Identification of a C-terminal cdc25 sequence required for promotion of germinal vesicle breakdown

Elaine A. POWERS*, David P. THOMPSON†, Peggy A. GARNER-HAMRICK*, Wanxia HE*, Anthony W. YEM‡, Carol A. BANNOW‡, Douglas J. STAPLES‡, Gregory A. WASZAK‡, Clark W. SMITH‡, Martin R. DEIBEL, JR. and Chris FISHER*¹

*Cell and Molecular Biology, Pharmacia and Upjohn, Inc., Kalamazoo, MI 49007, U.S.A., †Animal Health Discovery Research, Pharmacia and Upjohn, Inc., Kalamazoo, MI 49007, U.S.A., and ‡Protein Science, Pharmacia and Upjohn, Inc., Kalamazoo, MI 49007, U.S.A.

Glutathione S-transferase (GST)–cdc25B(31–566) induced germinal vesicle breakdown (GVBD) when microinjected into *Xenopus* oocytes. Purified, N-terminally truncated forms of cdc25B did not induce GVBD, even though many had phosphatase activity and activated cdc2 *in vitro*. N-terminally truncated forms of cdc25B inhibited induction of GVBD by longer forms of the enzyme suggesting a direct interaction *in vivo*. cdc25B(356–556), but not cdc25B(364–529), inhibited GVBD induction by GST–cdc25B(31–566) suggesting that a region of cdc25B near to the C-terminus was responsible for the inhibition. To determine the region of peptide sequence that was inhibitory, cdc25B(356–556) was subjected to proteolysis with endoproteinase lys-C. Following a demonstration that the resulting peptide mixture

inhibited GST–cdc25B-dependent GVBD, a series of peptides spanning amino acids at the C-terminus were synthesized. The peptide TRSWAGERSR inhibited GVBD induced by GST–cdc25B. An alanine scan of the peptide revealed residues critical for GVBD inhibition, and site-directed mutagenesis of the corresponding residues in GST–cdc25B(31–566) eliminated its ability to induce GVBD. These results demonstrate that a cdc25B C-terminal domain, involved in dominant-negative inhibition of GVBD-competent cdc25B, is required for induction of GVBD following microinjection into oocytes.

Key words: cdc2, cell cycle, cyclin-dependent kinase, oocyte, phosphatase.

INTRODUCTION

Cell cycle progression is regulated by the cyclin-dependent kinase (CDK) family. CDK activity throughout the cell cycle is highly regulated by association with cyclins and association with inhibitory proteins [1]. CDK activity is also regulated by phosphorylation. For example, phosphorylation of Thr-160/161 is required for kinase activity [2]. Phosphorylation of two highly conserved residues (Thr-14 and Tyr-15 of cdc2 and cdk2 respectively) by cell cycle inhibitory kinases, including Wee1, Mik1 and Myt1, is inhibitory [3]. The cdc25 phosphatase family activates CDKs and stimulates cell cycle progression by catalysing removal of CDK inhibitory phosphates [4].

Three members of the cdc25 family have been identified in mammalian cells [4]. The phosphatase domain of cdc25 resides in the conserved, C-terminal half of the protein. cdc25C promotes the G₂/M transition through its action on cyclin B/cdc2 [5,6,8], while cdc25A acts earlier in the cell cycle on cyclin E/CDK2 [9,10]. cdc25B has been implicated in both the G₂/M transition [11] and S-phase progression [12,13]. It is the least stable of the three family members, being rapidly degraded during G₂/M [13,14] by a proteasome-dependent mechanism [15]. Three cdc25B splice variants have been identified [16]. cdc25B1, the most studied of the three variants and the one used exclusively in this study, is a 566-amino-acid protein, with approximately the C-terminal one-third of the protein forming a conserved phosphatase domain [4,7,16].

cdc25 is itself regulated by phosphorylation. CDKs and polo-like kinases increase cdc25 phosphatase activity thus contributing to an amplification loop that ensures the faithful activation of CDKs during cell cycle transitions [8–10,17,18]. Other cdc25

kinases, including Chk1 (checkpoint kinase 1) [19–22] and Cds1 (checking DNA synthesis 1) [23], inhibit cdc25 function by inhibiting phosphatase activity [23] or through generation of a 14-3-3 binding site [20]. The latter modification results in nuclear exclusion of cdc25 [24,25]. The cdc25 N-terminus, therefore, exerts both positive and negative control over cdc25 function. Other proteins are also reported to associate or co-distribute with cdc25, including Ras, p13, Raf-1 and cyclin B [26–30].

Germinal vesicle breakdown (GVBD) is regulated by the direct cdc25-dependent activation of mitosis-promoting factor (MPF), the cyclin B–cdc2 complex of frog oocytes [31,32]. Several groups have demonstrated that exogenous cdc25 from different species will induce GVBD in *Xenopus* oocytes through the direct activation of cdc2 [8,32–35]. Previous studies reported that elimination of N-terminal cdc2 phosphorylation sites from the *Xenopus* cdc25C homologue does not block its ability to promote GVBD following microinjection into *Xenopus* oocytes [34]. Elimination does, however, inhibit cdc25 functional activity in cycling oocyte extracts [34]. Similarly, N-terminally truncated cdc25 activates cdc2 [33] and rescues cdc25 mutations in the fission yeast *Schizosaccharomyces pombe* [7,16].

