Oncogene (1999) 18, 4047–4054

http://www.stockton-press.co.uk/onc

Mammalian Chk2 is a downstream effector of the ATM-dependent DNA damage checkpoint pathway

Priya Chaturvedi[1], Wai K Eng[1], Yuan Zhu[2], Michael R Mattern[1], Rubin Mishra[1], Mark R Hurle[3], Xiaolong Zhang[4], Roland S Annan[4], Quinn Lu[5], Leo F Faucette[1], Gilbert F Scott[6], Xiaotong Li[2], Steven A Carr[4], Randall K Johnson[1], James D Winkler[1] and Bin-Bing S Zhou*[,1]

Departments of [1]Oncology Research, [2]Molecular Biology, [3]Bioinformatics, [4]Physical and Structural Chemistry, [5]Gene Expression Sciences, [6]Protein Biochemistry, SmithKline Beecham Pharmaceuticals, 709 Swedeland Road, King of Prussia, Pennsylvania, PA 19406, USA

In response to DNA damage and replication blocks, cells activate pathways that arrest the cell cycle and induce the transcription of genes that facilitate repair. In mammals, ATM (ataxia telangiectasia mutated) kinase together with other checkpoint kinases are important components in this response. We have cloned the rat and human homologs of *Saccharomyces cerevisiae* Rad 53 and *Schizosaccharomyces pombe* Cds1, called checkpoint kinase 2 (*chk2*). Complementation studies suggest that Chk2 can partially replace the function of the defective checkpoint kinase in the Cds1 deficient yeast strain. Chk2 was phosphorylated and activated in response to DNA damage in an ATM dependent manner. Its activation in response to replication blocks by hydroxyurea (HU) treatment, however, was independent of ATM. Using mass spectrometry, we found that, similar to Chk1, Chk2 can phosphorylate serine 216 in Cdc25C, a site known to be involved in negative regulation of Cdc25C. These results suggest that Chk2 is a downstream effector of the ATM-dependent DNA damage checkpoint pathway. Activation of Chk2 might not only delay mitotic entry, but also increase the capacity of cultured cells to survive after treatment with γ-radiation or with the topoisomerase-I inhibitor topotecan.

Keywords: Chk2; ATM; cdc25C; γ-radiation; hydroxyurea; topotecan;

Introduction

The integrity of chromosomal DNA is under constant surveillance during the cell cycle. In eukaryotic cells, when chromosomes become damaged or cannot be replicated completely, the cell cycle is arrested and the transcription of a set of genes that facilitate DNA repair is induced (Hartwell and Weinert, 1989; Murray *et al*., 1995; Elledge, 1996). Defects in these mechanisms of surveillance and arrest, known as DNA checkpoints have been linked with disease. In response to DNA damage, cells from humans with the recessive disorder ataxia telangiectasia (AT) fail to undergo the normal reduction in rate of DNA synthesis and mitotic delay (Painter and Young, 1980). In addition, AT cells are deficient in recovering from normally sublethal radiation damage, indicating defects in repair mechanisms (Weichselbaum *et al*., 1978). The gene that is mutated in AT was identified in 1995 and named ATM (AT mutated) (Savitsky *et al*., 1995).

ATM belongs to a distinct subfamily of kinases that are structurally and functionally conserved among eukaryotes, including mammals, *D. melanogaster*, *S. pombe* and *S. cerevisiae*, and share homology with phosphoinositide kinases (Elledge, 1996). While little is known about the pathway downstream of ATM in mammals, the DNA damage checkpoint pathway in yeast is better understood. In *S. cerevisiae* MEC1 is closely related to ATM and in *S. pombe* Rad3 is the ATM homolog (Savitsky *et al*., 1995; Bentley *et al*., 1996; Cimprich *et al*., 1996). These proteins appear to be involved in majority of checkpoint functions. MEC1 and Rad3 are required for both the transcriptional response and G1, S phase, and G2 arrest following DNA damage. In response to DNA damage and replication blocks, the Rad53 protein kinase is phosphorylated and activated in a MEC1-dependent manner (Sanchez *et al*., 1996; Sun *et al*., 1996). Mutations in Rad53 result in both defective transcriptional induction and cell cycle arrest (Allen *et al*., 1994; Weinert *et al*., 1994). The protein kinase Cds1 has 70% sequence similarity to the Rad53 protein, and is required for survival during cell cycle arrest induced by the ribonucleotide reductase inhibitor hydroxyurea (HU) (Murakami and Okayama, 1995). In *S. pombe*, cell cycle arrest by DNA damage requires the function of Chk1 protein kinase (Walworth *et al*., 1993). Both Cds1 and Chk1 are known to function downstream of Rad3 (Walworth and Bernard, 1996; Lindsay *et al*., 1998; Boddy *et al*., 1998). Thus, possible downstream effectors of ATM include mammalian homologs of *S. cerevisiae* Rad53, *S. pombe* Cds1 and (or) Chk1. Recently, human *chk1* was cloned and demonstrated to phosphorylate Cdc25C on serine-216, which is involved in negative regulation of Cdc25C (Sanchez *et al*., 1997; Peng, *et al*., 1997). It is unclear, however, whether Chk1 functions downstream of ATM.

Our studies of ATM−/− cells treated with γ-radiation and the topoisomerase-I inhibitor topotecan indicate the important role of ATM in the survival pathway and suggest the role of downstream checkpoint kinases as mediators of ATM function. We describe here the cloning of the rat and human homologs of *cds1* and *rad53* which have been named

*Correspondence: B Zhou, tel: (610) 270-6638; fax: (610) 270-5005; email: Bin-Bing_S_Zhou@sbphrd.com
Received 16 February 1999; revised 24 March 1999; accepted 7 April 1999

chk2 (Matsuoka *et al*., 1998) and show that Chk2 is an effector kinase downstream from ATM in the DNA damage checkpoint pathway. During the preparation of this manuscript, similar findings concerning mammalian Chk2/Cds1 (Matsuoka *et al*., 1998; Blasina *et al*., 1999) were reported. Our studies show that mammalian Chk2 can not only complement growth defects of *S. cerevisiae* Rad53 deficient strain (Matsuoka *et al*., 1998), but can also complement the checkpoint defects of an *S. pombe* Cds1 deficient strain. One intriguing and novel observation from this study is that mammalian Chk2 can be activated by the replication checkpoint in addition to the DNA damage checkpoint, but this activation is independent of ATM.

