Determinants for Activation of the Atypical AGC Kinase Greatwall During M Phase Entry

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The atypical AGC kinase Greatwall (Gwl) mediates a pathway that prevents the precocious removal of phosphorylations added to target proteins by M phase-promoting factor (MPF); Gwl is thus essential for M phase entry and maintenance. Gwl itself is activated by M phase-specific phosphorylations that are investigated here. Many phosphorylations are nonessential, being located within a long nonconserved region, any part of which can be deleted without effect. Using mass spectrometry and mutagenesis, we have identified 3 phosphosites critical to Gwl activation (pT193, pT206, and pS883 in *Xenopus*) located in evolutionarily conserved domains that differentiate Gwl from related kinases. We propose a model in which the initiating event for Gwl activation is phosphorylation by MPF of the proline-directed sites T193 and T206 in the presumptive activation loop. After this priming step, Gwl can intramolecularly phosphorylate its C-terminal tail at pS883; this site likely plays a similar role to the tail/Z motif of other AGC kinases. These events largely but do not completely explain the full activation of Gwl at M phase.
INTRODUCTION

Greatwall kinase (Gwl) plays a critical role in M phase entry and maintenance in *Drosophila, Xenopus* egg extracts, and human tissue culture cells (2, 4, 41, 44, 45). Gwl inactivates, specifically during M phase, PP2A phosphatase associated with B55-type regulatory subunits. Inactivation of PP2A/B55 prevents the premature removal of M phase-specific phosphorylations added to many target proteins by the cyclin dependent kinase MPF (M phase promoting factor; Cdk1/Cyclin B) (5, 22, 39). Gwl's role in PP2A/B55 inactivation is indirectly mediated through proteins of the Endosulfine family that are phosphorylated by Gwl (10, 23).

The mechanism by which Gwl is itself activated during M phase is only poorly understood, but clearly involves its hyperphosphorylation. MPF appears to be an upstream kinase important for Gwl activation (45), but the details are obscure and other kinases may also participate.

The evolution of Gwl is of considerable interest. Gwl proteins in flies, frogs, and humans are likely functionally orthologous (2, 44, 45). However, lineages including species such as *S. cerevisiae, C. elegans,* and plants lack true Gwl kinases (44).

In the human kinome, Gwl (MASTL) and its close relatives MAST1-4 form an unstudied branch of the AGC kinase family (20). Gwl lacks 6 of the 15 amino acids that distinguish AGC kinases from other eukaryotic protein kinases (17). Remarkably, at the position of the activation loop of other AGCs, Gwl contains an insertion of hundreds of amino acids that is poorly conserved and whose function is yet to be determined.

Here, we describe investigations designed to understand Gwl's structure and cell
cycle regulation. Our results suggest that Gwl follows the main logic of AGC activation, involving phosphorylations both of the presumptive activation loop and of a residue in the enzyme’s C-terminal tail [reviewed by (14, 27)]. The activation loop phosphorylations are proline-directed and targeted in vitro by CDKs, explaining at least in part how Gwl is turned on at M phase entry. The critical phosphosite in the C-terminal tail can then be generated by intramolecular autophosphorylation. This phosphorylated residue probably interacts with a basic patch on the enzyme's N-lobe to stabilize the active conformation. However, some aspects of Gwl regulation do not conform to the general AGC pattern and remain enigmatic.
MATERIALS AND METHODS

Antibodies and Xenopus egg extracts

Use of antibodies against Xenopus Gwl, Cdc25C, and Tyrosine 15 (Y15) of Cdk1 has been previously described (45). MPM-2 antibody against mitotic phosphoepitopes was the kind gift of J. Kuang [M.D. Anderson Cancer Center, Houston, TX; (42)].

CSF extracts were prepared according to (25, 26); frog care was monitored by the Institutional Animal Care and Use Committee at Cornell University.

Immunodepletion of CSF extracts was performed as described (37, 45) using Affi-prep protein A beads (Bio-Rad Laboratories, Hercules, CA) coated with affinity-purified anti-Greatwall. Mock-depleted extracts were treated with protein A beads alone.

To assess the biological activity of Gwl proteins prepared in Sf9 insect cells infected with baculovirus constructs (see below), the proteins were added to Gwl-depleted CSF extracts and then incubated at 25°C. Aliquots of the extracts were then analyzed by Western blotting with antibodies against Gwl, the interphase-specific inactivating phosphorylation at Y15 on Cdk1, and Cdc25C phosphatase (45).

Exogenous, active wild type Gwl made in Sf9 cells treated with okadaic acid (OA) is capable of rescuing the effects of Gwl depletion when added at concentrations greater than 0.25-0.5X that of the endogenous Gwl in CSF extracts, while kinase dead Gwl (with the G41S mutation) is unable to restore the extracts to M phase even at concentrations more than 10X that of the endogenous enzyme [Fig. 1A and (47)]. Fig. 1B displays the responsiveness of this assay to unactivated wild type Gwl made in Sf9 cells that had not been treated with OA. This exogenous Gwl becomes activated and
can promote M phase reentry in the extracts, but only slowly and only when added at concentrations 6X or higher than the endogenous levels.

Expression and purification of recombinant Gwl from baculovirus constructs

Mutant Gwl proteins (point mutations and deletion mutations) were generated from a Gwl cDNA clone by using the QuikChange Site Directed Mutagenesis Kit (Agilent, Santa Clara, CA). All proteins were expressed in Sf9 insect cells using the Bac-to-Bac system (Invitrogen, Carlsbad CA) and purified as previously described (45). Active Greatwall was produced by treating the infected cells with OA to a final concentration of 100 nM for 12 hr before harvesting. The kinase dead (KD) allele of *Xenopus* Gwl used was G41S, previously characterized in (44, 45).

All Gwl fusion proteins contained both a His$_6$ tag and a Z tag (the Z subdomain of protein A, which binds to IgG). The Z tag interacts with primary and secondary antibodies during Western blotting, accounting for approximately 50% of the band intensity seen on Western blots of recombinant Gwl proteins that contain it (47). In addition, active M phase Gwl behaves as a diffuse band and usually reacts less efficiently with anti-Gwl than does the unphosphorylated interphase form of Gwl. Because of these issues, the amounts of recombinant Gwl in all preparations were determined by Comassie blue staining of preparations that had been treated with λ protein phosphatase (LPP). Phosphatase treatment was carried out by incubating Gwl proteins in LPP buffer [50 mM Tris (pH 8.0), 2 mM MnCl$_2$, and 1 mg/ml acetylated bovine serum albumin (BSA)] with 40 U LPP (New England Biolabs) at 30°C for 30 min.
Gwl expression in Xenopus oocytes

mRNAs encoding FLAG-tagged S883E or S883D Gwl proteins were injected into immature G2 Xenopus oocytes, which were incubated overnight and then treated (or not) with the hormone progesterone as described (43). Untreated injected oocytes remained in G2; no germinal vesicle breakdown (GVBD) was observed, while GVBD occurred in nearly 100% of the oocytes treated with progesterone. Gwl was immunoprecipitated from injected oocytes using anti-FLAG antibody as described (43), and the immunoprecipitates were then subjected to standard kinase assays using the model substrate myelin basic protein (MBP).