The cdc25 catalytic domain resides in the C-terminal half of the protein, but little else is known about the biochemical functions of the cdc25 C-terminus. The crystal structures of cdc25A [36] and cdc25B [37] show that the C-terminus is disordered and extends away from the cdc25 catalytic cleft, suggesting that it is available to interact with other components, possibly substrates, in the cell.

The present study contains the surprising observation that purified forms of cdc25B missing their N-termini, are unable to induce GVBD, although they retain full cdc2-activating activity.

Abbreviations used: CDK, cyclin-dependent kinase; DTT, dithiothreitol; EKC, endopeptidase lys-C; Fmoc, fluorenyl-9-methoxycarbonyl; GST, glutathione S-transferase; GVBD, germinal vesicle breakdown; IPTG, isopropyl β -D-thiogalactoside; LB, Luria–Bertani medium; MPF, mitosis-promoting factor; NENT, Nonidet P40; EDTA, NaCl, Tris buffer; PNPP, *p*-nitrophenyl phosphate; TFA, trifluoroacetic acid.

¹ To whom correspondence should be addressed (e-mail cfisher@am.pnu.com).

The GVBD-incompetent forms of *cdc25*, and peptides derived from them, block the GVBD-promoting activity of longer forms of *cdc25B*, suggesting a direct interaction *in vivo*. The identification of a sequence in the *cdc25* C-terminus that mediates the inhibitory effect is described. Peptides from this region inhibit GVBD induced by glutathione S-transferase (GST)-*cdc25B*, and site-directed mutagenesis of critical residues in this region eliminates the ability of *cdc25B* to promote GVBD. These results suggest that this C-terminal sequence is critical to the GVBD-promoting function of *cdc25B*. The conservation of this domain among other vertebrate *cdc25* isoforms suggests that it plays an important role in cell cycle function.

EXPERIMENTAL

GST-*cdc25B* fusion proteins

The longest forms of *cdc25B* used in the present study were GST fusion proteins. Intact *cdc25B* lacking a fusion tag was not available because of degradation during preparation, due to the instability of the *cdc25B* N-terminus [14]. All forms of *cdc25B* used in the present study were derived from *cdc25B1*, a 566-amino-acid protein [7,16]. GST-*cdc25B*(31–566), encoding amino acids 31–566 of *cdc25B*, was expressed in bacteria and purified. A 2 kb *SmaI* fragment, encompassing all but 89 bp of the 5'-most region of the open reading frame of human *cdc25B* cDNA [7], was ligated into *SmaI*-cut pGEX-2T (Pharmacia). A GST fusion protein [GST-*cdc25B*(31–566)], which includes the full-length *cdc25B* protein minus the N-terminal 31 residues, was expressed in *Escherichia coli* strain JM109. Stock cultures were prepared in Luria-Bertani medium (LB) by overnight incubation at 37 °C with aeration. The cultures were then inoculated at 1:20 dilution into LB containing 100 µg/ml ampicillin, allowed to attain mid-exponential phase growth at 30 °C, and subsequently induced with 400 µM isopropyl β-D-thiogalactoside (IPTG). The temperature was reduced to 25 °C and the cultures were allowed to continue to grow overnight with aeration. The cells were collected by centrifugation, and lysed by passage through a syringe several times, following lysozyme treatment. The GST-*cdc25B* fusion proteins were purified away from the soluble portion of bacterial lysates with glutathione-Sepharose (Pharmacia) in NENT buffer [20 mM Tris (pH 8.0)/100 mM NaCl/1 mM EDTA/0.5% Nonidet P40, containing protease inhibitors 4 µg/ml aprotinin, 10 µg/ml leupeptin and 0.5 mM PMSF]. The bacterial lysates were incubated with glutathione-Sepharose, equilibrated in NENT. The beads were washed extensively, and the bound fusion proteins were eluted with 5 mM glutathione in NENT buffer. Protein concentrations, determined by the method of Bradford [38], ranged from 0.4–2.2 mg/ml.

Preparation of *cdc25B* minimal domains

cdc25B(356–556) was prepared from GST-*cdc25B*(302–566) by factor Xa cleavage of the fusion protein as described in [37]. Briefly, *cdc25B* [7] was used as a template for amplification of residues 976–1773 by PCR using the following primers containing *Bam*HI and *Xho*I restriction enzyme cleavage sites: GCG GAT CCA GCG GCT CTT CCG CTC TC (5' → 3') and GCC TCG AGT CAC TGG TCC TGC AGC CG (5' → 3'). The PCR reaction product was ligated into pCRII (Invitrogen, TA Cloning Kit), which was digested with *Bam*HI and *Xho*I. The liberated *cdc25B* fragment was purified following agarose gel electrophoresis and ligated into *Xho*I/*Bam*HI-cut pGEX-5X-3 (Pharmacia) for expression in *E. coli* strain JM109.