Results

ATM dependent survival pathway

Molecular details of the ATM pathway that determine the fate of cells which have sustained DNA damage are only just beginning to be understood. Surveillance of damage, cell cycle checkpoints, and DNA damage repair would be expected to increase chances of survival. To determine further the conservation between yeast and human in the DNA damage checkpoint and demonstrate the relevance of the ATM pathway to current cancer therapy, we compared the capacities of ATM-proficient (MRC-5) and ATM-deficient (AG04405) human fibroblasts to survive after treatments with topotecan and γ-radiation. Topotecan is an anticancer drug that transforms topoisomerase I-DNA transient intermediates into lethal DNA lesions, and has been suggested to activate a DNA damage checkpoint (Ryan *et al*., 1994; Kingsbury *et al*., 1991). Both ATM+/+ and ATM−/− cells were treated with increasing concentrations of topotecan (0.02–3 μM) and their viability was measured as explained in Materials and methods. As shown in Figure 1A, ATM-proficient cells (topotecan $IC_{50}=2$ μM) survived low concentrations of topotecan that inhibited the growth of ATM-deficient cells ($IC_{50}=0.3$ μM). Weichselbaum *et al*. (1978) have previously demonstrated the hypersensitivity of ATM-deficient cells to DNA damage induced by ionizing radiation. Moreover, ectopic expression of recombinant ATM in ATM deficient cells restores normal sensitivity to radiation in these cells (Ziv *et al*., 1997). To confirm that our experimental ATM-deficient cells had the anticipated hypersensitivity to DNA damage, their viability after exposure to ionizing radiation was determined. In comparison with repair-proficient diploid human fibroblasts (MRC-5), ATM−/− fibroblasts (AG04405) were 15-fold more sensitive to γ-radiation, as judged by reduction in growth to 50% of control (about 11 rads for ATM deficient cells and about 165 rads for ATM proficient cells) (Figure 1B). Thus the ATM pathway contributes to increased cellular resistance to some forms of DNA damage, similar to MEC1 and Rad3 pathways in yeast (Elledge, 1996). However, this ATM pathway does not increase cellular resistance to replication blocks caused by HU, as both ATM+/+ (MRC-5) and ATM−/− cells (AG04405) showed very similar sensitivities to HU killing (Figure 1C). Similar results were obtained using untransformed human fibroblast WI-38 as ATM+/+ cells and a different AT cell line CRL-7201 as ATM−/− cells (data not shown). Although the hypersensitivity of ATM−/− cells to γ-radiation is correlated with their defects in cell cycle checkpoints after irradiation (Kastan *et al*., 1992; Barlow *et al*., 1996), the molecular mechanism is poorly understood.

Identification of Chk2 as the mammalian homolog of S. cerevisiae *Rad53 and* S. pombe *Cds1*

To identify the novel DNA damage checkpoint kinase in mammals that might be the downstream effector of ATM, we searched for mammalian homologs of known yeast checkpoint kinases. Using the sequences of *cds1* and *rad53*, we performed blast searches against the expressed sequence tag (EST) database. An EST, AI030402, with significant sequence homology to the forked head-associated (FHA) domain (Hofmann and Bucher *et al*., 1995) of Cds1 and Rad53, was found. Figure 2A shows that EST AI030402 falls within the grouping made by several FHA-domain-containing kinases including Cds1 and Rad53. This grouping indicates the

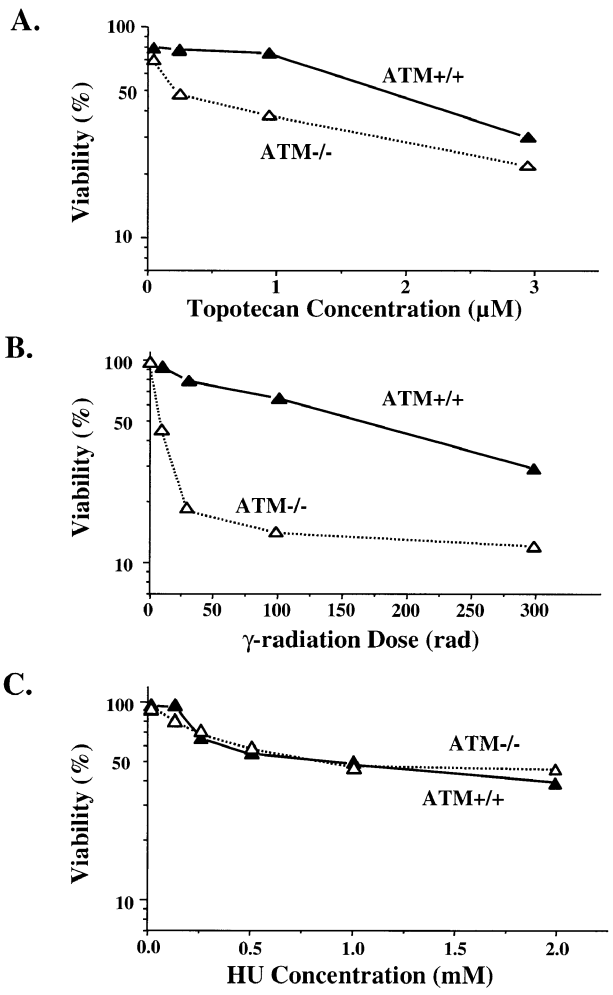

Figure 1 Inhibition of growth of fibroblasts cultured from a normal donor (ATM+/+ cells, MRC-5, ▲) and from a donor with Ataxia Telangiectasia (ATM−/− cells, AGO4405, △) by (**A**) topotecan (**B**) γ-radiation or (**C**) HU. Growth inhibition was determined as described in Materials and methods

homology in FHA domain is significant and EST AI030402 is part of a mammalian homolog of Cds1 and Rad53. We employed the EST to clone the full length rat homolog of these genes using the Marathon cDNA amplification kit (Clontech, CA, USA). The full length gene was cloned and named rat *chk*2. Rat *chk2* sequence has been deposited into GENBANK (accession number AF134054). Using the rat *chk2* sequence information, the human homolog was cloned. The rat *chk2* was found to be 86% identical to the human *chk2*. The sequence homology is strongest (more than 90%) in FHA (Figure 2B) and kinase domains. Rat Chk2 has 546 amino acids; the FHA domain includes amino acids 116–197, and the kinase domain includes amino acids 220–488. Mammalian Chk2 shows the same overall structure as Cds1 in that it lacks the long carboxyl-terminal extension found in Rad53. Northern blot analysis using RNA multi-tissue blots revealed that, in rat and mouse, the level of chk2 expression was the highest in spleen (data not shown), whereas in human tissues, it was the highest in testis (Matsouka *et al*., 1998; Blasina *et al*., 1999). Chk2 was also expressed at high levels in eight different human cancer cell lines examined (data not shown; Blasina *et al*., 1999).