Kinase assays

Standard kinase assays were performed for 10 min at 30°C in 10 μl of kinase buffer (20 mM HEPES, 10 mM MgCl₂, 0.1 mg/ml BSA, and 3 mM β-mercaptoethanol) supplemented with 100 μM cold ATP and 1 μCi [γ-³²P]ATP (3000 Ci/mmole) and with 0-250 ng of Gwl proteins (diluted into 1 μl of PBS + 50% glycerol) prepared from Sf9 insect cells infected with baculovirus constructs as above. In early experiments, 1.5 μg of myelin basic protein (MBP; Active Motif, Carlsbad, CA) was added to the reaction mix as a model substrate. The kinase reaction was terminated by addition of SDS sample buffer, and the samples were fractionated by SDS polyacrylamide gel electrophoresis. Radioactive Gwl and MBP bands were identified by autoradiography; the radioactivity incorporated into Gwl and MBP was quantified by cutting the bands out of the dried gel and analyzing them for ³²P in a scintillation counter. Figure 1C shows the linear range of this assay for active wild type (WT) Gwl made in OA-treated
In later experiments, 1.5 μg of a fragment of the verified Gwl substrate Endosulfine (23) was used instead of MBP in the in vitro kinase assays. This fragment (56KRLQKQKYFDSDYNMAKAK76 of Xenopus Endosulfine, with the Gwl site underlined) was fused to maltose-binding protein in the vector pMAL-C2x (New England Biolabs, Beverly, MA). The cloned Endosulfine fragment and MBP behaved identically as substrates for all Gwl variants tested.

For tests on phosphorylation of the Gwl C-terminal tail, 8.6 μg of the synthetic peptide RRNQAHLKVSGLS887 (500 μM final concentration) was incubated with 100 ng WT or KD Gwl (made in the presence of OA) and radioactive ATP in standard kinase assays. The entire reaction was then spotted on a phosphocellulose filter (Whatman P81). The filter was washed extensively in phosphoric acid, and the radioactivity remaining on the filter was measured in a scintillation counter as described (13).

**In vitro activation of Gwl**

250 ng Gwl made in the absence of OA was incubated in kinase buffer containing 100 μM ATP with 25 ng exogenous kinases for 1 hr at 30°C; for mass spectrometry of activated Gwl, the ATP concentration was increased to 1 mM. These kinases include MPF [Cdk1/Cyclin B; (45)], Cdk2/Cyclin A made according to (22), Plx1 (see below), recombinant active Aurora A (Sigma-Aldrich #A3483, St. Louis, MO), and Nek2 (Sigma-Aldrich #N4787). Activity of the exogenous kinases on Gwl was verified both by incorporation of radioactive phosphate from [γ-32P]ATP and by
mass spectrometry (MS) analysis. Aliquots corresponding to 90% of the in vitro phosphorylation reactions were examined after gel electrophoresis by Coomassie blue staining for quantification of Gwl. The remaining 10% of each sample (containing 25 ng of Gwl) was added to standard assays for Gwl kinase activity, some of which contained the CDK inhibitor Roscovitine (0.25 mM final concentration) as indicated.

To determine whether Suc1/Cks/p9 proteins can enhance CDK-driven activation of Gwl, Cks2 (Fitzgerald Industries, Acton, MA) was added at a 2.5X molar ratio to Cdk2/CyclinA prior to their incubation with Gwl made in the absence OA.

Incorporation of radioactivity into recombinant Endosulfine was measured either by autoradiography or on phosphocellulose filters. The latter assay accounted for radioactivity incorporated into Gwl by autophosphorylation or CDK action on Gwl using parallel reactions performed in the absence of the Endosulfine substrate.

**Plx1 preparations**

In contrast with a recent report (40) showing some activation of human Gwl with Polo-like kinase in vitro, we have not observed activation of *Xenopus* Gwl using several different preparations of Polo-like kinases. These include: (1) the constitutively active T201D mutant of Plx1 [isolated by ourselves from baculovirus-infected Sf9 cells not treated with OA (45)]; a similar preparation of T201D Plx1 made in the laboratory of Dr. James Maller (University of Colorado, Denver, CO); and a commercial preparation of active human Plk1 from Sigma-Aldrich (St. Louis, MO). We checked the activity of these preparations in several ways, including their ability to phosphorylate the known substrate Pin1 (e.g., Fig. 10A) and a model “Plktide” peptide substrate.
(CKKLGEDQAEISDDLLEDLSDEDE, SignalChem, Richmond, BC, Canada) (see Fig. 10B). The Plx1 preparation used in Fig. 3B had a specific activity of 6.4 nmole/min/mg when measured with this Plktide. Activity of the same Plx1 preparation was also demonstrated by its near quantitative phosphorylation of Gwl T416 (located in a reasonable Polo consensus within the NCMR; MS data not shown).

**Two-dimensional tryptic-phosphopeptide mapping**

Recombinant Greatwall proteins made in Sf9 cells in the absence of OA were incubated with CSF-arrested extracts containing 200 μCi [$\gamma$-32P]ATP at 23°C for 1 hr, followed by anti-Greatwall immunoprecipitation. The immunoprecipitates were then subjected to two-dimensional tryptic-phosphopeptide mapping as described (46). The same mapping procedure was also performed with wildtype active recombinant Gwl (made in Sf9 cells in the presence of OA) incubated in a standard kinase assay in the presence of [$\gamma$-32P]ATP.

**Mass spectrometry**

Mitotic Gwl was immunoprecipitated from CSF extracts by protein A beads coated with Gwl antibody; alternatively, recombinant Gwl was expressed and purified from Sf9 cells, and in some cases activated with Cdk2/Cyclin A, as described above. Gwl proteins made by any of these methods were then separated on SDS-polyacrylamide gels, identified by staining with PageBlue Coomassie stain (Fermentas, Glen Burnie, MD), and destained in water. Excised gel bands (~500 ng) were subjected to in-gel digestion by trypsin (or in selected cases with chymotrypsin or
endopeptidase GluC) and subsequent extraction as previously described (45). The digest was reconstituted in 10 μL of 2% acetonitrile (ACN) with 0.5% formic acid (FA) for nanoLC-ESI-MS/MS analysis using a LTQ-Orbitrap Velos (Thermo-Fisher Scientific, San Jose, CA) mass spectrometer equipped with a “Plug and Play” nano ion source device (CorSolutions LLC, Ithaca, NY). The nanoLC was carried out by Dionex UltiMate3000 MDLC system (Dionex, Sunnyvale, CA). The Orbitrap Velos was operated in positive ion mode with nano spray voltage set at 1.5 kV and source temperature at 275°C. Further details are available upon request.

All MS and MS/MS raw spectra were processed using Proteome Discoverer 1.1 (PD1.1, Thermo-Fisher Scientific, San Jose, CA), and the spectra were searched using in-house license Mascot Deamon (version 2.3, Matrix Science, Boston, MA) against NCBInr, taxonomy: *Drosophila melanogaster* and *Xenopus laevis*. The database searches were performed with one missed cleavage site by trypsin allowed. The peptide tolerance was set to 10 ppm and MS/MS tolerance was set to 0.8 Da for CID and 0.05 Da for HCD. Only significant scores for the peptides defined by Mascot probability analysis (www.matrixscience.com/help/scoring_help.html#PBM) greater than “identity” were considered for the peptide identification and phosphorylation site determinations. All MS/MS spectra for identified phosphorylation peptides were manually inspected, validated and quantified using PD1.1 and Xcalibur 2.1 software. For certain phosphosites, the relative abundance of the phosphopeptide and unphosphorylated peptide ions at one or two different charged states (doubly charged and triply charged ions) were estimated based on the peak areas of the extracted ion profiles from the nanoLC/MS/MS analysis.
Gwl homology modeling

The protein kinase A (PKA) catalytic subunit (PDB ID 3DND) structure was used as the template for modeling; the sequence alignment between Gwl and PKA was determined with the CLUSTALW2 program (19). The homology model for the kinase domain of Gwl was built using the computational Modeller 9V4 program (http://salilab.org/modeller/9v4/release.html).
RESULTS

Gwl phosphorylation and activation

Gwl activation during M phase involves its hyperphosphorylation, affecting Gwl’s electrophoretic mobility (Figs. 1 and 2) and leading to the labeling of more than 20 tryptic phosphopeptides (data not shown). One or more phosphosites in M phase Gwl constitute epitopes for the MPM-2 monoclonal antibody (Fig. 2A). This antibody reacts with many mitotic phosphoproteins, often at motifs targeted by cyclin-dependent kinases (CDKs) that contain phosphoserine or phosphothreonine followed by proline (42).