IPTG-induced *E. coli* cell paste (2 l) was lysed in the presence of lysozyme (1 mg/ml) and fresh dithiothreitol (DTT) (20 mM) in TEN buffer [50 mM Tris/HCl (pH 8.0)/0.5 mM EDTA/300 mM NaCl/0.2% Nonidet P40]. The lysate was centrifuged to pellet cell debris and insoluble protein, and the supernatant was collected. This solution was mixed with 20 ml of glutathione-Sepharose, which had been pre-equilibrated with factor Xa digestion buffer [50 mM Tris/HCl (pH 8.0)/100 mM NaCl/1 mM CaCl₂]. The fusion protein was purified away from *E. coli* proteins by affinity chromatography on the glutathione-Sepharose affinity column. After collection of the non-bound pool, the resin, which contained bound GST-*cdc25B*(302–566), was incubated with equilibration factor Xa digestion buffer [50 mM Tris/HCl (pH 8.0)/100 mM NaCl/1 mM CaCl₂] in the same manner as that described by Abielovich and Shlomai [39]. The eluate from this step was pooled and concentrated by ultrafiltration (Amicon YM10 filter; Millipore, Bedford, MA, U.S.A.). After Q fast-flow anion-exchange chromatography to resolve the protein product from contaminants, including the factor Xa protease, appropriate fractions were collected and concentrated by ultrafiltration (Amicon). Finally, glycerol was added to provide a 50% solution, which was then stored at –20 °C. Demonstration of homogeneity was accomplished by standard methods. Validation of the sequence was completed by N-terminal sequencing and MS to show that the product was *cdc25B*(356–556).

cdc25B(364–529) was prepared from GST-*cdc25B*(364–529) by factor Xa cleavage of the fusion protein. Cloning and expression were completed in a manner similar to that described above for GST-*cdc25B*(302–566). GST-*cdc25B*(364–529) was purified from frozen *E. coli* cell paste by glutathione-Sepharose affinity chromatography, and the product *cdc25B*(364–529) was recovered by factor Xa cleavage as described above for *cdc25B*(356–556). The protein was analysed by sequencing and MS to confirm the predicted sequence.

cdc2 kinase activation assays

Inactive, phosphorylated *cdc2* in the form of preMPF was prepared by a modification of the procedures of Cyert and Kirschner [40]. Ovaries from *Xenopus laevis* were collected and treated with 5 mg/ml bacterial collagenase (Life Technologies, Rockville, MD, U.S.A.) overnight at 16 °C. Interphase-arrested oocytes were prepared by isolation of the stage VI oocytes by reverse flow in cold 100 mM NaCl. Oocytes were lysed by vigorous pipetting in 1 × vol. extraction buffer (80 mM sodium glycerophosphate/20 mM EGTA/15 mM MgCl₂/10 mM DTT/protease inhibitors), and centrifuged at 160 000 g for 1 h. The cytoplasmic fraction was precipitated with 0.5 × vol. of saturated ammonium sulphate in extraction buffer on ice for 30 min, and precipitated material collected following centrifugation at 200 000 g for 15 min. The resulting pellet was resuspended in extraction buffer with protease inhibitors.

Activation of *cdc2*-cyclin B (preMPF) by *cdc25* was examined in the presence of an ATP-regenerating system [50 µg/ml creatine phosphokinase (Boehringer Mannheim, Indianapolis, IN, U.S.A.)/10 mM creatine phosphate (also from Boehringer Mannheim)/1 mM ATP (Sigma)]. These conditions were found to produce the maximal *cdc25*-dependent activation effect, as reported recently by others [41]. *cdc25* was added for 1 h at room temperature prior to measurement of histone H1 phosphorylation. This incubation time had been determined by a previous time-course to be optimal for *cdc2* activation. Each MPF sample was incubated after activation with an equal volume of the histone phosphorylation buffer [0.6 mg/ml histone H1

(Sigma)/0.2 mM ATP/10 μ M cAMP-dependent protein kinase inhibitor (Sigma)/approx. 5 μ Ci/assay [32 P]ATP (Amersham; approx. 6000 Ci/mmol)/40 mM Hepes/10 mM EGTA/20 mM MgCl_2 (pH 7.3)] for 10 min at room temperature. The reaction was stopped by the addition of Laemmli [42] buffer and the samples were examined following electrophoresis using SDS/PAGE (12.5% polyacrylamide) minigels. One gel was dried and exposed to X-OMAT AR X-ray film (Kodak, Rochester, NY, U.S.A.), while another was stained for 30 min in 0.05% Coomassie Brilliant Blue (Life Technologies). The histone bands were excised and counted for radioactivity in Beckman Ready Safe scintillation cocktail, while the autoradiograms were analysed on a PhosphorImager (Molecular Dynamics, Sunnyvale, CA, U.S.A.).

