Monoclonal Antibodies Against *Xenopus* Greatwall Kinase

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Mitosis is known to be regulated by protein kinases, including MPF, Plk1, Aurora kinases, and so on, which become active in M-phase and phosphorylate a wide range of substrates to control multiple aspects of mitotic entry, progression, and exit. Mechanistic investigations of these kinases not only provide key insights into cell cycle regulation, but also hold great promise for cancer therapy. Recent studies, largely in *Xenopus*, characterized a new mitotic kinase named Greatwall (Gwl) that plays essential roles in both mitotic entry and maintenance. In this study, we generated a panel of mouse monoclonal antibodies (MAbs) specific for *Xenopus* Gwl and characterized these antibodies for their utility in immunoblotting, immunoprecipitation, and immunodepletion in *Xenopus* egg extracts. Importantly, we generated an MAb that is capable of neutralizing endogenous Gwl. The addition of this antibody into M-phase extracts results in loss of mitotic phosphorylation of Gwl, Plk1, and Cdk1 substrates. These results illustrate a new tool to study loss-of-function of Gwl, and support its essential role in mitosis. Finally, we demonstrated the usefulness of the MAb against human Gwl/MASTL.

Introduction

ACTIVATION OF MITOTIC KINASES, particularly maturation promoting factor (MPF, Cdk1/Cyclin B), polo-like kinase 1 (Plk1), and Aurora A, occurs in a coordinative manner, and is a signature event in mitosis.⁽¹⁾ Although a great deal has been learned about these kinases, many important aspects of their regulation and function still await further investigation. Importantly, some of these kinases, such as Plk1 and Aurora kinases, exhibit oncogenic characteristics and are potentially targetable for cancer therapy.⁽²⁾

Greatwall (Gwl), initially identified in *Drosophila* as a protein required for proper chromosome condensation and mitotic progression,⁽³⁾ has been extensively studied in *Xenopus* egg extracts during the past few years. Like that of many other mitotic kinases, phosphorylation and activation of Gwl oscillate during the cell cycle and peak in mitosis. It has been shown that Cdk activity is required for mitotic activation of Gwl, possibly through direct phosphorylation of Gwl at its activation sites. Importantly, Gwl activation is essential for induction of mitosis.⁽⁴⁻⁶⁾ Further analysis indicated that this function of Gwl is attributed to inhibition of PP2A/B55 δ , the principal protein phosphatase complex that reverses Cdk1-mediated phosphorylation events. Gwl-dependent inhibition of PP2A/B55 δ is achieved not through direct interaction or phosphorylation of the phosphatase complex *per se*, but rather by phosphorylating two key substrates, α -endosulfine (Ensa) and Arpp-19, which then bind and inhibit PP2A/B55 δ .⁽⁷⁻¹⁴⁾ The function of Gwl characterized in *Xenopus* has also been shown to be conserved in its human homologue, microtubule-associated serine/threonine kinase-like (MASTL). Human cells

treated with MASTL RNAi either fail to enter mitosis or exhibit multiple defects while progressing through mitosis, depending on the efficiency of MASTL depletion.^(15,16) Moreover, our recent study also found that Gwl functions as a negative regulator for DNA damage checkpoint activation. In *Xenopus* egg extracts, Gwl is required for the efficient de-activation of checkpoint signaling, as well as re-activation of Cdk1.^(17,18)

Despite progress, we still know relatively little about some important aspects of Gwl function. For example, recent studies suggested that Gwl activity can be induced *in vitro* through its phosphorylation by Cdk1 and Plk1 and interaction with another AGC kinase,^(5,19) but how Gwl is activated and regulated in cells or egg extracts is largely unclear. Continuous efforts in revealing Gwl function and regulation can be greatly facilitated by the availability of immunological reagents.

Materials and Methods

Cell culture

A human oral squamous cell carcinoma cell line (UM-SCC-11B), as previously characterized,⁽²⁰⁾ was maintained in Dulbecco's modified Eagle's Medium (DMEM, Sigma, St Louis, MO) supplemented with 10% fetal bovine serum (FBS, Sigma).

Construction, expression, and purification of antigen

Antibodies were generated as described previously.^(21,22) PCR was used to generate a cDNA encoding amino acids 1-205 of *Xenopus* Gwl kinase (XGwl1: 5' CGCGAATTCAAATG GGGATTGTGGCTG 3' and XGwl2: 5' CGCCTCGAGTTA ACGTGAATAGTCCCG 3'). The PCR product was cloned into

pCR2.1TOPO and sequenced to ensure that no errors occurred, and the EcoRI-XhoI fragment was ligated into pMBP-parallel 2.⁽²³⁾ The Greatwall fusion protein was expressed in *E. coli* and purified by affinity chromatography using amylose resin (New England BioLabs, Ipswich, MA).