We have reconstituted a significant fraction of the Gwl activation process in vitro by treating interphase Gwl (made from baculovirus constructs in Sf9 cultured cells) with CDKs [Fig. 3; see also (40, 45)]. Consistent with the general lack of CDK specificity in vitro (15), Cdk1-Cyclin B (MPF), Cdk2-Cyclin E, and Cdk2-Cyclin A are equally efficient in activating Gwl (Fig. 3A and data not shown). CDK-treated interphase Gwl attains at most only ~25% of the kinase activity of M phase Gwl [made in Sf9 cells arrested with the phosphatase inhibitor okadaic acid (OA); see (45, 47)]. However, following the prescient suggestion of a reviewer, we added the CDK-associated protein Cks2 (Suc1/p9) to the in vitro activation reaction. This factor was previously reported to promote CDK phosphorylation of other G2/M regulators such as Cdc25 (29). The addition of Cks2 enhanced CDK activation of Gwl by ~4X, producing an enzyme with activity more comparable to that of Gwl made in the presence of OA (Fig. 3C and D). Gwl activation may therefore not require upstream kinases other than CDKs. Indeed, in our hands the M phase kinases Plx1, Aurora A,
and Nek2 do not contribute to Gwl activation alone or in synergism with CDKs (Fig. 3B); the same is true of PKA and p42 MAPK (data not shown).

Gwl undergoes autophosphorylation; that is, active preparations of wild type (WT) Gwl (either endogenous protein purified from M phase extracts or recombinant enzyme made in OA-treated Sf9 cells) become labeled by radioactive ATP. This labeling represents autophosphorylation rather than action of a contaminating kinase because kinase dead (KD) Gwl does not incorporate $^{32}$P [(45); see also Figs. 3A and 4A] and because a linear correlation exists between the phosphorylation of mutant forms of Gwl and their activities on exogenous substrates (see below). At least 15 tryptic peptides become labeled by Gwl autophosphorylation (data not shown); as explained later, most of these sites are not critical to Gwl activation, but at least one of the sites does play a key role.

To determine whether Gwl autophosphorylation is intra- or inter-molecular, we performed two experiments in which a small amount of active, WT Gwl was added to an excess of inactive Gwl (Fig. 4). $^{32}$P incorporation was almost exclusively into active Gwl, showing that the autophosphorylation is primarily intramolecular. $^{32}$P labeling of active Gwl displays first-order kinetics typical of unimolecular reactions (Fig. 1C and D), supporting the intramolecular autophosphorylation model.

**Specific sequences within the long intervening region are not essential for Gwl function**

Gwl's highly conserved kinase domain is split by a poorly conserved ~500 amino acid insertion (residues 223-717 in *Xenopus*) (Figs. 5 and 6). To establish
whether this nonconserved middle region (NCMR) is critical for Gwl activity, we made
truncated proteins, including Gwl(Δ241-549), Gwl(Δ400-699), and Gwl(Δ300-650),
that collectively remove almost all of the NCMR (Fig. 7A and data not shown). The
truncated proteins, as well as WT and KD Gwl, were expressed in Sf9 cells that were
either arrested in M phase by OA or untreated and thus mostly in interphase.
The purified proteins were adjusted to the same molar concentration and assayed
for kinase activity (Fig. 7B). Figure 1C shows the assay’s dynamic range. The model
substrates myelin basic protein (MBP) and Endosulfine yielded similar results. The
interphase enzymes displayed little or no kinase activity. Surprisingly, when expressed
in OA-treated cells, Gwl(Δ241-549), Gwl(Δ400-699), and Gwl(Δ300-650), as well as
smaller NCMR deletions, exhibit wild-type levels of autophosphorylation and kinase
activity (Fig. 7B and C, and data not shown; summarized in Fig. 5). Apparently, no
specific sequence in the NCMR has an essential role.
We also tested the biological function of the truncated Gwl proteins in Xenopus
egg extracts. Depletion of endogenous Gwl from CSF extracts leads to a loss of mitotic
status that can be prevented by adding active exogenous Gwl (45). Fig. 1A shows the
dynamic range of this functional assay. All deleted Gwl proteins just described (made
in OA-treated cells) can maintain Gwl-depleted CSF extracts in M phase (Fig. 7D),
verifying their biological activity.
Another indication that the NCMR’s particular amino acid sequence is
non-essential is the finding that Drosophila Gwl, which has no sequence homology with
frog Gwl in the NCMR, can substitute for the Xenopus enzyme in CSF extracts (data not
shown). Fly and frog Gwl are thus functionally orthologous, a conclusion earlier
implied by phenotypic similarities (44, 45, 47). *Drosophila* Gwl’s autophosphorylation is less pronounced than that of *Xenopus* Gwl (Fig. 2D), suggesting that some autophophorylation sites in the frog enzyme are nonessential.

The NCMR is still likely to be important for Gwl regulation, even if particular NCMR sequences are not. For example, Gwl(Δ241-699), which removes almost the entire NCMR (Fig. 5), is inactive in both the kinase and extract assays (Fig. 7B-D). Interestingly, however, interphase Gwl deleted for half the NCMR is more easily activated in extracts than WT (Fig. 1B, right panel). The Discussion suggests possible explanations for these paradoxical results.

**Mutagenesis of potential phosphorylation sites**

To investigate how phosphorylation mitotically activates Gwl, we mutated individually all conserved S and T residues outside of the NCMR to nonphosphorylatable A. We altered selected MS validated sites within the NCMR to serve as controls, but we did not comprehensively analyze all NCMR phosphosites because proteins collectively lacking almost all the NCMR remain functional. Gwl’s mitotic regulation must therefore rely on phosphorylations elsewhere, either within the kinase domain or in the short conserved regions adjacent to the kinase domain (blue in Fig. 5) that distinguish Gwl from other AGC kinases.

We tested 33 sites by preparing mutant proteins from OA-treated Sf9 cells and analyzing their kinase activity in vitro (Fig. 8A-D). The Gwl mutants S39A, S89A, S98A, S101A, S119A, T193A, T206A, S212A, T748A, T868A, and S883A exhibited significantly reduced autophosphorylation and kinase activity towards Endosulfine or

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MBP substrates. Among this set of 33 mutants, a linear correlation exists between the phosphorylation of exogenous substrates and of Gwl itself ($r = 0.892, p < 1 \times 10^{-6}$), verifying that Gwl labeling represents autophosphorylation rather than a contaminating kinase (Fig. 8D). The regression line's slope is less than 1, likely because a few mutations such as S98A and S119A disrupt substrate recognition but not intrinsic enzyme activity.