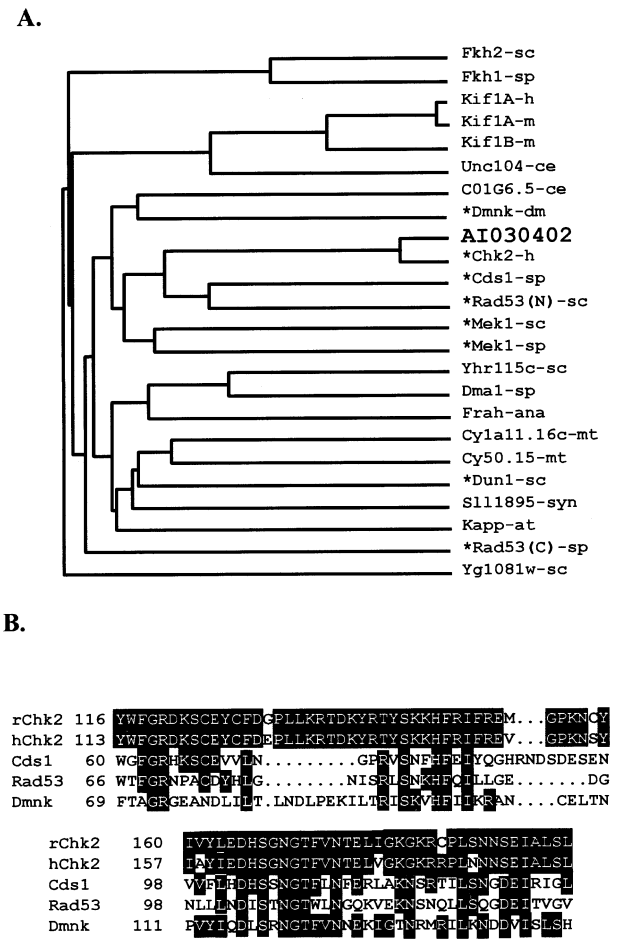

Figure 2 (**A**) Dendrogram showing the relationship of FHA domains for all known FHA containing kinases (signified by *), all FHA-containing proteins in SwissProt, and rat EST AI030402. Dendrogram was generated using the UPGMA clustering method in PILEUP (Wisconsin Package Version 8.1, Genetics computing Group, Madison, WI, USA). (**B**) Alignment of Chk2 homologs in the FHA domains. Identical amino acids are shown as black boxes

Mammalian chk2 can complement the phenotype of S. pombe cds1 *deletion*

HU can activate a replication checkpoint causing cells to arrest in the S phase. Contrary to the wild-type *S. pombe* cells, cells lacking *cds1* (Δ*cds1*) are highly sensitive to killing by HU; this sensitivity can be reversed by transfecting the null cells with wild-type *S. pombe cds1* (Murakami and Okayama, 1995). To determine whether mammalian Chk2 is a functional homolog of *S. pombe* Cds1, we expressed the rat Chk2 in *S. pombe* cells and examined their response to HU. The rat *chk2* gene was expressed from a NMT1 promoter in *S. pombe*, and its expression can be suppressed in the presence of thiamine. The HU sensitivity was measured in yeast cells which were adapted to medium without thiamine. Figure 3 shows that while the expression of *S. pombe cds1* gene from the NMT1 promoter rendered Δ*cds1* cells virtually resistant to 7.5 mM HU, the expression of rat Chk2 partially complemented *S. pombe* Δ*cds1* defect. On the other hand, the cells transfected with vector alone could not survive with 7.5 mM HU treatment. In addition, expression of rat Chk2 resulted in a slow growth phenotype manifested as small colonies on

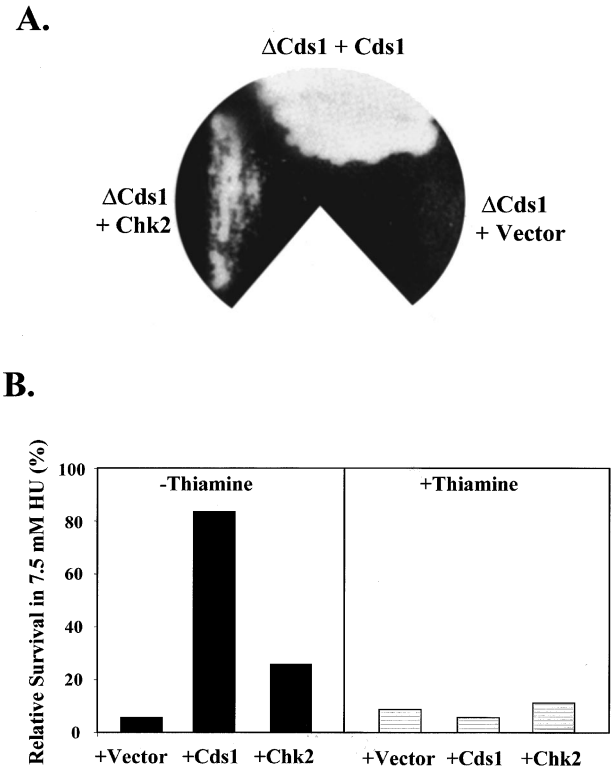

Figure 3 Partial complementation of *S. pombe* Cds1 by rat Chk2. (**A**) TE700 (Δcds1) yeast cells carrying plasmid with either the rat Chk2 cDNA, the *S. pombe* Cds1 gene or vector only were streaked onto EMM medium with 6 mM HU and incubated at 30°C for 72 h. (**B**) Assay for HU sensitivity in liquid culture was performed as described in Materials and methods. Cells were grown in the presence or absence of 100 μM thiamine, as indicated. OD_{595} of wells with cells grown in 7.5 mM HU, normalized to OD_{595} of cells grown in 0 mM HU was expressed as relative survival

thiamine free medium. The addition of 50–100 μM thiamine suppressed the abilities of Cds1 and Chk2 plasmids bearing cells to survive HU (Figure 3B). We also have generated a kinase-defective mutant rat Chk2 (D350A). When this mutant was introduced into Δ*cds1* strain, no slow growth phenotype was observed, suggesting that the kinase activity was responsible for the slow growth phenotype associated with the wild-type Chk2. Δ*cds1* cells carrying the mutant behaved similarly as cells carrying a vector, and were killed by 7.5 mM HU (data not shown).

