Phosphatase Type 2A-dependent and -independent Pathways for ATR Phosphorylation of Chk1

Ge Li, Robert T. Elder, Kefeng Qin, Hyeon Ung Park, Dong Liang, and Richard Y. Zhao

ATM and Rad3-related (ATR) is a regulatory kinase that, when activated by hydroxyurea, UV, or human immunodeficiency virus-1 Vpr, causes cell cycle arrest through Chk1-Ser345 phosphorylation. We demonstrate here that these three agents only Vpr requires protein phosphatase type 2A (PP2A) to activate ATR for Chk1-Ser345 phosphorylation. A requirement for PP2A by Vpr was first shown with the PP2A-specific inhibitor okadaic acid, which reduced Vpr-induced G2 arrest and Cdk1-Tyr15 phosphorylation. Using small interference RNA to down-regulate specific subunits of PP2A indicated that the catalytic β-isofrom PP2A(ββ) and the A regulatory α-isofrom PP2A(αα) are involved in the G2 induction, and these down-regulations decreased the Vpr-induced, ATR-dependent phosphorylations of Cdk1-Tyr15 and Chk1-Ser345. In contrast, the same down-regulations had no effect on hydroxyurea- or UV-activated ATR-dependent Chk1-Ser345 phosphorylation. Vpr and hydroxyurea/UV all induce ATR-mediated γH2AX-Ser139 phosphorylation and foci formation, but down-regulation of PP2A(αα) or PP2A(ββ) did not decrease γH2AX-Ser139 phosphorylation by any of these agents or foci formation by Vpr. Conversely, H2AX down-regulation had little effect on PP2A(αα/ββ)-mediated G2 arrest and Chk1-Ser345 phosphorylation by Vpr. The expression of vpr increases the amount and phosphorylation of Claspin, an activator of Chk1 phosphorylation. Down-regulation of either PP2A(ββ) or PP2A(αα) had little effect on Claspin phosphorylation; but the amount of Claspin was reduced. Claspin may then be one of the phosphoproteins through which PP2A(αα/ββ) affects Chk1 phosphorylation when ATR is activated by human immunodeficiency virus-1 Vpr.

To ensure accurate transmission of genetic information, eukaryotic cells have developed an elaborate network of checkpoints to monitor the successful completion of every cell cycle step and to respond to cellular abnormalities such as DNA damage and replication inhibition as they arise during cell proliferation. The ATR kinase, a member of the phosphoinositide-3-kinase-related kinase family (1), is a central regulator for the replication checkpoint that is induced by treatment with DNA replication inhibitor hydroxyurea (HU).2 When ATR is activated, it initiates a regulatory cascade of phosphorylation events. One of the intermediate steps in this cascade is the activating phosphorylation of the Chk1 effector kinase (2). This activated Chk1 in turn phosphorylates and inactivates the Cdc25 phosphatases. Cdc25 phosphatases control the activity of cyclin-dependent kinases by removing the inhibitory phosphates on Tyr15 and Thr16 (1). Specifically for the G2 to M transition, which is controlled by activation of Cdk1, activation of ATR leads to inactivation of the Cdc25 phosphatases, in particular Cdc25C (3), ultimately resulting in the persistence of the phosphorylated Tyr15 and Thr16 on Cdk1, which causes a G2 arrest (4).

ATR can be activated by many agents including HU, which blocks replication, and DNA damaging agents such as UV and ionizing radiation. The agents capable of activating ATR share the common property of generating long single-stranded DNA regions either by blocking DNA replication, such as occurs with HU, to give stalled replication forks or by nuclease processing of the initial DNA damage (5). The signal thought to activate ATR comes from the single-stranded DNA-binding protein RPA (replication protein A), which coats these abnormally long regions of single-stranded DNA (6). A number of other factors, such as ATRIP, Rad1, Rad9, Hus1, and Rad17, are thought to play various roles in activating ATR at these RPA-coated regions (1, 5).

The actual mechanism of ATR activation does not appear to involve phosphorylation or any other covalent modification of ATR, and in vitro assays of immunoprecipitated ATR before and after activation generally do not show an increase in activity (7). Instead of a covalent modification, most models propose that the principal mechanism of activation is the formation of macromolecular assemblies bringing substrates in close proximity to ATR. If these macromolecular assemblies are large enough, they can be visualized as foci in the nucleus by immuno-fluorescence. For example, ATR and RPA form nuclear foci in response to HU or UV (8), and these foci may represent the

---

1. This study was supported in part by National Institute of Health Grants AI40891 and GM63080 (to R. Y. Z.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

2. The on-line version of this article (available at http://www.jbc.org) contains supplemental Figs. S1–S5.

3. To whom correspondence should be addressed: Dept. of Pathology, University of Maryland School of Medicine, 10 South Pine St., MSTF700A, Baltimore, MD 21201. Tel.: 410-706-6301; Fax: 410-706-6302; E-mail: rzhao@som.umaryland.edu.
initial formation of macromolecular complexes where substrates are brought to be phosphorylated by ATR. Recently an alternative to this model of ATR activation by relocalization has been proposed. Kumagai et al. (9) showed that direct binding of TopBP1 was sufficient to activate ATR and proposed that this transient association with TopBP1 is the initial step in activation of ATR (9).