We also tested the mutant proteins for biological activity in extracts (Fig. 8E and data not shown). These functional analyses were in general consistent with the in vitro kinase assays. With the exception of S119A, all of the mutant proteins with disrupted kinase activity were also unable to sustain M phase in Gwl-depleted CSF extracts. S119A is probably a borderline case in which the mutant enzyme’s reduced activity is still sufficient to cross the threshold needed to sustain M phase in the extracts, between 25 and 50% of the activity of endogenous WT Gwl (Fig. 1A).

We also altered 9 potentially important sites to D or E to mimic phosphorylated S or T, respectively. When expressed in OA-treated cells, most of these mutants had reduced activity in vitro, excepting the hyperactive S101D (Fig. 9A and B). Importantly, however, proteins containing T193E, T206E, and S883D, phosphomimetic mutations of the sites most critical to Gwl regulation (see below), are functional in extracts (Fig. 9C) and have only a mild reduction in kinase activity in vitro (Fig. 9A and B). The inactivity of the corresponding original T193A, T206A, and S883A mutants thus likely reflects the inability of these sites to be phosphorylated, rather than specific structural requirements for T or S at these positions.

Because one purpose of making the phosphomimetic mutants was the generation
of constitutively active Gwl, we also expressed combinations of these mutations in Sf9 cells not treated with OA. None of the many variations we tried exhibited full constitutive activity. However, the interphase S101D T193E double mutant has about 10-15% of M phase WT Gwl’s kinase activity (normalized for protein amount), even though this double mutant remains hypophosphorylated (Fig. 9D). S101D T193E made in the absence of OA cannot rescue the M phase status of Gwl-depleted CSF extracts except in considerable excess (Fig. 9E). The limited constitutive activity of this mutant enzyme is stimulated in vitro by CDK treatment (data not shown); CDK targeting of sites other than S101 and T193 is thus needed for full Gwl activation.

Mass spectrometry of mitotic phosphorylation sites

We conducted several MS experiments to identify the M phase phosphosites in frog and fly Gwl. In some experiments, endogenous mitotic Gwl was immunoprecipitated from *Xenopus* CSF extracts; in others, recombinant Gwl was prepared by expression in OA-treated Sf9 cells. Finally, because CDKs can activate Gwl in vitro (Fig. 3), recombinant Gwl expressed in Sf9 cells in the absence of OA (and thus lacking M phase phosphorylations) was treated with MPF or Cdk2/Cyclin A. Gwl prepared by these methods (Fig. 2C) was digested with trypsin or, to increase coverage, with chymotrypsin or endopeptidase GluC. MS was used to find phosphorylated peptides (Fig. 6). Peptides representing 89% of Gwl were seen at least once, although individual experiments covered only 45-80% of the protein. MS identified 23 phosphoamino acids in fly Gwl and >50 phosphoamino acids in frog Gwl, of which ~25 were seen consistently. These numbers roughly correspond with the
number of phosphopeptides seen by 2-dimensional tryptic mapping (not shown), indicating that MS accounts for most M phase phosphorylation sites. With the exception of phosphotyrosine at positions 727 and 751 in *Xenopus* Gwl, all of the phosphorylated sites are S or T. More than half are proline-directed; that is, S/T P motifs likely targeted by CDKs. Excepting T725, all of the phosphosites we identified in conserved parts of Gwl (that is, outside of the NCMR) were previously documented as M phase-specific sites in previous studies of human cells [(7, 8, 28); summarized in Fig. 6]. Phosphorylations of Y727 or Y751 are unlikely to play critical roles in Gwl regulation, because fly Gwl has F at the Y727 cognate position, while all AGC kinases have Y at the 751 cognate position.

Only a small subset of MS-detected phosphorylations intersects with the critical phosphorylation sites identified by the mutagenesis experiments above; these are pT193, pT206, pS212, pT748, and pS883 in *Xenopus* Gwl. We estimated the phosphate occupancy of these sites by comparing the peak areas for all phosphorylated and nonphosphorylated forms of the corresponding peptides (Table 1). The phosphate occupancy of all of these sites is higher in active frog Gwl from OA-treated cells than in inactive Gwl from untreated cells. T193 and S883 are likely nearly stoichiometrically occupied by phosphate in active samples; Table 1 probably underestimates occupancies because MS detection efficiencies for phosphopeptides are often >10X lower than for nonphosphorylated forms (12). The phosphate occupancy of pT206 also appears to be relatively high (>25%), while those for pS212 and pT748 are more modest. Similar results were observed for occupancy of the corresponding sites in *Drosophila* Gwl made in OA-treated cells (Table 1).
Kinases targeting critical phosphorylation sites

T193 and T206 are proline-directed sites likely recognized by CDKs. Indeed, MPF can target both sites in vitro, when either a fragment of Gwl containing these sites (Fig. 10A) or full length inactive Gwl (Table 1) is used as a substrate. A fragment containing T193 but with the T206A mutation is a better substrate than a fragment with T193A and T206, indicating that T193 is more efficiently targeted by MPF (Fig. 10A).

S883 is not proline-directed, and a fragment including this site is not phosphorylated in vitro by MPF (Fig. 10A). Indeed, the region surrounding S883 does not conform to the consensus sequence for any known kinase. We thus tested the idea that Gwl itself can target S883 through autophosphorylation. As shown in Table 1, pS883 is produced when inactive WT frog Gwl (made in the absence of OA) is partially activated in vitro with MPF, but not when KD Gwl is used instead. Furthermore, WT Gwl (but not KD Gwl, Plx1, or Cdk1/Cyclin A) can utilize a short peptide containing S883 as an exogenous substrate (Fig. 10B), and MS analysis demonstrates that pS883 is the only site Gwl phosphorylates in this peptide (data not shown). The S883-containing peptide is phosphorylated ~200-fold less efficiently than a similarly-sized peptide containing the Gwl target site in Endosulfine (data not shown), consistent with experiments on other kinases using peptides containing autophosphorylation sites as exogenous substrates (11).
DISCUSSION

Gwl: an unconventional AGC kinase

The common core mechanism of AGC kinase activation involves phosphorylation at three conserved sites: in the activation loop, in the hydrophobic motif (HF) at the end of the C terminal tail, and in the middle of the tail (called the turn motif in PKA or the tail/Z site in growth factor-stimulated AGC kinases) [reviewed by (14, 27, 30); see Fig. 11]. Activation loop phosphorylation (often by PDK1) increases phosphoryl transfer catalysis and in some cases substrate access to the active-site cleft (1). HF phosphorylations (added either autokally, by exogenous kinases such as mTOR, or phosphomimicked by acidic amino acids) promote the enzyme’s active conformation. The phosphorylated HF becomes anchored to the "HF pocket" in the kinase's N-terminal lobe [reviewed in (27, 30); see also Fig. 12]. This binding stabilizes the α−C helix that forms one side of the pocket, and thus enhances phosphotransferase activity. Phosphorylations of the activation loop and the HF have synergistic effects because they promote mutually stabilizing interactions on the α−C helix (14).

The third phosphorylation (added autokally or by other kinases) near the middle of the C-terminal tail helps the tail to loop around the kinase core. This third phosphorylation can occur at either of two adjacent sites (the "tail/Z site" and the "turn motif") that promote looping by distinct mechanisms. In growth factor-stimulated AGC kinases, phosphorylation of the tail/Z site allows it to bind to a positively charged patch on the N-terminal lobe. In PKA, turn motif phosphorylation causes a bend in the tail due to interactions of this pS338 with nearby residues (Figs. 11 and 12).
looping facilitated by both mechanisms allows the phosphorylated HF to locate its binding pocket in the N-terminal lobe (14, 17).