Mammalian Chk2 can phosphorylate serine-216 in Cdc25C in vitro

The functional overlap between *S. pombe* Chk1 and Cds1 in the yeast replication checkpoint (Lindsay *et al*., 1998) suggests that they have common regulatory targets. Both Cds1 and Chk1 phosphorylate Cdc25 in similar sites in *S. pombe* (Zeng *et al*., 1998). Thus we analysed the ability of mammalian Chk2 to phosphorylate Cdc25C using GST-Cdc25C as a substrate in the kinase assay. In our assay,

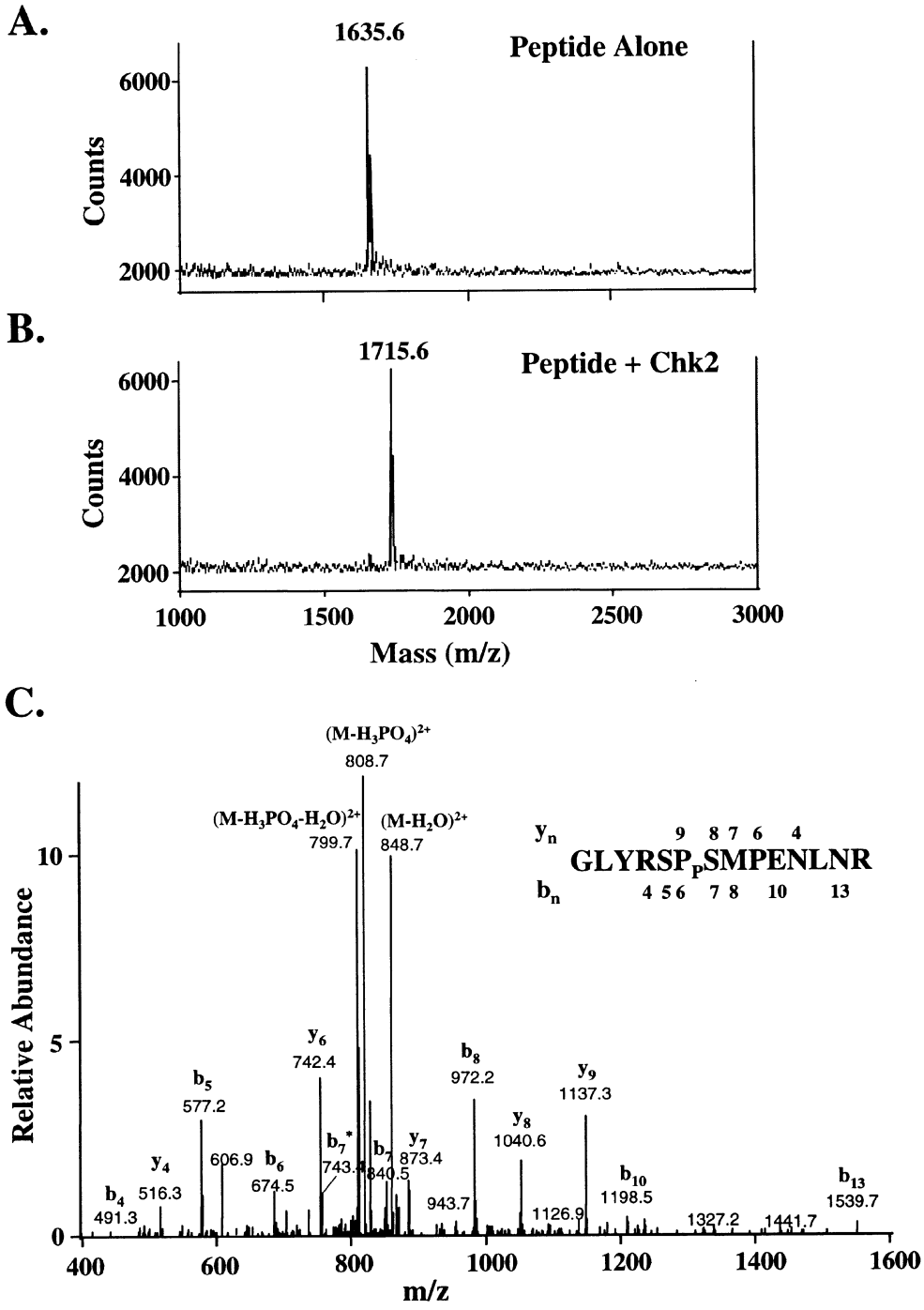

Figure 4 Chk2 phosphorylates a Cdc25C peptide at serine-216. Linear MALDI-TOF spectrum of the Cdc25 peptide 210–223 (GLYRSPSMPENLNR) before (**A**) and after (**B**) *in vitro* kinase reaction with Chk2 kinase. (**C**) Identification of the phosphorylated residue in the Cdc25C peptide 210–223 (GLYRSPSMPENLNR) by on-line LC-MS/MS using ES collision induced dissociation (CID) tandem MS (see Materials and methods) of the doubly charged precursor (m/z 857.5) for the phosphopeptide shown in (**b**) above. (Nomenclature is according to Biemann, 1990). Ions labeled b_n* have the structure b_n-H_3PO_4

purified GST-Chk2 could phosphorylate GST-Cdc25C but not GST alone (data not shown). Furthermore, we designed a 14-mer peptide, GLYRSPSMPENLNR (amino acids 210–224 of Cdc25C) with three potential phosphorylation sites: serine 214, serine-216 and tyrosine-212, and used it to test whether GST-Chk2 can phosphorylate the same serine residue (serine-216) in Cdc25C as Chk1. By matrix-assisted laser desorption/ionization time-of-flight (MALDI TOF) mass spectrometry, we observed the addition of one mole of phosphate (mass shift from 1635.6 to 1715.6 Da) when we incubated the peptide with the purified GST-Chk2 (Figure 4A and B). Using on-line liquid chromatography-electrospray tandem mass spectrometry (LC-ES-MS/MS), we mapped the site of phosphorylation to serine-216. The product ion spectrum of the phosphorylated Cdc25C peptide is shown in Figure 4C. That the mass of the N-terminal fragments b_4, b_5 and b_6 ions are unshifted by the mass of phosphate addition (80 Da) indicates that neither tyrosine-212 nor serine-214 can be phosphorylated, leaving only serine-216 as a potential phosphorylation site. The presence of both the b_7 ion (the first N-terminal fragment to contain serine-216) and $b_7 - H_3PO_4$ proves phosphorylation at serine-216. Consistent with this is the fact that all of the remaining b_n ions are shifted in mass by 80 Da. A similar argument can be detailed for the C-terminal fragment y_n ion series. Taken together, these data indicate unambiguously that phosphorylation occurs at serine-216.

