoclonal detects both the mouse and human Bub1 proteins. All three monoclonals were able to immunoprecipitate Bub1 from mouse whole cell lysate. 2B8 was also able to immunoprecipitate Bub1 from human cell lysates (data not shown). In addition, the mBub1 monoclonals were able to immunoprecipitate the Bub1 kinase in an active form (unpublished data, O.V.G).

It has previously been suggested in the literature that antibodies raised against the Bub1 protein recognize a band of the correctly predicted molecular weight for mammalian Bub1 of ~120 kDa on SDS-PAGE. However, the Western blot presented in one of these articles appears to show that the antibody actually detects a slower migrating band as we have observed for our mBub1 monoclonal antibodies. Similarly, the Xenopus Bub1 homolog has a predicted molecular weight of 131 kDa, but antibodies raised against this protein have been shown to detect a protein migrating at 150 kDa on SDS-PAGE. When Bub1 immunoprecipitates of mitotic extracts were treated with lambda protein phosphatase, the apparent molecular weight of Bub1 was reduced to 140 kDa, indicating that phosphorylation during mitosis was partly responsible for the slower migration of the Xenopus Bub1 protein. The antibody specificity experiments described here verify that when compared to the prestained markers, Bub1 migrates slower on SDS-PAGE than its predicted molecular weight of 120 kDa. However, we found that the migration of Bub1 protein does not change under conditions where pRB is dephosphorylated; thus, the cause of this slow migration remains to be determined.

All three mBub1 monoclonal antibodies were also shown to be applicable to immunofluorescence studies in mouse cells and were used to demonstrate Bub1 localization in cells expressing LT throughout the cell cycle. These experiments suggested that the subcellular localization of Bub1 is not disrupted by the presence of LT at any point during the cell cycle.
FIG. 6. (A) NIH 3T3 cells (expressing LT) stained with propidium iodide (for DNA localization) and NT mBub1 monoclonal 2B8. The arrowed cell has entered mitosis (prophase) and shows punctate spots of Bub1, a protein that is known to be localized to the kinetochores at this stage of the cell cycle in normal cells. Scale bar, 10 μm. (B) A prometaphase NIH 3T3 cell (expressing LT) stained with propidium iodide (DNA) and NT mBub1 monoclonal 2B8 Bub1. The Bub1 antibody recognizes a protein that is localized to the center of a chromosome rosette, at the same apparent location as the kinetochores in this structure. Scale bar, 5 μm. (C) Double staining of prometaphase NIH 3T3 cells (expressing LT) using pairs of mBub1 monoclonal antibodies to demonstrate co-localization of epitopes. The upper panels show double staining of NT mBub1 monoclonal 2B8 and CT mBub1 monoclonal 13G9; the lower panels show double staining of the two CT mBub1 monoclonals (13G9 and 17E9). Scale bar, 10 μm.
The mBub1 monoclonals will be useful reagents for future experiments to study the role that SV40 LT plays in compromising spindle checkpoint function via Bub1. Comparisons of Bub1 expression and complex formation, as well as subcellular localization in cells with or without LT, in the presence or absence of the microtubule-depolymerizing drug nocodazole will be used to address these questions.

Mutations in the Bub1 gene are related to the chromosome instability phenotype in human neoplasias,\(^{(13)}\) and hBub1 defects have also been observed in leukemia and lymphoma cells.\(^{(21)}\) The mBub1 monoclonal antibodies that we have generated will provide useful tools in the study of mouse models of cancer, in addition to their use for research into the spindle checkpoint. Cross-reaction of monoclonal 2B8 to humans also makes it applicable for the investigation of human pathologies.

ACKNOWLEDGMENTS

We thank R. Freire for providing valuable reagents and S. Klishcies for development of IF protocols. This work was supported by the Ludwig Institute for Cancer Research (PSJ), a Wellcome Trust Project Grant 072672 (PSJ), and a BBSRC doctoral studentship (RLL).

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Address reprint requests to: Parmjit S. Jat

Department of Neurodegenerative Diseases
Institute of Neurology
University College London
Queen Square
London WC1N 3BG
United Kingdom

E-mail: p.jat@prion.ucl.ac.uk