After this manuscript’s original submission, another paper addressing Gwl regulation appeared in print (40). The authors concluded that phosphorylation of a single site in human Gwl (corresponding to S883 in *Xenopus*) is essential for activation, that this site is the direct target of both MPF and Plk1, and that this phosphosite plays a role in Gwl analogous to that of the tail/Z site in other AGC kinases. We agree that S883 is critical and probably functions similarly to the tail/Z site, but otherwise we view Gwl regulation differently. If S883 was the sole essential phosphosite, then Gwl with a S883D or S883E phosphomimetic substitution should exhibit constitutive activity, when made either in interphase cells in the absence of OA, or in immature *Xenopus* oocytes. However, mutant enzymes synthesized by either method are inactive (Figs. 9D,E and 13), even though the same proteins acquire biologically relevant kinase activity in OA-treated cells (Fig. 9A-C) or in eggs induced to mature by progesterone (Fig. 13). Other events in addition to S883 phosphorylation must therefore occur to activate Gwl during M phase.

In the model we present below and in Fig. 14, Gwl activation is initiated by CDK phosphorylation of T193 and T206, which act as activation loop phosphorylations.

In contrast with the other report (40), we do not think that either MPF or Plk1 directly targets S883; instead, CDK-primed Gwl autophosphorylates S883.

Critical phosphosites in Gwl regulation

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Although Gwl is phosphorylated during M phase at many sites, our results focus attention on five candidates: pT193, pT206, pS212, pT748, and pS883 (*Xenopus* numbering). These residues are located in evolutionarily conserved regions characteristic of Gwl but not other AGC kinases (blue in Fig. 5). In fact, these regions discriminate Gwl from the closely related Rim15p kinase in *S. cerevisiae*, which has the same substrate specificity (it targets a yeast homolog of Endosulfine) but is regulated by a different mechanism (38). Mutation of any of these five sites to A severely compromises Gwl function (Fig. 8). MS has detected all five phosphosites in *D. melanogaster*, *X. laevis*, and *H. sapiens* [Fig. 6; (7, 8, 28, 40)].

The data implicating pT193, pT206, and pS883 are particularly strong. Enzymes made in OA-treated cells with the phosphomimetic mutations T193E, T206E, and S883D rescue Gwl activity in extracts (Fig. 9C), arguing that inactivity of the corresponding non-phosphorylatable mutants does not reflect structural requirements for T or S in those positions. The phosphate occupancies at T193, T206, and S883 are very high in active WT frog or fly Gwl (Table 1). Finally, the equivalents of pT193 and pT206 are known to be highly enriched in human M phase cells (8), while the human equivalent of pS883 is highly correlated with Gwl activity as shown by a phospho-specific antibody (40).

The roles of pS212 and pT748 are less clear. Because the phosphomimetics S212D and T748E display little or no function (Fig. 9A-C), the inactivity of S212A and T748A (Fig. 8) might be explained by intolerance of Gwl’s structure for amino acid substitutions at these positions. pS212 is difficult to quantify because it is found in a multiply phosphorylated peptide, while the phosphate occupancy of pT748 in active...
Gwl is only about 2% (Table 1).

Our results demonstrate the importance of phosphorylations at T193, T206, and S883 for Gwl activation. However, because we have yet to find a combination of phosphomimetic mutations with high constitutive activity, other sites likely play subsidiary roles. As just mentioned, pS212 and pT748 are potential contributors. Other conserved sites (S215, T221, T725, T729, and S886) are consistently phosphorylated during M phase (Fig. 6). Although S/T-to-A mutations of these latter sites have no obvious consequences, the functional tests in Fig. 8 may have missed small effects of specific mutations. Several nonconserved residues within the NCMR are also phosphorylated during M phase in fly, frog, or human Gwls. None of these phosphosites is individually needed, but they could be redundant such that Gwl activity might require multiple NCMR phosphorylations.

Finally, alanine mutations at S39, S89, S98, S101, and T868 strongly disrupt Gwl function (Fig. 8), although evidence for the phosphorylation of these sites is lacking. The absence of such evidence is particularly surprising for S101, because the S101D mutation confers limited constitutive activity to Gwl, particularly in the double mutant S101D T193E (Fig. 9D and E). S101D thus appears to stabilize a partially active conformation of Gwl for unknown structural reasons that may or may not reflect actual S101 phosphorylation.

The remainder of this discussion focuses on T193, T206, and S883 phosphorylations as key regulatory steps in Gwl activation, but it must be remembered that these events are unlikely to represent the entire story.
Kinases involved in Gwl activation

We propose that CDKs prime Gwl at the critical sites T193 and T206, consistent with our findings that: (1) Gwl is specifically activated during M phase; (2) the CDK inhibitors roscovitine and p21\textsuperscript{Cip} prevent Gwl activation in frog oocytes and egg extracts (47); (3) CDKs potentiate interphase Gwl’s enzymatic activity in vitro [Fig. 3; see also (40, 45)]; (4) both T193 and T206 are evolutionarily conserved S/T P motifs (the minimal CDK consensus); and (5) MPF phosphorylates both T193 and T206 in vitro (Fig. 10A and Table 1). It is highly likely that MPF is the kinase targeting T193 and T206 during M phase proper, but a different CDK such as Cdk1-cyclin A or a related enzyme such as Cdk1-Speedy/Ringo (6, 33) could target these sites during M phase entry prior to full MPF mobilization. In this way, Gwl could act not only downstream of MPF (by preventing the premature dephosphorylation of MPF effectors), but also upstream as part of the MPF triggering mechanism [by helping control MPF regulators such as Cdc25 phosphatase and Myt1/Wee1 kinases; reviewed by (31)].

S883 is the other site critical for Gwl activation identified in our studies. We disagree with the recent proposal that S883 is directly targeted by CDKs (40). In contrast with almost all known CDK substrates, S883 is not followed by proline, and we have failed to observe CDK phosphorylation of a fragment or peptide containing S883 (Fig. 10A and B). We believe instead that pS883 results from Gwl autophosphorylation primed by prior CDK phosphorylation at T193 and T206. This model explains why CDKs cause the appearance of pS883 when added to purified WT, but not KD, interphase Xenopus Gwl in vitro (Table 1). pS883 autophosphorylation also explains the general correlation seen between this site's phosphorylation and Gwl...
activity (40). Direct evidence for this hypothesis is the ability of active but not inactive Gwl to phosphorylate a peptide containing S883 (Fig. 10B). Although the effect is clear, this peptide is only a poor exogenous substrate for Gwl, consistent with the finding that Gwl autophosphorylation is normally intramolecular (Fig. 4). Several precedents exist for autophosphorylation of S883-analogous sites. In PKA and some PKC isotypes, the turn motif/tail/Z site is autophosphorylated intramolecularly (3, 16, 27, 32); in fact, bacterially synthesized PKA autophosphorylates the turn motif (S338) to a phosphate occupancy of >98% (36). Interestingly, the sequences preceding pS883 in Gwl (880LKVpS883) and pS338 of PKA (335IRVpS338) are quite similar.