Generation of monoclonal antibodies

Three 8- to 9-week-old female Balb/C mice were injected subcutaneously with 50–150 mg antigen per mouse. Intraperitoneal booster injections of 50–150 mg were given at 2-week intervals, followed by daily injections 3 days prior to sacrifice. Splenocytes were isolated, incubated 10 min on ice in Sigma red cell lysis buffer to remove the red blood cells, and fused with the mouse myeloma cell line P3/NS1/1-Ag4-1 (ATCC, Manassas, VA) in the presence of polyethylene glycol (ATCC). The complete fusion was plated in 30 96-well plates, and medium containing hypoxanthine, aminopterin, and thymidine was added the following day to eliminate unfused myeloma cells. Hybridoma supernatants were screened by immunoblot analysis. Positive hybridomas were cloned by limiting dilution and maintained in Sigma HY medium supplemented with 20% FBS.

Immunoblotting

Protein samples were denatured by boiling in 2X Laemmli sample buffer (Bio-Rad, Hercules, CA) for 3 min, and then resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Proteins were then electrotransferred to polyvinylidene difluoride (PVDF) membranes (Millipore, Billerica, MA). Membranes were blocked with 5% non-fat dry milk in TBST (10 mM Tris HCl [pH 7.5], 150 mM NaCl, 0.05% Tween-20) for 1 h at room temperature. Hybridoma-conditioned medium was used at 1:100 dilution in TBST and incubated with the membranes for 2 h. Other primary antibodies, including anti-Plx1 (from Dr. James Maller, University of Colorado, Denver), anti-phospho-Plk1 Thr 210 (corresponding to Thr 201 in Plx1, Abcam, Cambridge, MA), anti-phospho-CDK substrate antibody (Cell Signaling, Beverly, MA), anti- β -actin (Abcam), anti-MASTL (Abcam), anti-GST (Sigma), and anti-Gwl polyclonal (from Dr. Michael Goldberg, Cornell University), were used at 1:1000 dilution. Membranes were then washed three times in TBST before horseradish peroxidase (HRP) conjugated anti-mouse secondary antibody (Sigma) diluted 1:2000 in TBST was added. After 1 h incubation with the secondary antibody, membranes were washed three times in TBST and immunoreactive signals detected using enhanced chemiluminescence (ECL) substrate kit (Pierce, Rockford, IL).

Immunodepletion

For Gwl immunodepletion, anti-mouse magnetic beads (New England BioLabs) were prewashed three times in washing buffer (50 mM HEPES [pH 7.5], 150 mM NaCl, 1 mM DTT, and 0.5% Tween-20) and then incubated for 1 h with the antibody-containing media. Beads conjugated to the antibody were washed three times in washing buffer and then mixed with *Xenopus* egg extracts. After 20 min incubation, the beads were removed with a magnet and the remaining extract collected. The above steps of immunodepletion can be repeated for better efficiency.

Immunoprecipitation

For immunoprecipitation, anti-mouse magnetic beads (New England BioLabs) were prewashed three times in washing buffer (50 mM HEPES [pH 7.5], 150 mM NaCl, 1 mM DTT, and 0.5% Tween-20) and incubated for 1 h with the antibody-containing media. Beads conjugated to the antibody were washed three times in washing buffer and then mixed with *Xenopus* egg extracts. After 20 min incubation, the beads were removed with a magnet and washed three times in washing buffer before elution with 2X Laemmli sample buffer and analysis by immunoblotting.

Xenopus egg extracts

Cytostatic factor (CSF) extracts were prepared as previously described.⁽²⁴⁾ Briefly, adult female frogs (NASCO, Fort Atkinson, WI) were injected with pregnant mare's serum gonadotropin (PMSG, Sigma) at 100 U/frog, 3–8 days before the second injection with human chorionic gonadotrophin (HCG, Sigma) at 225 U/frog. After HCG injection, frogs were kept in water with 100 mM NaCl overnight to lay eggs. Eggs were collected and dejellied in 2% cysteine solution and washed four times in 1X extract buffer (100 mM KCl, 1 mM $MgCl_2$, 10 mM HEPES [pH 7.7], 50 mM sucrose) and two times in modified extract buffer (100 mM KCl, 1 mM $MgCl_2$, 10 mM HEPES [pH 7.7], 50 mM sucrose, 5 mM EGTA). During the process, eggs with abnormal appearance were removed. Remaining eggs were then collected in modified extract buffer with Cytochalasin B (100 ng/mL, Sigma) and piled up by brief centrifugation. Excess buffer was removed, and a few drops of Versilube oil were added from the top. Eggs were then crushed by centrifugation (10,000 g for 10 min), and the extract layer recovered for another round of centrifugation (10,000 g for 15 min). CSF extracts were recovered from the second centrifugation. To obtain interphase egg extracts, CSF extracts were stably released into interphase by supplementation with 0.4 mM $CaCl_2$ and 100 μ g/mL cycloheximide, followed by incubation for 30 min at room temperature.