Recombinant, phosphorylated cdc2–cyclinB complex was purified from Sf9 cells that had been triple-infected with baculoviruses expressing cdc2, cyclin B, and Myt1 at a multiplicity of infection (m.o.i.) of 2 (virus particle:cell ratio). The cells were grown for 66 h in a rotary flask at 100 rev./min at a temperature of 27 °C. The cells were harvested by centrifugation, and the cell pellet was resuspended in extraction buffer [10 mM Hepes/NaOH (pH 7.4)/10 mM NaCl/1 mM EDTA/0.1 mM PMSF/20 μ g/ml aprotinin/1 μ g/ml leupeptin/1 μ g/ml soyabean trypsin inhibitor]. The suspension was incubated on ice for 30 min and then sonicated five times for 15 s at 0 °C with a Branson Sonifier 450. The NaCl concentration was then increased to 50 mM, the sonicate centrifuged at 12000 *g* for 30 min in a SS34 rotor at 4 °C, and the supernatant was loaded onto a Q-Sepharose column (1.6 cm $\times$ 10 cm). The column was washed with at least five column volumes of equilibration buffer [10 mM Hepes/NaOH (pH 7.4)/10 mM NaCl/1 mM EDTA]. Bound proteins were eluted with consecutive linear NaCl gradients in equilibration buffer (0.01–0.4 M with a volume of 250 ml, followed by 0.4–1 M with a volume of 20 ml). A final wash with 30 ml of 1 M NaCl was used to ensure complete dissociation of bound protein. Absorbance was monitored at 280 nm and 4 ml fractions were collected at a flow rate of 4 ml/min. The column fractions were run on duplicate SDS/12% polyacrylamide gels, transferred to Immobilon P transfer membranes (Millipore), and probed with antibodies against cyclin B and phosphotyrosine. The peak fractions were pooled based on Western blots, concentrated, and the protein concentration was determined by using the Bradford assay.

The Myt1-inhibited cdc2 complex (50 ng) was preincubated with equimolar amounts of either GST–cdc25B(31–566) (30 ng) or cdc25B(356–556) (7.565 ng) in 35 μ l of phosphatase buffer [20 mM Tris/HCl (pH 8.3)/150 mM NaCl/5 mM EDTA/5 mM DTT/0.1% (w/v) Triton X-100] at room temperature for 0, 2, 4, 6, 8, 10, 15, 30 and 60 min. Phosphatase inhibitor (0.5 μ l of 50 mM Na_3VO_4) was added to stop the reaction on ice prior to the kinase assay. Kinase activity was measured following the addition of 2.5 μ l of 2 mCi/ml [γ - 32 P]ATP and 32.5 μ l of histone H1 kinase buffer [0.6 mg/ml histone H1/0.2 mM ATP/40 mM Hepes/10 mM EDTA/20 mM MgCl_2 , pH 7.3] per assay for 17 min at room temperature. The reaction was stopped by adding 70 μ l of 2 $\times$ SDS sample buffer. Phosphorylation of histone was analysed following electrophoresis on SDS/15% acrylamide gels followed by autoradiography and PhosphorImager analysis.

Phosphatase assay and kinetic analyses

Assays of *p*-nitrophenyl phosphate (PNPP) hydrolase activity associated with cdc25B were conducted in a manner similar to that described by Horiguchi et al. [43]. The assay was conducted

in a volume of 125 μ l which included 25 mM Hepes, 10 mM DTT, 0.1 mg/ml BSA and variable concentrations of PNPP (pH 8.0). For assays where a single concentration of substrate was used at saturation, a final concentration of 20 mM PNPP was used. For the determination of K_m and V_{max} , multiple PNPP concentrations were used at a constant enzyme concentration. Rates at each concentration of substrate were determined and the K_m and V_{max} were calculated by line fitting to the Michaelis–Menten equation.

Peptides

Stepwise, solid phase peptide synthesis was performed on an ABI 430A peptide synthesizer (Applied Biosystems, Foster City, CA, U.S.A.) or an Advanced Chemtech ACT357 peptide synthesizer. The fluoren-9-ylmethoxycarbonyl (Fmoc) group was used as the N-amino protecting group. Temporary side-chain-protecting groups were Arg(2,2,5,7,8-pentamethylchroman-6-sulphonyl), Glu(*t*-butyl ester), Ser(*t*-butyl), Thr(*t*-butyl) and Trp(*t*-butyloxycarbonyl). Each residue was single-coupled using a 2-(1-*H*-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate/1-methyl-2-pyrrolidinone (HBTU/NMP) protocol [43a]. For the ACT357, each residue was double-coupled, first with diisopropylcarbodiimide/NMP and then with HBTU/NMP. After removal of the N-terminal Fmoc group, temporary side-chain-protecting groups were removed and the peptide was cleaved from the resin by treatment with 95% trifluoroacetic acid (TFA)/5% scavengers (ethyl methylsulphide/anisole/1,2-ethanedithiol, 1:3:1, by vol.) for 2 h at room temperature. The crude peptide was precipitated from the cleavage solution with cold diethyl ether. The precipitated peptide was collected on a sintered glass funnel, washed with diethyl ether, dissolved in dilute acetic acid and freeze-dried. The crude peptide was purified by preparative reverse-phase chromatography on a Vydac C_{18} column (27.5 mm $\times$ 250 mm) using a water/acetonitrile gradient, each phase containing 0.1% TFA. Clean fractions, as determined by analytical HPLC, were pooled and the acetonitrile was evaporated under reduced pressure; the aqueous solution was then lyophilized. The purified peptide was characterized by electrospray ionization MS. Each of the peptides was reconstituted in water prior to testing.