CDKs together with Cks2 (Suc1/p9) produce substantial activation of interphase Gwl (Fig. 3). Suc1 is needed for M phase entry of egg extracts, a requirement ascribed to Suc1’s ability to enhance CDK phosphorylation of G2/M regulators such as Cdc25, Myt1, and Wee1 (29). Our results indicate that Gwl should be added to this target list. At present we see no compelling reason to invoke roles for upstream kinases other than CDKs in Gwl activation, although we cannot exclude the possibility. For example, in our hands abrogation of the function of several kinases with cell cycle involvement, including Mos, MEK, MAPK, CaMKII, and p90Rsk, does not interfere with Gwl activation [(47) and data not shown]. Studies of drug-treated Xenopus oocytes suggest that neither PI-3, JNK, mTOR1, S6K, nor PKBβ is required to turn Gwl on during maturation (24, 35). The results shown in Fig. 3B provide no evidence for participation of the mitotic kinases Plx1, Aurora, or Nek2; similar experiments (not shown) also appear to exclude PKA and p42 MAPK.

Vigneron et al. (2011) have recently reported activation of human Gwl with the
Polo-like kinase Plk1, and suggest that Plk1 (as well as CDKs) can directly target the human equivalent of S883 (40). We regard the idea that Polo-like kinases are critical Gwl activators as unsettled. First, S883 is unlikely to be directly phosphorylated by Plk1. S883 does not reside in an obvious Plk1 consensus in frog or human Gwl [Plk1's selectivity for E/N/D/Q at the -2 position relative to the S/T is very strong; (34)], nor does Plx1 target a fragment or peptide containing Gwl S883 in vitro (Fig. 10). Second, we have repeatedly failed to activate interphase frog Gwl with any of several preparations of human Plk1 or frog Plx1. One potential explanation for the differing results is that Plk1 phosphorylates human Gwl in vitro at several locations within the NCMR that are not found in frog Gwl and that have never been detected as phosphosites even in M phase human Gwl (39). Plk1 targeting of these nonconserved sites might partially activate human Gwl in vitro, yet not reflect a fundamental aspect of Gwl regulation in vivo. Third, hypermorphic mutations of gwl in Drosophila are dominant enhancers of polo loss-of-function mutations (2). The direction of this genetic interaction is exactly the opposite of expectations if Polo were a Gwl activator. Finally, in starfish, Gwl can be activated during oocyte maturation in the absence of Polo activity (18).

**A model for Gwl activation**

Our current working model (Fig. 14) has strong precedents in PKA’s activation mechanism. The initial step is activation loop phosphorylation by an exogenous kinase: PDK1 for PKA (30) and MPF for Gwl. The pT193 and pT206 Gwl presumptive activation loop phosphorylations are in fact found in positions analogous to the pT197...
activation loop site in PKA (Fig. 11). Primed Gwl can now intramolecularly
autophosphorylate pS833, which is located in the same relative position in the
C-terminal tail as PKA's turn motif (S338) (Figs. 11 and 12). In PKA, S338 is
autophosphorylated intramolecularly to >98% occupancy (36). Our depiction of Gwl
pS883 phosphorylation in Fig. 14 is based on structural analyses indicating that PKA's
tail is flexible enough so that the turn motif can access the active site within the same
molecule (32).

Other phosphorylations, particularly within the NCMR, probably play
subsidiary or redundant functions in Gwl activation. One possibility suggested in Fig.
14 is that the NCMR in interphase Gwl blocks substrates from the active site, while M
phase NCMR phosphorylations relieve this inhibition. A smaller NCMR could require
fewer phosphorylations, explaining the easier activation of interphase Gwl deleted for
part of the NCMR relative to the WT enzyme (Fig. 1B). However, the evidence for
such function of the NCMR is at present only fragmentary. Several NCMR
phosphorylations observed by MS (Fig. 6) are proline-directed and could result from
CDK action, but others are not proline-directed and could be the targets of exogenous
kinases or perhaps more likely, autophosphorylation.

The steps subsequent to the phosphorylations involved in Gwl activation raise a
curious paradox. Gwl's N-terminal lobe appears to contain a "pocket" like those that
interact with the phosphorylated hydrophobic motif (HF) of other AGC kinases (Fig.
12). Gwl shares the residues within such pockets that make the strongest contacts with
the HF, and mutations of the corresponding amino acids in human Gwl to A abrogate
enzyme function (40). The paradox is that Gwl’s C-terminal tail does not appear to be
long enough to wrap around the N lobe and contact the HF binding pocket in the same polypeptide (Fig. 12).

Gwl’s C terminus has very weak similarities with classical HF motifs, so we have entertained the notion that Gwl might multimerize during M phase, with the pS883 phosphorylated tail of one subunit inserting into the HF binding pocket of another. However, we have to date seen no evidence for Gwl multimerization (data not shown).

Vigneron et al. (2011) have proposed a provocative alternative explanation for Gwl’s retention of an HF binding pocket but lack of an HF: Perhaps other AGC kinases can help activate Gwl by inserting their phosphorylated HFs into Gwl’s pocket (40). We regard this hypothesis as very attractive, but we have not been able to verify that HF motif phosphopeptides elevate Gwl function.

What does the pS883 phosphorylation in Gwl’s tail do if it does not act as an HF? Most likely, pS883 plays a role similar to that of the tail/Z site found in the analogous position of other AGC kinases, ~10-20 residues upstream of the C-terminal HF (Figs. 11 and 12). As mentioned previously, the phosphorylated tail/Z site interacts with a patch of basic amino acids on the N lobe, helping deliver the HF to the HF binding pocket (14, 30). Gwl has 3-4 of the N-lobe amino acids defining the basic patch [R40, K45, K64, and possibly N104 in frog Gwl (14)], and mutations in these basic residues disrupt Gwl function (40). However, the putative contact between pS883 and the basic patch (depicted in Fig. 14) could not accelerate binding of the HF to the pocket as in other AGC kinases, because Gwl’s tail is too short. Instead, the pS883/basic patch interaction would need to promote Gwl activation in some other fashion not easily discerned from known AGC structures. Gwl’s activation thus still presents puzzles and
paradoxes worthy of future research.

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Table 1. Occupancy of key phosphorylation sites. MS analyzed samples are shown in Fig. 2C. An equals sign (=) connects corresponding sites in *Xenopus* and *Drosophila* Gwl. (-OA): WT or KD *Xenopus* Gwl grown in the absence of OA that were either untreated or treated in vitro with Cdk2/Cyclin A. (+OA): active WT or KDF frog or fly Gwl grown in the presence of OA. Numbers are in percentages, and are calculated as the ratio of the peak areas for all phosphorylated species / all species (phosphorylated plus unphosphorylated) of the peptide with the indicated residue. All peptides were generated by trypsin digestion, except the peptide including pT206 (*Xenopus*; chymotrypsin) and that including pS883 (*Xenopus*; endoproteinase Glu-C). Dashes (-): phosphorylated forms of the peptide were not detected. NF (not found): neither phosphorylated nor unphosphorylated forms of the peptide were detected. *Xenopus* Gwl (-OA) was activated by Cdk2/Cyclin A to approximately 10% of the specific activity of Gwl (+OA). Values for pT227 and pS233 in fly Gwl were

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Table 1 - Occupancy of key phosphorylation sites.
determined from a single peptide containing a mixture of the two monophosphorylated forms.
ACKNOWLEDGMENTS

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FIGURE LEGENDS

Figure 1. Assays for Gwl kinase activity and biological function. (A and B)