Results

Immunoblotting analysis using selective monoclonal antibodies

To better study the function and regulation of Gwl in *Xenopus* egg extracts, we sought to develop mouse monoclonal antibodies (MAbs) using a recombinant protein that contains N-terminal *Xenopus* Gwl (amino acids 1–205) as antigen. As described in the Materials and Methods section, initial selection of hybridomas was based on the ability of antibody preps to recognize the recombinant purified fusion protein and a lack of reactivity with the fusion partner. Twenty-six hybridoma lines were initially saved for further analysis (data not shown). Positive clones from the initial screen were further characterized by immunoblotting interphase *Xenopus* egg extracts. A previously characterized rabbit polyclonal antibody against *Xenopus* Gwl was used as a positive control,⁽⁵⁾ which, as expected, recognized Gwl as a single band at 110 kDa (Fig 1A). Five representative mouse monoclonal antibodies (24G5, 19C5, 24B12, 11E4, and 19G8) recognized a strong band at the same molecular size as the positive control. 24G5 and 19C5 antibodies recognize a single band, whereas

FIG. 1. Characterization of MAbs for immunoblotting. (A) *Xenopus* egg extracts were prepared and analyzed by immunoblotting using a rabbit polyclonal antibody and five newly generated MAbs, as indicated. (B) *Xenopus* egg extracts were immunodepleted using the rabbit polyclonal antibody or mock-treated. The extracts were then analyzed by immunoblotting using 19C5, 24G5, and 11E4 antibodies to confirm their specificity, and Plx1 antibody to demonstrate equal protein loading. (C) Diagram of Gwl domain structure. The kinase domain is shown in black and other highly conserved regions are in gray. Three fragments of the N-terminal Gwl were tagged with GST, expressed and purified from bacteria. These proteins were then analyzed by immunoblotting using the indicated antibodies.

blotting with 24B12, 11E4, and 19G8 antibodies also exhibits several non-specific bands at various molecular weights.

To confirm that the main band observed in Figure 1A using our antibodies is indeed *Xenopus* Gwl, we utilized the previously characterized rabbit antibody to immunodeplete XGwl. Convincingly, 19C5, 24G5, and 11E4 antibodies recognize a major band at 110 kDa in the control extract, but not in XGwl-depleted extract, thus confirming specificity toward XGwl in immunoblots (Fig. 1B).

As illustrated in Figure 1C, the N-terminal segment of XGwl used as antigen contains a partial kinase domain and its

flanking regions that are well conserved through evolution. To further map the epitope recognized by our XGwl antibodies, we expressed three fragments of the N-terminus, including N1 that contains amino acids 1-39; N2 that contains aa 34-190, the kinase domain region; and N3 that contains aa 186-340. We found that 19G8 antibody targets the N1 region, whereas all other MAbs recognize N2, the kinase domain in N-terminal XGwl. Interestingly, among those that recognize the kinase domain, 24G5 does not react with an apparently proteolytic fragment of the kinase domain, suggesting that this antibody recognizes a unique region within the kinase domain (Fig. 1C).

FIG. 2. Utilization of MAbs in immunoprecipitation. As described in the Materials and Methods section, MAbs were used to immunoprecipitate (IP) Gwl from *Xenopus* egg extracts, and the immunoprecipitated proteins were analyzed by immunoblotting using the indicated antibodies.

MAbs immunoprecipitate *Xenopus* Gwl

To further characterize these MAbs, we tested their ability to immunoprecipitate XGwl from *Xenopus* egg extracts. As described in the Materials and Methods section, antibodies were conjugated to beads and incubated with extracts. Beads were then washed and immunoprecipitated proteins were analyzed by immunoblotting. Four antibodies (24B12, 19C5, 11E4, and 19G8) were able to efficiently immunoprecipitate XGwl, whereas almost no XGwl was recovered with 24G5 antibody (Fig. 2).

Immunodepletion of XGwl using the MAb

Immunodepletion of specific proteins from *Xenopus* egg extracts allows convenient assessment of their biochemical functions. We sought to determine if our antibodies were able to efficiently immunodeplete endogenous Gwl, and picked 24B12 as the best candidate based on its performance in immunoprecipitation assays. Anti-mouse beads with 24B12 antibody were added to incubate with extracts and then removed. The remaining extracts were compared with control, mock-treated extracts for the protein level of Gwl. As shown in Figure 3, after one round of immunodepletion, the level of Gwl present in the extract was clearly reduced, whereas almost no Gwl was detectable in the remaining extract after another round of depletion. Antibody 24B12 is thus capable of immunodepleting XGwl from *Xenopus* egg extracts.