Oocyte injections

Stage VI *Xenopus laevis* oocytes were isolated for injection by treatment of ovary follicles with 2 mg/ml collagenase (Sigma) in Ca^{2+} -free ND 96 buffer [5 mM Hepes/96 mM NaCl/2 mM KCl/1 mM MgCl_2 /2.5 mM sodium pyruvate (Life Technologies), pH 7.5] for 30 min at 16 °C, after which individual oocytes were manually removed from their follicular sheaths. The oocytes were allowed to recover for 2–18 h in ND 96 (with 1.8 mM CaCl_2) after which damaged cells were removed.

The oocytes were rinsed three times in Ca^{2+} -free ND 96 medium before injection. The injected oocytes (10–15 oocytes per test sample) were incubated in ND 96 at 19 °C and periodically scored for GVBD. Oocytes were generally injected with 16 ng of GST–cdc25B(31–566) in a volume of 46 nl. When proteins and peptides were tested for GVBD inhibitory activity, the peptide was mixed 1:1 with GST–cdc25B(31–566) prior to injection and 92 nl was injected. For comparison between experiments, % control GVBD refers to data normalized to induction of GVBD promoted by microinjection of GST–cdc25B(31–566). Results expressed as % GVBD represent the actual counts.

Concentrations given for the peptides indicate the molarity at which they were injected. Since the final volume injected (46 nl) for each test solution is approx. 5% of the total volume of the

Figure 1 cdc25B promotes GVBD in *Xenopus* oocytes following micro-injection

(A) Oocyte maturation curve plotting GVBD induced by GST-cdc25B(31-566). GVBD was completed by 2 h following microinjection. (B) GST alone has little or no effect on GVBD. Cycloheximide does not inhibit GVBD induced by GST-cdc25B(31-566) indicating a lack of dependence on protein synthesis.

oocytes, these concentrations are approx. 20 times greater than the final concentrations within the oocytes following injection. Solutions of water, various buffers, GST, and inhibitors alone were injected as negative controls, and were found to induce little or no GVBD.

Site-directed mutagenesis of cdc25B

A two-step PCR protocol was used to introduce codon changes in cdc25B for amino acid residues Ser-549 and Glu-553. The changes resulted in a change to alanine in both cases. Two overlapping PCR products were generated for each desired mutation in the first round of PCR. The first product contained the desired mutation in the antisense 3' primer and included the endogenous cdc25B *Eco*RI site in the sense 5' primer. The second product contained the mutation in the sense 5' primer and

Figure 2 A minimal cdc25B domain [cdc25B(356-556)] activates MPF histone kinase activity *in vitro* but does not promote GVBD

GST-cdc25B(31-566) promotes GVBD whereas cdc25B(356-556) does not, although both polypeptides activate MPF *in vitro*. Higher concentrations of cdc25B(356-556) did not improve its effectiveness. The concentrations given on the Figure are for the material that was injected. The final intracellular concentrations are approx. one-twentieth of the injected material. The inset to the figure shows that MPF is activated following addition of GST-cdc25B(31-566) and cdc25B(356-556). 'None' refers to MPF sample receiving no cdc25B.

included the cdc25B stop codon and an additional *Eco*RI site in the antisense 3' primer. The overlapping PCR products were then annealed and amplified in the second round of PCR, creating a mutated fragment of cdc25B that included sequence from the endogenous *Eco*RI site through to the stop codon. These secondary PCR products were subcloned into pCR2.1 using the TA cloning kit (Invitrogen, Carlsbad, U.S.A.). Clones containing the insert were sequenced to confirm the presence of the appropriate mutation using Amersham's Sequenase 2.0 sequencing kit (Arlington Heights, IL, U.S.A.). After confirmation of the sequence, *Eco*RI-digested fragments and cdc25B(31-566)/pGEX-2T were ligated together, replacing the endogenous cdc25B sequence with the mutated sequence. Orientation of the clones was confirmed, and several clones were examined to confirm expression of the mutant protein with the appropriate mass.

Combinations of PCR primers (Genosys, The Woodlands, TX, U.S.A.) were used (in each case, the antisense primer is listed first).

The *Eco*RI site-containing primers used were: cdc25B-5R1, 5'-GGAATTCTCACTGGTCCTGCAGCC; cdc25B-23R1, 5'-GGAATTCTCATCTGAGCGTGGGC.

The site-specific mutation primers (with bold nucleotides indicating a change from the endogenous sequence) used were: S549A-1, 5'-AGCCCAGGCGCGAGTCTTGAGGCG; S549A-2, 5'-AAGACTCGCGCCTGGGCTGGGGAGC; E553A-1, 5'-GGCTCCGCGCCCCAGCCAGCTGC; E553A-2, 5'-GGCTGGGGCGCGGAGCCGGCGGGA.

RESULTS

Truncated versions of cdc25B do not promote GVBD but activate cdc2 *in vitro*

Microinjection of 16 ng (approx. 4 μ M) of GST--cdc25B(31–566) from different preparations into *Xenopus* oocytes consistently promoted GVBD in more than two-thirds of the oocytes within 2 h (Figure 1A). GVBD was not induced by GST alone (Figure 1B), nor was it inhibited by 0.5 μ g/ml cycloheximide, indicating that protein synthesis is not required for cdc25-dependent maturation. These data are consistent with GVBD promotion by cdc25 through a direct effect on cdc2.