Mammalian Chk2 is activated in response to DNA damage in an ATM-dependent manner

To assess the function of Chk2 in the DNA damage checkpoint, the effect of γ-radiation on Chk2 activation and phosphorylation was studied. Further, to examine the role of ATM in Chk2 activation in response to DNA damage, these studies were performed using ATM−/− and wild-type fibroblasts. Experiments were done with MRC-5 (ATM+/+) and AG04405 (ATM−/−) cells. Both cell types were irradiated with 20 Gy of γ-radiation and either lysed instantly or allowed to recover for 15 min at 37°C before lysate preparation. As control, lysates were prepared from cells that had been left untreated. Firstly, total cell lysates containing equal amounts of protein from γ-irradiated or untreated cells were immunoprecipitated with anti-Chk2 antibody or with preimmune serum. The proteins were then separated on SDS–PAGE and immunoblotted with anti-Chk2 antibody. Figure 5A shows that even though the amount of Chk2 was almost the same in untreated and treated samples, the Chk2 protein in irradiated ATM+/+ samples migrated slower (lanes 3, 4) than in the control (lane 5). It is interesting to note that such change in protein mobility was not seen in irradiated ATM−/− cells (lanes 6 and 7). The change in mobility is attributed to the phosphorylation of Chk2 in an ATM-dependent manner. Similar findings have been reported by Matsuoka *et al*. (1998), where they have demonstrated that the mobility shift could be reversed by phosphatase treatment. Next the immunoprecipitated Chk2 from each sample was assayed for its kinase activity using GST-Cdc25C as the substrate. Figure 5B indicates that there was basal level of activity in both irradiated and unirradiated control cells (lanes 5 and 8). However, the activity was enhanced about sixfold (lane 3) in ATM+/+ cells which were γ-irradiated and allowed to recover for 15 min and about threefold (lane 4) in γ-irradiated cells compared to control cells (lane 5). In addition, no change in Chk2 kinase activity was seen in ATM−/− cells in response to γ-irradiation (lane 8). These results were confirmed when similar experiments were repeated using WI38 (ATM+/+) and CRL-7201 (ATM−/−) cells (data not shown). Our results indicate that mammalian Chk2 is activated in response to DNA damage in an ATM-dependent manner, and that activation of Chk2 by ATM is required, in turn, for phosphorylation of Cdc25C.

Effects of topotecan-induced DNA damage and HU-induced replication blocks on Chk2

As topotecan can activate a DNA damage checkpoint (Ryan *et al*., 1994; Kingsbury *et al*., 1991), its effect on activation of Chk2 was investigated. Also, to determine whether Chk2 is activated in response to

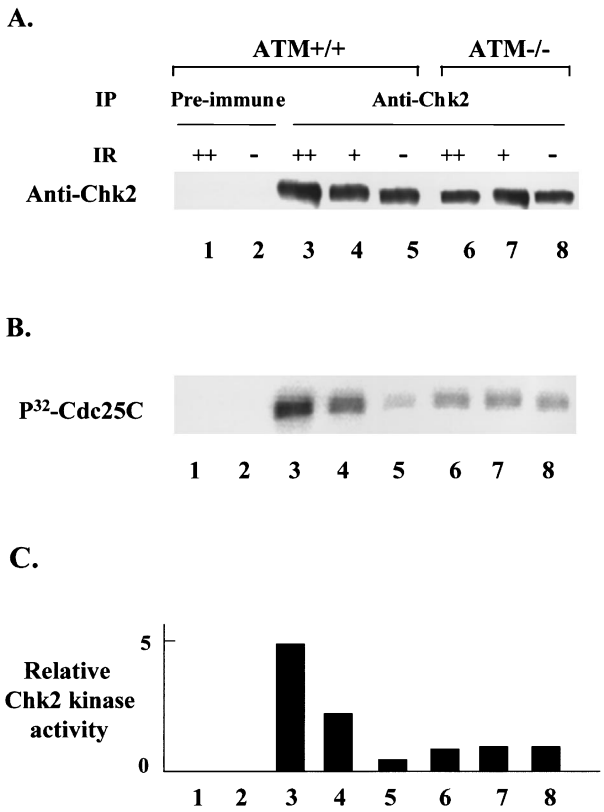

Figure 5 Chk2 activation in response to γ-irradiation is ATM dependent. MRC-5 cells (ATM+/+) and AGO4405 cells. (ATM−/−) cells were treated with 20 Gy of γ-radiation (IR), collected immediately (+) or after 15 min post-irradiation incubation (++) or left untreated (−). The cells were lysed on harvesting and equal amount of protein in each sample was resolved by 10% SDS–PAGE and immunoblotted with anti-Chk2 antibody (**A**). (**B**) The cell lysates were immunoprecipitated (IP) with anti-Chk2 antibody or with preimmune-sserum. The immunoprecipitates were washed and kinase assays were performed using GST-Cdc25C as a substrate. The reaction mixtures were resolved by SDS–PAGE and the labeled GST-Cdc25C was visualized by autoradiography (**C**) the relative Chk2 activities are shown graphically