Extract assays. CSF extracts were Gwl- or mock-depleted and then supplemented with WT or KD Gwl (from OA-treated Sf9 cells) or with buffer. 0.25X etc. indicate the concentrations of exogenous Gwl relative to normal endogenous levels. After 30 min at 25°C, the extracts were analyzed for Gwl, Cdc25C, and Tyr15 (pY15) on Cdk1. M phase Gwl and Cdc25 (black arrows) migrate slower than their interphase counterparts (gray arrows). (A) WT Gwl at 0.5X maintains depleted extracts in M phase, but KD Gwl cannot prevent M phase exit even at 3X. (B) Gwl depleted CSF extracts were supplemented with Gwl proteins or buffer alone (Gwl Depl). After 10, 20, and 30 min of incubation, the extracts were analyzed as in A; here, Cdc25 is a doublet. WT Gwl made in the absence of OA restores M phase only at 6X and only after 30 min (presumably after cyclin accumulation). Right: Gwl-depleted extracts were supplemented with Gwl deleted for residues 300-650 (see Fig. 4) made in the absence of OA. The Δ300-650 enzyme is more easily activated than WT by the MPF activity that briefly remains immediately after depletion (45). (C) Standard kinase assays. Both autophosphorylation and phosphorylation of the model substrate MBP are linear from 5 ng to 250 ng of WT Gwl made in the presence of OA. (D) Gwl autophosphorylation is a unimolecular reaction. The initial rate of autophosphorylation (from panel C; circles) is independent of Gwl concentration. Squares show expectations if the reaction was instead bimolecular (between two Gwl molecules or Gwl and a contaminating kinase).

Figure 2. Gwl phosphorylation. (A) M phase but not interphase Gwl carries an
MPM-2 epitope. CSF extracts were directly analyzed (M) or treated with calcium (interphase extracts; I). Input lanes show 10% of the extract subjected to Gwl immunoprecipitation (IP). (B) Kinase activity of WT *Xenopus* (X) and *Drosophila* (D) Gwl (made in OA-treated Sf9 cells). Kinases assays contained 10 ng (left lanes) or 5 ng of Gwl (right lanes). Autophosphorylation activity of the fly enzyme is 30-40% of frog Gwl, but both phosphorylate Endosulfine to similar extents. (C) Coomassie staining of samples analyzed by MS in Table 1. KD Gwl in OA-treated Sf9 cells is less phosphorylated than WT because the KD enzyme does not autophosphorylate. In vitro activation of WT interphase Gwl (made in the absence of OA) by Cdk2/Cyclin A produces a smear of hyperphosphorylated Gwl. Fly Gwl is 41 amino acids shorter than frog Gwl.

Figure 3. Activation of Gwl by CDKs. (A) Left: 250 ng of WT Gwl made in Sf9 cells without OA was treated with 50 ng of Cdk2/Cyclin A as indicated. In the right lane, the CDK inhibitor Roscovitine was added at 0.25 mM. 1/10 of each reaction was added to a standard kinase assay, monitoring Gwl autophosphorylation or phosphorylation of Endosulfine. Right: a similar experiment using 50 ng of MPF (Cdk1/Cyclin B). Roscovitine was added to each standard kinase assay to inactivate the MPF. To resolve phosphorylated Gwl, the gel in the right panel were run longer than that at the left; arrows indicate the position of active Gwl (made in the presence of OA) in a separate lane. (B) Testing possible roles of other M phase-specific kinases in activating Gwl. In the tabulation at the top, + means 250 ng of WT inactive Gwl (made in the absence of OA) and/or 50 ng of the other indicated kinases; # indicates 50 ng of
active WT Gwl made in the presence of OA (arrows).  (C and D) The Suc1 paralog Cks2 enhances CDK-driven activation of Gwl.  Cks2 was added to reactions containing Cdk2/Cyclin A and Gwl made in the absence of OA.  In D (measured on phosphocellulose filters), Cks2 enhances CDK’s activation of Gwl from 12.5% to 49% of the specific activity of Gwl made in the presence of OA.

Figure 4.  **Gwl autophosphorylation is primarily intramolecular.**  (A) 25 ng of WT or KD Gwl (both containing a Z tag and made in OA-treated Sf9 cells) was incubated in kinase assays with 250 ng of either heat inactivated Δ300–650Gwl or heat inactivated Myc-tagged WT Gwl (the Myc tag is smaller than the Z tag) as indicated.  Despite the 10X molar excess of the heat-inactivated substrates, most $^{32}$P incorporation is into the active WT Z-tagged enzyme.  (B) WT Gwl made in the presence or absence of OA was quantified by Coomassie staining (top).  Bottom panels: 100 ng of active Gwl and/or 250 ng of inactive Gwl were added as indicated to standard kinase reactions that contained Endosulfine or no substrate.  The active enzyme (Gwl+OA) did not phosphorylate the inactive one (Gwl-OA).

Figure 5.  **Schematic view of Gwl structure and mutant analysis.**  In WT *Xenopus* Gwl, the two halves of the conserved kinase domain are orange, the NCMR is yellow, and blue shows regions that distinguish Gwl from other AGC kinases.  In the top row, red tick marks indicate S/T-to-A mutants with lower function than WT, while green tick marks are mutants with near-normal activity.  Asterisks (*) are sites essential for Gwl function whose M phase phosphorylation has been MS verified.  In subsequent rows,
thin lines indicate deleted parts of the NCMR. Excepting Gwl(Δ241-699), all these deletions exhibit near wild type activities in kinase and CSF extract rescue assays (Fig. 7 below and data not shown).

Figure 6. Gwl phosphorylation sites and mutagenesis. *Xenopus* Gwl aligned with human, mouse and *Drosophila* counterparts. "In vivo" mitotic *Xenopus* Gwl was purified by IP from CSF extracts or by expression in OA-treated Sf9 insect cells with near identical results; "in vitro" Gwl was made in Sf9 cells in the absence of OA and then treated in vitro with CDKs. Amino acids covered by MS analysis with both "in vivo" and "in vitro" Gwl are in bold italics; those found only with "in vivo" enzymes are in bold; those found only in the "in vitro" Gwl are in italics; and regions of Gwl not detected in any MS studies are in normal font. Yellow ("in vivo" and "in vitro"), aqua ("in vivo" only), or gray ("in vitro" only) shading indicates phosphorylated S, T, or Y residues identified here or in published studies of human Gwl (7, 8, 28). Underlined letters represent S or T-to-A mutations made in frog Gwl. Mutations that disrupt Gwl function in extracts are shown in red; mutations without effect are in green. Blue letters indicate sites of known loss-of-function mutations in fly (G66S, G69E, S127L, A728V) or frog (G41S, D173A) Gwl (44, 45).

Figure 7. NCMR deletions. (A) WT, KD, and deleted Gwl were purified from Sf9 cells in the presence or absence of OA. Proteins were stained with Coomassie; those purified from OA-treated cells were treated with λ phosphate (PPase) before electrophoresis because hyperphosphorylated Gwl runs as a smear. Numbers in
parentheses indicate concentrations relative to WT. (B) Kinase activity of Gwl deletions. 50ng of the indicated Gwl proteins were added to standard kinase assays monitoring both autophosphorylation and the labeling of myelin basic protein (MBP).

(C) Graphic representation of the results in B. The y-axis depicts relative specific kinase activities (that is, kinase activity per nanogram of Gwl) normalized with respect to the specific activity of WT Gwl made in the presence of OA. (D) Function of Gwl deletions in egg extracts. CSF extracts were immunodepleted for Gwl and then supplemented with recombinant Gwl at the original endogenous levels. Extracts were incubated at 25°C for 30 min before immunoblotting. Cdc25C becomes hyperphosphorylated in M phase (black arrow; the gray arrow indicates the interphase form), while Y15 of Cdk1 is phosphorylated during interphase but not M phase.