A truncated version of cdc25B containing the catalytic domain and spanning amino acid residues 356–556 [cdc25B(356–556)] did not induce GVBD. Various concentrations of cdc25B(356–556), ranging from equimolar concentrations to amounts far exceeding the effective molar concentration of GST--cdc25B(31–566), did not induce GVBD following microinjection (Figure 2). These results were surprising because both proteins are apparently equivalent in their ability to activate MPF *in vitro* (inset to Figure 2). Identical results were obtained with human cdc25C (results not shown).

Figure 3 cdc25B(356–556) is equivalent to GST--cdc25B(31–566) in activation of cdc2

Activation of purified, Myt1-inhibited cyclin B–cdc2 complex by GST--cdc25B(31–566) and cdc25B(356–556) was examined over time using equivalent amounts of cdc25 (5 nM). GST--cdc25B(31–566) and cdc25B(356–556) showed a similar time course of cdc2 activation, with cdc25B(356–556) giving the more rapid activation of the two enzymes. The lower panel depicts the quantification of the autoradiographic data by PhosphorImager analysis.

Figure 4 Minimal cdc25B proteins, unable to promote GVBD, inhibit GVBD when co-injected with GST--cdc25B(31–566)

Several truncated versions of cdc25B were examined for their ability to inhibit the GVBD induced by full-length GST--cdc25B(31–566). cdc25B(356–556), GST--cdc25B(405–566) and peptides derived from the minimal domain inhibited the GVBD-inducing activity of the full-length protein. The concentrations shown are those of the starting material and the final intracellular concentrations were approx. one-twentieth that of the starting material.

Kinetic comparisons of GST--cdc25B(31–566) and cdc25B(356–556) activity using the phosphatase substrate PNPP demonstrated that the shorter version possesses a lower K_m than GST--cdc25B(31–566). The K_m of cdc25B(356–556) was 5–6 mM, in contrast with > 12 mM for GST--cdc25B(31–566). cdc25B(356–556) was also equivalent, or superior, to GST--cdc25B(31–566) in its activation of the purified Myt1-phosphorylated cyclinB–cdc2 complex (Figure 3). These results are surprising because they indicate that microinjected cdc25B(356–556) is unable to induce GVBD, even though it retains the ability to activate cdc2 *in vitro*. Furthermore, the lack of GVBD-promoting activity is not attributable to increased degradation of cdc25B(356–556), since the protein was recoverable from the oocytes several hours after microinjection (results not shown).

Truncated versions of cdc25B inhibit GVBD induced by the full-length protein

Since cdc25B(356–556) is not competent to induce GVBD, its effects upon promotion of GVBD by functionally competent forms of the protein is of interest. Interestingly, co-injection of cdc25B(356–556) with GST--cdc25B(31–566) inhibited GVBD induction (Figure 4). This observation, reproduced in numerous experiments, suggests a direct interaction between these proteins *in vivo*. Other truncated versions of cdc25B, which failed to promote GVBD by themselves, also inhibited the GVBD-inducing activity of GST--cdc25B(31–566). GST--cdc25B(405–566) inhibited GVBD induction, but cdc25B(364–529) did not (Figure 4 and Table 1) suggesting that an inhibitory sequence is

Table 1 Summary of numerous experiments investigating the biological activity of purified cdc25B following microinjection into *Xenopus* oocytes

cdc25B1 Form		GVBD Induction	Inhibition of GST-cdc25B(31-566) Induced GVBD
GST-cdc25(31-566)		Yes	—
cdc25(356-556)		No	Yes
cdc25(364-529)		No	No
GST-cdc25(405-566)		No	Yes
EKC Digested cdc25(356-566)		No	Yes
TRSWAGERSR		No	Yes

Figure 5 The cdc25B peptide TRSWAGERSR inhibits cdc25B-dependent GVDB

(A) Peptide TRSWAGERSR inhibited induction of GVBD by GST-cdc25B(31-566) to a similar extent as cdc25B(356-556). The concentrations of material injected were 200 μM cdc25B(356-556) and 100 μM TRSWAGERSR, with a final intracellular concentration of approx. one-twentieth of this starting concentration. (B) Inhibition of GVBD by TRSWAGERSR was concentration-dependent. The concentrations shown are those of the starting material and the final intracellular concentrations were approx. one-twentieth of the starting concentration.

located in the cdc25B C-terminus. Data from numerous experiments are summarized in Table 1.

Identification of a cdc25B peptide that inhibits cdc25B *in vivo*

A mixture of peptides produced by cleavage of the cdc25B minimal domain with endopeptidase lys-C (EKC) also showed GVBD inhibitory activity (Figure 4 and Table 1). Peptides were

Figure 6 An alanine-substitution scan of TRSWAGERSR reveals residues critical for inhibition of cdc25B-induced GVBD

Each of the amino acid residues of TRSWAGERSR was substituted with alanine (shown by A in bold), and the resulting peptides were tested for their ability to inhibit GVBD induced by GST-cdc25B(31-566). Three residues were critical (Arg-3, Glu-7 and Arg-10), three N-terminus residues were important (Thr-1, Arg-2 and Trp-4), and residues Gly-6, Arg-8 and Ser-9 were unimportant in maintaining the inhibitory nature of the peptide. The results are shown as mean % control GVBD, with the range as the error shown.

then synthesized systematically from the C-terminus using knowledge of EKC specificity for lysine residues to dictate peptide size. A C-terminal peptide (TRSWAGERSR) reproducibly inhibited GVBD when co-injected with GST-cdc25B(31-566) (Figure 5A). A dose-response curve indicates that 50% inhibition is achieved with a final intracellular concentration of approximately 5 μM TRSWAGERSR (Figure 5B).