replication blocks, cells were treated with HU and Chk2 activation was studied by both gel shift and immunoprecipitate kinase assays. In addition, to examine the role of ATM in Chk2 activation in response to DNA damage or replication blocks caused by topotecan or HU, we assessed the activity in both ATM−/− and ATM+/+ cells. Firstly, to determine the amount of Chk2 in each of the samples, equal amounts of protein from total cell lysates prepared from drug treated and untreated cells were separated on SDS–PAGE and immunoblotted with anti-Chk2 antibody. Next Chk2 was immunoprecipitated from all the samples using the anti-Chk2 antibody and tested for its kinase activity using GST-Cdc25C as the substrate. Figure 6 shows that even though the levels of Chk2 in all the samples were comparable, in HU and topotecan treated ATM+/+ cells, the migration of Chk2 protein was slower (Figure 6A, lanes 2 and 3) and phosphorylation was stronger (Figure 6B, lanes 2 and 3) as compared to untreated control samples (Figure 6A and B, lane 1). In ATM−/− cells, a clear shift in Chk2 mobility and an increase in phosphorylation could be seen in HU treated cells (Figure 6A and B, lane 5); in contrast, there was no change observed in mobility or phosphorylation in topotecan treated cells (Figure 6A and B, lane 6) as compared to control cells (Figure 6A and B, lane 4). These results indicate that topotecan can activate Chk2 in the same ATM-dependent manner as γ-irradiation, whereas the modification of Chk2 in response to HU is independent of ATM control.

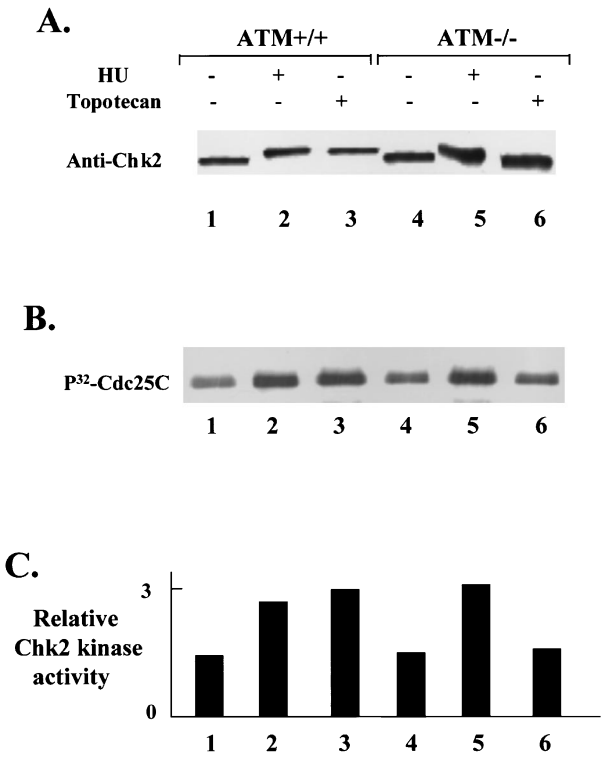

Figure 6 Chk2 activation in response to topotecan and HU treatments MRC-5 cells (ATM+/+) and AGO4405 cells. ATM−/− cells were treated with topotecan and HU (as described in Materials and methods). Total cell lysate from each sample was prepared and resolved on 10% SDS–PAGE and immunoblotted with anti-Chk2 antibody (**A**). (**B**) The cell lysates were immunoprecipitated (IP) with anti-Chk2 antibody. The immunoprecipitates were washed and kinase assay was performed using GST-Cdc25C as a substrate. The reaction mixtures were resolved by 10% SDS–PAGE and the labeled GST-Cdc25C was visualized by autoradiography. (**C**) The relative Chk2 activities are shown graphically

Discussion

In this communication, we report the cloning of mammalian Chk2 and provide evidence for its role in the DNA damage as well as the replication checkpoint. Mammalian Chk2 has been shown to have sequence homology as well as functional similarity to *S. cerevisiae* Rad53 and *S. pombe* Cds1. Recently, Matsuoka *et al.* (1998) demonstrated the growth defect complementation of the rad53 null mutation by human Chk2. The complementation assay studies reported here using Cds1 deficient *S. pombe* strains demonstrate that mammalian Chk2 can complement the checkpoint defects of yeast strains (Figure 3). Thus, mammalian Chk2 is a functional homolog of both Cds1 and Rad53.

Fission yeast Cds1 is involved in the replication checkpoint, but not in the DNA damage checkpoint (Murakami and Okayama, 1995; Lindsay *et al.*, 1998), whereas the budding yeast homolog Rad53 has a role in both the replication and DNA damage responses (Sanchez *et al.*, 1996; Sun *et al.*, 1996). To investigate the effect of DNA damage on Chk2 activation we used γ-irradiated and topotecan-treated cells and found that the Chk2 is modified, activated and capable of phosphorylating Cdc25C in response to DNA damage (Figure 5). Similar findings have been reported by Matsuoka *et al.* (1998) and Blasina *et al.* (1999). From the HU treatment studies, it seems clear that the mammalian Chk2 can respond to replication blocks as well (Figure 6).

ATM appears to be important in the regulation of Chk2 kinase responding to DNA damage (Figures 5 and 6), while the activation of Chk2 by replication block seems to be ATM-independent (Figure 6). The ATM pathway apparently increases cellular resistance to DNA damage caused by γ-radiation and topotecan, but it does not have much effect on replication blocks caused by HU (Figure 1). These results imply that the ATM pathway is important to the DNA damage checkpoint but not to the replication checkpoint. As reported by Matsuoka *et al.* (1998), the Chk2 function is regulated in an ATM-independent manner in the presence of high levels of DNA damage. These findings indicate the possibility of other regulators of Chk2; one of them could be ATM related kinase ATR (Keegan *et al.*, 1996). How ATM or ATR regulates Chk2 is unclear. It could be by direct phosphorylation or by a series of interactions. Chk2 has a potential protein-protein interacting domain, the FHA domain. In *S. cerevisae*, Rad9 interacts with the FHA2 domain in Rad53 and leads to its phosphorylation; the deletion of this domain leads to inactivation of Rad53 (Sun *et al.*, 1998). It will be interesting to investigate the role of the FHA domain of Chk2 in mediating the ATM dependent activation of this kinase.

Using a peptide assay, mass spectrometry showed that Chk2 can directly phosphorylate Cdc25C on serine-216, a site known to be involved in negative regulation of Cdc25C (Figure 4). This is the same site

phosphorylated by another checkpoint kinase Chk1 (Peng *et al*., 1997; Sanchez *et al*., 1997), which suggests that in response to DNA damage and DNA replicational stress Chk1 and Chk2 phosphorylate Cdc25C to prevent entry into mitosis. It is certainly interesting that despite the lack of structural homology between Chk1 and Chk2, they have overlapping functions. Also, it is intriguing whether both Chk1 and Chk2 kinases are required to prevent mitosis by inhibiting Cdc25C or in the absence of one the other can suffice the function.