FIG. 3. Utilization of 24B12 antibody in immunodepletion. As described in the Materials and Methods section, 24B12 antibody was used to immunodeplete Gwl from *Xenopus* egg extracts. The remaining extract after each round of depletion was analyzed by immunoblotting using 24G5 (Gwl) and Plx1 antibodies. Mock-treated extract was loaded as a control.

Neutralizing XGwl using the MAb

Antibodies that neutralize their endogenous targets provide unique opportunities for functional studies. We sought to determine if our MAbs, upon addition into egg extracts, are capable of disrupting XGwl function. In M-phase egg extracts, XGwl is activated by phosphorylation, which can be monitored by slower migration in SDS-PAGE. As shown in Figure 4, antibody 11E4, but not other MAbs tested (as represented by 24B12 here), abolished mitotic phosphorylation of XGwl. Interestingly, loss of XGwl activation caused by addition of 11E4 antibody accompanies reduced phosphorylation of Plx1 at its T-loop activating site, as well as the spectrum of Cdk1 substrates, as indicated by immunoblots using a phospho-specific antibody. This result underscores the essential role of Gwl in maintaining mitosis, and is, to our knowledge, the first characterization of a neutralizing antibody of Gwl.

Utilization of MAbs in human cells

Studies in flies, frogs, and human cells define Gwl as a well-conserved gene with similar functions in different organisms. Our MAbs against *Xenopus* Gwl would have a broader spectrum of applications if they cross-react with Gwl homologues in other species, especially human. Prompted by these notions, we sought to investigate if our antibodies also recognize human Gwl, MASTL. We constructed an expression vector containing MASTL and ectopically expressed it in human squamous carcinoma SCC11B cells. As shown in Figure 5A, 19G8 antibody clearly recognizes the overexpression of human MASTL. When utilized to detect endogenous MASTL, this antibody reacts with a band at the expected size, along with some non-specific signals. We then compared it to a commercially available rabbit anti-MASTL antibody (Fig. 5B); while both antibodies seem to

FIG. 4. 11E4 antibody neutralizes Gwl in extracts. 24B12 and 11E4 IgG purified from hybridoma-conditioned media was incubated with M-phase (CSF) extracts for 1 h at room temperature. Control extract with no added antibody was similarly incubated. All extracts were analyzed by immunoblotting as indicated.

FIG. 5. 19G8 antibody recognizes human MASTL. (A) MASTL, the human homologue of *Xenopus* Gwl, was expressed in SCC11B cells. MASTL-overexpressing and mock-treated cells were analyzed by immunoblotting using the indicated antibodies. (B) SCC11B cell lysate was immunoblotted using 19G8 or a commercially available MASTL antibody. The arrow points to a band at the expected size of MASTL.

react with endogenous MASTL, 19G8 exhibits fewer non-specific bands that are also much further distanced from the expected molecular weight of MASTL. Therefore 19G8 is useful for immunoblot analysis of human MASTL, but perhaps not for immunohistochemistry or immunofluorescence, due to the strong non-specific bands of lower molecular weights.

Discussion

Specific immunologic reagents, especially MAbs, are of great value to the molecular analysis of gene functions. For *Xenopus* egg extract-based cell-free systems, being able to immunoblot, immunoprecipitate, and immunodeplete the target protein using specific antibodies represents crucial advantages of the experimental system. To aid in further functional analysis of Gwl kinase, we generated monoclonal antibodies against the N-terminal segment of *Xenopus* Gwl, and evaluated their usefulness in multiple applications in *Xenopus* egg extracts. We show here that five MAbs (24G5, 19C5, 24B12, 11E4, and 19G8) efficiently recognize endogenous Gwl in *Xenopus* egg extracts; immunoblots using two of these MAbs exhibit one single band, indicating excellent specificity in this application. Four antibodies (19C5, 24B12, 11E4, and 19G8) are able to immunoprecipitate XGwl from extracts, among which 24B12 is also useful for immunodepletion. Interestingly, we characterized here the first neutralizing antibody of XGwl: addition of 11E4 antibody into M-phase egg extracts resulted in inactivation of XGwl and mitotic exit, as judged by dephosphorylation of Plx1 and Cdk1-substrates. We therefore, are very satisfied with the usefulness of these antibodies in *Xenopus*. Finally, we show that at least one of these antibodies, 19G8, also cross-reacts with the human homologue of *Xenopus* Gwl, MASTL, suggesting potential utilization of this antibody beyond its desired applications in *Xenopus* systems.

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Author Disclosure Statement

The authors have no financial interests to disclose.

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