A set of peptides, in which each residue of TRSWAGERSR was substituted with an alanine residue, was then prepared and tested at a final intracellular concentration of approx. 5 μM. Three peptide residues were found to be critical for inhibitory activity (Figure 6). Alanine substitutions for these residues (peptide residues Ser-3, Glu-7 and Arg-10) resulted in the loss of 80% or more of the inhibitory activity of TRSWAGERSR. Alanine substitutions for another set of amino acids (peptide residues Thr-1, Arg-2 and Trp-4), clustered towards the N-terminal end of the peptide, also resulted in reduced inhibitory

HS cdc25B	K	T	R	S	W	A	G	E	R	S	R	R	E
M cdc25B	K	T	R	S	W	A	G	E	R	S	R	R	E
HS cdc25A	K	S	R	T	W	A	G	E	K	S	K	R	E
M cdc25A	K	S	R	T	W	A	G	E	K	S	K	R	E
XL cdc25A	K	S	R	T	W	A	G	E	R	S	K	R	E
HS cdc25C	Q	S	K	V	Q	E	G	E	R	Q	L	R	E
M cdc25C	Q	S	K	A	Q	E	G	E	R	Q	L	Q	G
XL cdc25C	K	C	K	T	S	V	G	D	R	K	R	R	E

Figure 7 Alignment of conserved vertebrate (human, mouse and frog) cdc25 N-terminal regions containing the TRSWAGERSR sequence

Non-conservative amino acid changes are boxed and shaded grey, and the two residues of cdc25B selected for site-directed mutagenesis are underlined. High amino acid sequence similarity exists among cdc25 isoforms, and among vertebrate species with the highest degree of conservation shared between cdc25A and cdc25B. HS, *Homo sapiens*; M, mouse; XL, *Xenopus laevis*.

Table 2 Site-directed mutation of TRSWAGERSR residues eliminates GVBD-promoting ability

Xenopus oocytes were injected with equimolar amounts of GST-cdc25B(31–566), GST-cdc25B(E553A), and GST-cdc25B(S549A) and scored for GVBD. Other experiments utilized higher amounts of the mutant proteins, but yielded identical results. Both forms of cdc25B carrying mutations of critical residues within the TRSWAGERSR sequence did not induce GVBD.

Protein Injected	GVBD/Total*	% GVBD
GST-cdc25B(31–566)	9/12	75
GST-cdc25B(E553A)	0/12	0
GST-cdc25B(S549A)	0/11	0

* Total no. of oocytes that had undergone GVBD 3 h after injection.

activity (Figure 6). In contrast, substitutions at Gly-6, Arg-8 and Ser-9 resulted in little change in peptide inhibitory activity.

Site-directed mutagenesis of cdc25B TRSWAGERSR domain eliminates GVBD inducing activity

A comparison of the TRSWAGERSR sequence among the three known vertebrate homologues from various species shows that it is highly conserved (Figure 7), particularly between cdc25A and cdc25B from various species. The sequence is 100% conserved in the *Xenopus* cdc25A homologue. Comparison with the frog cdc25B homologue is not possible as its sequence has not yet been published. We subsequently changed residues Ser-549 and Glu-553 (corresponding to residues Ser-3 and Glu-7 of TRSWAGERSR) of GST-cdc25B(31–566) to alanine by site-directed mutagenesis. The resulting fusion proteins activated cdc2 *in vitro* demonstrating that their catalytic function *in vitro* is not altered (results not shown). However, both mutant fusion proteins failed to induce GVBD, while the parent GST-cdc25B(31–566) protein, prepared in parallel, exhibited typical GVBD-inducing activity (Table 2). Higher concentrations of the mutant proteins also failed to induce GVBD (results not shown). These results directly demonstrate an important role for the TRSWAGERSR domain in cdc25B function *in vivo*.

DISCUSSION

The present study provides evidence that truncated versions of cdc25B containing the catalytic domain are insufficient for inducing GVBD. This is surprising, considering that the truncated cdc25B proteins retain phosphatase activity and activate cdc2. Previously published studies demonstrated that the C-terminus of cdc25, containing the consensus phosphatase domain, is

sufficient to activate cdc2 in oocyte extracts, and induce mitosis and complement cdc25 in yeast [7,16]. Others have shown that cdc25 is activated by cdc2 phosphorylation [8–10]. Mutation of cdc2 phosphorylation sites, however, is insufficient for inhibiting GVBD induction following microinjection (although it reduces the ability of cdc25 to promote mitosis in oocyte extracts) [34]. Our results provide evidence that the cdc25 N-terminus is required for induction of GVBD, but is not required for activating cdc2 *in vitro* as determined by several criteria. These criteria include activation of MPF isolated from *Xenopus* oocytes, activation of purified Myt1-inhibited recombinant cdc2–cyclinB complex, and kinetic comparisons made with versions of cdc25B that contain the N-terminus and are competent for promoting GVBD.