S. pombe Cds1 has been suggested to be a S phase checkpoint kinase (Lindsay *et al*., 1998). The complementation of checkpoint defects of the ΔCds1 strain by Chk2 suggest an active role of mammalian Chk2 in the S phase checkpoint. Unlike the situation in yeast, there is not much known about the mammalian S phase checkpoint. Contrary to normal cells, ATM deficient human cells fail to reduce their rate of DNA synthesis in response to DNA damage (Painter and Young, 1980). The ATM deficient cells used in this study were more sensitive to topotecan and radiation treatment than normal cells (Figure 1), consistent with defects in DNA damage survival pathways and S phase checkpoints. These properties are shared with MEC1 and Rad53 in *S. cerevisiae*, in which downstream events are better characterized. Rad53 is required for preventing initiation of late origins of replication, slowing of DNA synthesis and transcriptional induction of repair proteins when DNA is damaged (Poulovich and Hartwell, 1995; Santocanale and Diffley, 1998; Shirahige *et al*., 1998; Huang *et al*., 1998). If mammalian Chk2 is truly a Rad53 homolog, one may hypothesize that Chk2, functioning downstream of ATM, will play a direct role in regulating the rate of DNA synthesis and in the transcriptional induction of repair proteins in response to DNA damage. It is likely that Chk2 phosphorylates substrates in addition to Cdc25C. Finally, the demonstration that Chk2 is part of the ATM signaling pathway leads to the hypothesis that interfering with this enzyme in repair proficient cancer cells will increase their sensitivity to agents like topotecan and γ-radiation.

Materials and methods

Full length cDNA cloning

The full length rat *chk2* was cloned from rat liver and spleen cDNA using the Marathon kit (Clontech, Palo Alto, CA, USA). PCR was performed using the gene specific primers corresponding to the EST clone and the AP primers (Clontech). The PCR products were cloned into pCRIITOPO vector (Invitrogene, Carlsbad, CA, USA) and sequenced. Subsequently, the entire coding region of rat *chk2* was isolated by RT–PCR. Similar approaches (Marathon PCR and RT–PCR) were used to clone the full length human *chk2*.

Cell lines

MRC5, WI-38 and CRL-7201 were obtained from American Type Culture Collection (ATCC), (VA, USA); AGO4405 was obtained from Coriell Cell Repositories, (NJ, USA). Cultures were maintained in Dulbecco's modified minimal essential medium (DMEM) as recommended by the cell providers.

Compound incubation, irradiation, and growth inhibition assays

Approximately 2×10^7 cells growing exponentially in 150 cm^2 flasks were irradiated, incubated for 20 h at 37°C in the presence of 1 μM topotecan (Department of Medicinal Chemistry, SmithKline Beecham) or 2 mM HU (Sigma Chemical Co, St. Louis, MO, USA), or left untreated. Cultures were γ-irradiated in growth medium by exposure to a gammacell 40 cesium source (Atomic Energy of Canada Ltd) (dose rate 90 rads/min; total dose = 20 Gy). For each experiment, two identical flasks were irradiated; immediately thereafter, one was put at ice temperature and the other was incubated for 15 min at 37°C to permit post-irradiation responses. A sham-irradiated control was kept at ambient temperature during irradiation and processed immediately thereafter. Inhibition of growth of ATM−/− and +/+ human fibroblast cultures by radiation, topotecan and hydroxyurea were assayed by a standard XTT protocol (Scudiero *et al*., 1988).

Yeast complementation experiments

S. Pombe strain TE700 (cds1::ura4+ Leu1-32 ura4-D18 h^-) was used for the complementation experiments (Zeng *et al*., 1998). The rat Chk2 cDNA was subcloned into *S. pombe* vector rep1 (Maundrell, 1990) to generate plasmid pWE832, in which the kinase-defective mutation (D350A) was also made. HU sensitivity was assayed by scoring growth on EMM solid medium containing 6 mM HU. Alternatively, cells were grown in microtiter wells with medium containing 7.5 mM HU. After 24–48 h incubation, the turbidity (OD_{595}) of wells was measured. The blank-subtracted normalized density was used as a measurement of growth/survival in 7.5 mM HU.

Antibodies

Rabbit polyclonal antibodies were raised against a C-terminal peptide of human Chk2: (C)QPSTSRKRPREGEAEGAE-TTKR-COOH and affinity purified. The purified antibody can recognize both endogenous and recombinant Chk2 of the right molecular weight, and its recognition can be blocked by the peptide (data not shown).

Recombinant protein production

The full length human Chk2 cDNA fragment was cloned into pGEX-4T-2 bacterial expression vectors. Recombinant Chk2 was purified as a GST fusion protein through affinity step followed by a gel filtration column. GST-Cdc25C was purified by the same protocol.

MALDI TOF MS and LC-ESI-MS/MS

Peptide molecular weights were measured using a linear MALDI TOF mass spectrometer with delayed extraction (Perseptive Biosystems, MA, USA). Phosphopeptides were sequenced by tandem MS (Carr *et al*., 1996) using an electrospray ion trap mass spectrometer (Finnigan MAT, CA, USA) coupled on-line with a capillary HPLC (Beckman, CA, USA) as previously described (Zhang *et al*., 1998).

Western blotting and kinase assay

For Chk2 kinase and Western blot assays, the cells were lysed in buffer containing 20 mM Tris-HCl (pH 8.0), 100 mM NaCl, 1 mM Na_3VO_4 and protease inhibitors for 30 min at 4°C. 150 μg total protein was immunoprecipitated with affinity purified anti-Chk2 antibody at 4°C for 2 h followed by incubation with protein A-Sepharose for 1 h at 4°C. Kinase reactions contained immunoprecipitated Chk2 and GST-Cdc25 in 20 mM HEPES, 50 mM KCl, 10 mM $MgCl_2$, 1 mM EGTA, 0.5 mM DTT, 40 μM ATP and 15 μCi

[γ-^{32}]ATP for 1 h at 37°C. The samples were analysed by SDS–PAGE and visualized by autoradiography.