Figure 8. Analysis of S/T-to-A mutations. (A) Summary showing specific kinase activities of S/T-to-A mutants expressed in OA-treated cells. Sites marked with asterisks were observed by MS to be phosphorylated in M phase. Sites in bold are required for Gwl function in extracts (see E). Normalization to the specific activity of WT Gwl was as in Fig. 7C. (B and C) Kinase assays. 50ng of the indicated proteins were assayed in vitro for autophosphorylation and for kinase activity against MBP (B) or Endosulfine (Endos; C) as in Fig. 7B. Gwl was quantified by Western blot in B or by Coomassie staining after λ protein phosphatase treatment in C. (D) Relationship between autophosphorylation and phosphorylation of exogenous substrates, based on B and C. Numbers on each axis represent the relative specific activities of mutant proteins (+OA) relative to WT Gwl (+OA); each point represents one mutant protein.
(E) Functional analysis of S/T-to-A mutant proteins in CSF extracts. Physiological activities of the mutant proteins were determined as in Fig. 7D; concentrations of exogenous recombinant Gwl proteins were equivalent to that of endogenous Gwl.

Figure 9. Phosphomimetic mutations. (A-C) S/T residues of importance were mutated to D/E, and the proteins expressed in OA-treated Sf9 cells. Mutants were assayed for kinase activity (A and B) and function in CSF extracts (C) as in Fig. 7. (D-F) Phosphomimetic Gwl mutants with limited constitutive activity, made in Sf9 cells minus OA. (D and E) Standard kinase assays with 5-20 ng of mutant Gwl. In E, 2 ng of active WT Gwl (+OA) was the control. S101D and S101D T193E mutants made in the absence of OA respectively display 3% and 15% of the control’s specific activity, even though the mutants remain hypophosphorylated (in E, the arrow indicates active M phase Gwl). (F) Biological assays of S101D T193E double phosphomimetic mutant Gwl made in the absence of OA. This protein rescues M phase in Gwl-depleted extracts, but only in 4X excess.

Figure 10. Kinases targeting key phosphosites in Gwl. (A) The indicated Gwl fragments were expressed as GST fusions in E. coli, and then treated with MPF or Plx1 in the presence of $^{32}$P ATP in standard kinase assays. The fragment containing residues 185-213 contains two proline-directed phosphosites (T193 and T206). T-to-A mutations show that both sites are targeted by MPF, although T193 is the more efficient substrate. MPF does not target the S883-containing fragment. Plx1 does not phosphorylate any of these Gwl fragments, though it is active against Pin1, a previously demonstrated substrate (9). (B) Gwl but not Plx1 or CDK can phosphorylate a peptide containing...
The C-terminal peptide RRNNAQHLKVSGFSL (CT) was incubated with WT or KD Gwl (made in the presence of OA), or with Plx1 (PL) or Cdk1/CyclinA (CD) in standard kinase assays. Incorporation of $^{32}$P into the peptide (in counts per minute) was measured (see Materials and Methods). Two independent experiments are shown; black and gray bars are independent trials of each experiment. Activity of the Plx1 preparation is demonstrated by phosphorylation of a “Plktide” peptide substrate (PT).

**Figure 11.** Gwl is an atypical AGC kinase. Primary sequences of *Xenopus* Gwl and human PKCθ, PKBα, and PKA. Aqua: residues in Gwl conserved in other enzymes. Gray: residues shared by two or more AGC kinases but not Gwl. Yellow: residues found in Gwl and other AGC kinases not depicted (PKBβ, PKCα, PKCβ, PKCτ, PKCζ, or PDK1). The red-shaded is the site in most AGC activation domains phosphorylated by PDK1; to aid orientation this single site is shown on both sides of the NCMR. Red dots: phosphorylation sites in the C-terminal tails of AGC kinases that commonly participate in activation. Underlined amino acids in Gwl were mutated; red underlined letters are S/T-to-A changes that significantly reduce Gwl activity, while green underlined amino acids are nonessential. Structural annotations are from (17, 21).

**Figure 12.** Homology structural modeling. (A) *Xenopus* Gwl (red) modeled relative to human PKA (blue). The Gwl structure lacks the first 25 N-terminal amino acids and the NCMR, which have no equivalents in PKA. Gwl’s C terminal tail (“C” in red) is shorter than PKA’s (“C” in blue) and thus cannot extend as far. The arrow points to PKLA’s turn motif. Alpha helices B and C ($α−B$ and $α−$) demarcate two sides of
the pocket in the N lobe (boxed region) that normally binds the hydrophobic domain (HF) of AGC kinases.  (B) Close-up of the box in part A, showing the binding of PKA's C-terminal HF (green) to its corresponding pocket, which stabilizes the active configuration of α−.  Residues within the pocket important for HF interactions are labeled.  (C) Close-up of the region of Gwl boxed in A.  Gwl’s N lobe has residues likely to form an HF binding pocket, but the C terminal tail is too short to contact this pocket intramolecularly.

**Figure 13.** Gwl proteins with phosphomimetic mutations of S883 do not exhibit constitutive activity in vivo.  mRNAs for FLAG-tagged S883E or S883D Gwl were injected into immature G2 *Xenopus* oocytes that were treated (or not) with progesterone (43).  (A) Untreated injected oocytes remained immature (IM) with Gwl in its interphase form; expression of S883E or S883D did not induce germinal vesicle breakdown (data not shown).  Germinal vesicle breakdown was induced in ~100% of oocytes treated with progesterone (GV); Gwl acquires M-phase phosphorylations.  (B and C) Gwl was immunoprecipitated from injected oocytes and subjected to standard kinase assays with MBP substrate or in the substrate’s absence (C).  S883D and S883E Gwl proteins made in immature interphase oocytes have no intrinsic constitutive activity, but they are strongly activated during progesterone-induced maturation.

**Figure 14.** A model for Gwl activation.  Gwl is depicted with four domains: the N lobe, the C lobe, the nonconserved middle region (NCMR), and the C terminal tail (thick black line).  During M phase entry, MPF phosphorylates Gwl at the presumptive
activation loop sites T193 and T206. MPF, and perhaps other kinases, may also target several phosphosites within the NCMR that could have redundant functions. NCMR phosphorylations could conceivably change its conformation to allow substrates access to Gwl's active site between the N and C lobes. After Gwl is primed by MPF, S883 in the C-terminal tail can now be autophosphorylated at the active site. It is likely that pS883 can subsequently interact with a patch of basic residues in the N lobe (dark triangle) to help stabilize active Gwl (40). The round hole in the N lobe depicts the conserved HF binding pocket (Fig. 12), whose role is not yet clear.
Figure 1
Figure 2
Figure 3
**Figure 4**

(A) Table showing protein expression levels:
- WT Z-Gwl: + + + + +
- KD Z-Gwl: + + + + +
- Inactive Myc-Gwl: - - + + +
- Inactive ΔGwl: + + + + +

(B) Table showing protein expression levels:
- WT Gwl (+OA): + + + + +
- WT Gwl (-OA): - + + + +
- Endos: - - + + +

**Images**
- Coomassie staining of proteins
- Phosphorus incorporation assay
Figure 7
Figure 8
Figure 9
Figure 10
Figure 11
Figure 13
Figure 14