The *Xenopus* oocyte model has proved to be useful in studies of the role of cdc25 in cell cycle progression. Full-length forms of human cdc25, as well as cdc25 from several other species, promote GVBD in this model [8,32–35]. *Xenopus* homologues of two of the three mammalian cdc25s (cdc25A and cdc25C) have been identified to date, and both promote oocyte maturation [8,34,35,44]. Obviously the mechanism of GVBD promotion, as well as the CDK specificity, is highly conserved among diverse cdc25 types. For this reason we believe that the C-terminal domain described here has important implications for function in all isoforms of cdc25. The high degree of conservation of TRSWAGERSR among cdc25 isoforms is consistent with this belief.

cdc25B(356–556) (and other GVBD-incompetent forms of cdc25) interferes with GVBD promotion by GST-cdc25B(31–566) suggesting a direct interaction within the oocyte. For this reason we do not favour the hypothesis that loss of the N-terminus alters the subcellular distribution or substrate recognition of the microinjected protein as an explanation for loss of function *in vivo*. Because GST-cdc25B(405–566) also inhibited cdc25B-induced GVBD, whereas cdc25B(364–529) did not, we focused our search for the inhibitory sequence element within the cdc25B C-terminus. The synthetic C-terminal peptide TRSWAGERSR (cdc25B amino acids 547–556) inhibits GVBD in the low micromolar range, suggesting that it is mediating the inhibitory effect of the cdc25 minimal domain. The concentration of peptide required for inhibition in the present study is equivalent to, or less than, that required for inhibition of GVBD in other studies, where GTPase-activating protein peptides were used to inhibit ras-mediated GVBD [45,46].

The TRSWAGERSR peptide does not inhibit activation of MPF by cdc25 *in vitro*, suggesting that it does not simply compete for binding to the cdc25 substrate *in vitro*. Likewise, substitution of alanine for residues within TRSWAGERSR in cdc25B does not produce a measurable effect on enzymic activity *in vitro*, but dramatically inhibits the GVBD-inducing function of the protein *in vivo*. For this reason we believe that the TRSWAGERSR sequence is required for interaction with cellular components, which may include cdc2 in a supramolecular complex *in vivo*, and that are not required for cdc2 activation *in vitro*. However, it is difficult to directly test whether the TRSWAGERSR sequence influences cdc2 activation *in vivo*, because activation of cdc2 may occur following lysis of microinjected oocytes. Nevertheless, experiments conducted to date indicate that the TRSWAGERSR peptide prevents activation of cdc2 *in vivo* when co-injected with cdc25B. Experiments designed to introduce TRSWAGERSR into mammalian cells via addition of a signal sequence have failed to successfully demonstrate a cell cycle blockade. At present, we are unsure if this negative result is due to a failure in peptide delivery or to a lack of peptide activity in these cells.

Since the removal of the cdc25 N-terminus results in loss of GVBD-inducing activity, it is unlikely that that it plays an inhibitory role in this system. cdc25 is regulated in a variety of ways through its highly phosphorylated N-terminus. CDKs activate cdc25 via phosphorylation, an event believed to contribute to a CDK amplification loop that ensures cell cycle progression [8–10]. CDK phosphorylation also regulates cdc25 binding to the peptidyl-prolyl *cis-trans* isomerase Pin1 via a Trp-Trp domain [47–49]. cdc25 phosphorylation by the checkpoint regulatory kinases checkpoint kinase 1 and checking DNA synthesis 1 also has pleiotropic effects. These include direct inhibition of cdc25 activity [23], and creation of 14-3-3 phosphoserine-binding sites, which inhibit cdc25 function and cell cycle transition [20–23]. This is apparently accomplished by 14-3-3 protein binding resulting in nuclear exclusion [24,25]. Our results are most consistent with an activating role for the N-terminus in regulating cdc25-dependent GVBD, since its removal clearly eliminates GVBD-inducing activity.

Out of the ten TRSWAGERSR residues, three were essential for maintaining the inhibitory activity of the peptide. To directly test whether these residues are important for function in the full-length protein, we substituted two of the amino acids in GST-cdc25B(31–566). GST-cdc25B(S549A) and GST-cdc25B(E553A) both failed to initiate GVBD following micro-injection, even though both mutant proteins activated cdc2 *in vitro*. The role of the C-terminal region of cdc25B in mediating GVBD is unknown, but its close proximity to the cdc25 catalytic domain indicates that it may play a role in substrate binding or recruitment. Another possibility is that the C-terminus plays a regulatory role *in vivo*, where it mediates the interaction of cdc25B with cdc2, either directly or through other interacting proteins or complexes.

Structural studies of cdc25 may help elucidate the function of the C-terminus. The solution of the cdc25A [36] and cdc25B [37] crystal structures has provided an insight into the function and catalytic mechanism of the enzyme. The recombinant, truncated proteins used for crystallization of both proteins included the TRSWAGERSR domain, but the C-terminus, including the sequence of interest, was disordered and was therefore not mapped in the structure [36,37]. The disordered C-terminus is closely associated with the catalytic cleft, but is presumably freely available for interaction with other molecules, since it extends away from the main body of the folded protein. For this reason we believe that the cdc25 C-terminus may play an important role in regulating interaction with other components, and perhaps substrates, within the cell.

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