Acknowledgements
We thank Jeff Jackson and Amy Roshak for discussion and suggestions during the course of this work, John D Martin for peptide synthesis, Mike Hansbury for technical help, Jackson Wong and Doug Fecteau for assistance in tissue culture work. We thank Kristi Forbes, Hiroto Okayama and Paul Russell for providing *S. pombe* Cds1 constructs ΔCds1 strains. P Chaturvedi is a postdoctoral fellow supported by National Cancer Institute grant (CA-50771-09).

References

Allen JB, Zhou Z, Siede W, Friedberg EC and Elledge SJ. (1994). *Genes Dev.,* **8,** 2401–2415.
Barlow C, Hirotsune S, Paylor R, Liyanage M, Eckhaus M, Collins F, Shiloh Y, Crowley JN, Ried T, Tagle D and Wynshaw-Boris A. (1996). *Cell,* **86,** 159–171.
Biemann K. (1990). In: McCloskey JA. (ed.). *Methods in Enzymology*, Vol 193, Academic Press. New York: pp. 886–887.
Bentley NJ, Holtzman DA, Flaggs G, Keegan KS, DeMaggio A, Ford JC, Hoekstra M and Carr AM. (1996). *EMBO J.,* **15,** 6641–6651.
Blasina A, Van de Weyer I, Laus MC, Luyten WHML, Parker AE and McGowan CH. (1999). *Curr. Biol.,* **9,** 1–10.
Boddy MN, Furnari B, Mondesert O and Russell P. (1998). *Science,* **280,** 909–912.
Carr SA, Huddleston MJ and Annan RS. (1996). *Anal. Biochem.,* **239,** 180–192.
Cimprich KA, Shin TB, Keith CT and Schreiber SL. (1996). *Proc. Natl. Acad. Sci. USA,* **93,** 2850–2855.
Elledge SJ. (1996). *Science,* **274,** 1664–1672.
Hartwell LH and Weinert TA. (1989). *Science,* **246,** 629–634.
Hofmann K and Bucher P. (1995). *Trends Biochem Sci.,* **20,** 347–349.
Huang M, Zhou Z and Elledge SJ. (1998). *Cell,* **94,** 595–605.
Kastan MB, Zhou Q, El-Diery WS, Carrier F, Jacks T, Walsh WV, Plunkett BS, Vogelstein B, Fornace Jr AJ. (1992). *Cell,* **71,** 587–597.
Keegan KS, Holtzman DA, Plug AW, Christenson ER, Brainerd EE, Flaggs G, Bentley NJ, Taylor EM, Meyn MS, Moss SB, Carr AM, Ashley T, Hoekstra MF. (1996). *Genes Dev.,* **10,** 2423–2437.
Kingsbury WD, Boehm JC, Jakas DR, Holden KG, Hecht SM, Gallagher G, Caranfa MJ, McCabe FL, Faucette LF, Johnson RK and Hertzberg RP. (1991). *J. Med. Chem.,* **34,** 98–107.
Lindsay HD, Griffiths DJF, Edwards RJ, Christensen PU, Murray JM, Osman F, Walworth N and Carr AM. (1998). *Genes Dev.,* **12,** 382–395.
Matsuoka S, Huang M and Elledge SJ. (1998). *Science,* **282,** 1893–1897.
Maundrell K. (1990). *J. Biol. Chem.,* **265,** 10857–10864.
Murakami H and Okayama H. (1995). *Nature,* **374,** 817–819.
Murray AW. (1995). *Curr. Opin. Genet. Dev.,* **5,** 5–11.
Painter RB and Young BR. (1980). *Proc. Natl. Acad. Sci. USA,* **77,** 7315–7317.
Peng CY, Graves PR, Thoma RS, Wu Z, Shaw AS and Piwnica-Worms H. (1997). *Science,* **277,** 1501–1505.
Ryan AJ, Squires S, Strutt HL, Evans A, Johnson RT. (1994). *Carcinogenesis,* **15,** 823–828.
Sanchez Y, Desany BA, Jones WJ, Liu Q, Wang B and Elledge SJ. (1996). *Science,* **271,** 357–360.
Sanchez Y, Wong C, Thoma RS, Richman R, Wu Z, Piwnica-Worms H and Elledge SJ. (1997). *Science,* **277,** 1497–1501.
Santocanale C and Diffley JFX. (1998). *Nature,* **395,** 615–617.
Savitsky K, Bar-shira A, Gilad S, Rotman G, Ziv Y, Vanagaite L, Tagle DA, Smith S, Uziel T, Sfez S, Ashkenazi LP, Frydman M, Harnik R, Sankhavaram RP, Simmons A, Clines GA, Sartiel A, Gatti RA, Chessa L, Sanal O, Lavin MF, Jaspers NGJ, Taylor MR, Arlett CF, Miki T, Weissman SM, Lovett M, Collins F and Shiloh Y. (1995). *Nature,* **268,** 1749–1753.
Scudiero DA, Shoemaker RH, Pauli KD, Monks A, Tierney S, Nofziger TH, Currens MJ, Seniff D and Boyd MR. (1988). *Cancer Res.,* **48,** 4827–4834.
Shirahige K, Hori Y, Shiraishi K, Yamashita M, Takahashi K, Obuse C, Tsurimoto T and Yoshikawa H. (1998). *Nature,* **395,** 618–621.
Sun Z, Fay DS, Marini F, Foiani M and Stern DF. (1996). *Genes Dev.,* **10,** 395–406.
Sun Z, Hsiao J, Fay DS and Stern DF. (1998). *Science,* **281,** 272–274.
Walworth N, Davey S and Beach D. (1993). *Nature,* **363,** 368–371.
Walworth N and Bernard R. (1996). *Science,* **271,** 353–356.
Weichselbaum RR, Nove J and Little JB. (1978). *Nature,* **271,** 261–262.
Weinert TA, Kiser GL and Hartwell LH. (1994). *Genes Dev.,* **8,** 652–665.
Zeng Y, Forbes KC, Wu Z, Moreno S, Piwnica-Worms H and Enoch T. (1998). *Nature,* **395,** 507–510.
Zhang X, Herring CJ, Romano PR, Szczepanowska J, Breska H, Hinnebusch and Qin J. (1998). *Anal Chem.,* **70,** 2050–2059.
Ziv Y, Bar-Shira A, Pecker I, Russell P Jorgensen TJ, Tsarfate I and Shiloh Y. (1997). *Oncogene,* **15,** 159–167.