HIV-1 Vpr protein has recently been shown to be another agent that induces a G₂ arrest through ATR (10). Early results showed that Vpr induces cell cycle G₂ arrest through inhibitory Tyr¹⁵ phosphorylation of Cdk1 in mammalian and yeast cells, suggesting that this viral protein exerts a highly conserved effect on a basic cellular function (11–13). Consistent with an ATR pathway, the inhibitory Tyr¹⁵ phosphorylation of Cdk1 is achieved by inhibition of Cdc25 phosphatase (14, 15), although induced G₂ arrest through phosphorylation and activation of one or more phosphoinositide-3-kinase-related kinase shows that Vpr induces cell cycle G₂ arrest through inhibitory Tyr¹⁵ phosphorylation of Cdk1 in mammalian and yeast cells. Further studies have shown numerous similarities between the ATR pathway activated by Vpr and by other agents such as HU and UV. These similarities include a requirement for Rad17 and Hus1 (17), the induction of phosphorylation on Chk1 (10, 17), and the formation of nuclear foci by RPA, 53BP1, BRCA1, and γH2AX (17, 18).

The variant histone H2AX is a protein commonly used to monitor the nuclear foci formed during DNA damage and replication checkpoints. In response to replication arrest or many forms of DNA damage, H2AX is phosphorylated on Ser¹³⁹ to form γH2AX foci, and this phosphorylation is dependent on one or more phosphoinositide-3-kinase-related kinase including ATR (19). The γH2AX foci have been shown to retain DNA repair factors at the site of DNA damage, and it has been suggested that one of the roles of γH2AX is to concentrate DNA repair factors at sites of DNA damage (20, 21). Recently, protein phosphatase 2A (PP2A) has been shown to be recruited to DNA damage foci by binding to γH2AX (22). Chowdhury et al. (22) proposed that the role of the PP2A at the γH2AX foci was to dephosphorylate γH2AX during recovery after DNA repair had been completed.

PP2A is one of the major Ser/Thr phosphatases implicated in the regulation of many cellular processes including regulation of signal transduction pathways, cell cycle progression, DNA replication, gene transcription, and protein translation (23–26). The PP2A holoenzyme is composed of a 36-kDa catalytic C subunit (PP2A(C)), a 65-kDa scaffolding A subunit (PP2A(A) or PR65), and a regulatory B subunit (PP2A(B)). The core enzyme consists of PP2A(C) and PP2A(A), and one of the many B regulatory subunits associates with this core enzyme (A/C) to form a holoenzyme with specific properties and substrates (27). There are two isoforms of the catalytic core of PP2A, i.e., PP2A(Cα) and PP2A(Cβ), which share 97% identity in their primary amino acid sequences (28–30). PP2A(Cα) is more abundant than PP2A(Cβ). PP2A(A) is also present in two isoforms in mammalian cells, α and β, which share 86% sequence identity (31). PP2A(Aβ) is less abundant than PP2A(Aα), and it does not bind strongly to the catalytic C and regulatory B subunits (32, 33). Much of the diversity of PP2A holoenzymes comes from the four major classes of PP2A(B) regulatory subunits, PR55/B, PR61/B’, PR72/B”, and PR93/PR110/B”. Each exists in at least four isoforms leading to many different holoenzymes, which partially explains the multiple and diverse cellular functions of PP2A (23, 34).

A role for PP2A in Vpr-induced G₂ arrest was originally suggested by studies with the inhibitor okadaic acid in mammalian and yeast cells (12, 13). Further evidence came from fission yeast where deletion of the gene for a catalytic subunit (ppa2) and a regulatory subunit (pab1) of PP2A reduced Vpr-induced G₂ arrest (14, 35). However, specific involvement of PP2A in Vpr-induced G₂ arrest in mammalian cells is controversial. A report showing Vpr induces G₂ arrest in mammalian cells by interacting with the B55 regulatory subunit of PP2A (36) was retracted (37).

In this study we investigated the role of PP2A in the activation of ATR by Vpr during the induction of cell cycle G₂ arrest. We measured the Vpr-induced phosphorylation of Chk1 and cell cycle G₂ arrest when PP2A enzymatic activity was down-regulated either by the potent PP2A inhibitor okadaic acid (OA) or by specific small interference RNA (siRNA) to PP2A. We show here that the CB and Aα subunits of PP2A, but not the CA subunit, are required for the Vpr-induced and ATR-dependent phosphorylation of Chk1. However, depletion of the CB and Aα subunits did not decrease the extent of nuclei with γH2AX foci or the amount of γH2AX formed even though these events are ATR-dependent. In contrast to the PP2A(Aα/Cβ) dependence of Chk1 phosphorylation when ATR is activated by Vpr, depletion of the Aα or CB subunit of PP2A has no effect on the ATR-dependent phosphorylation of Chk1 when ATR is activated by HU or UV. Thus, PP2A has a positive role in the ATR-dependent phosphorylation of Chk1 activated by Vpr but not when ATR is activated by HU or UV.

**EXPERIMENTAL PROCEDURES**

Cell Culture—HeLa cells were grown in Dulbecco’s modified Eagle’s medium (Cellgro) supplemented with 10% fetal bovine serum (Invitrogen). The DL-3 cell line is a derivative of the HEK293VE-632 cell line stably transfected with an inducible vpr expression plasmid (pZRH-vpr) (38). DL-3 cells were maintained in Dulbecco’s modified Eagle’s medium containing 10% fetal bovine serum, 100 μg/ml zeocin, and 200 μg/ml hygromycin (Invitrogen). The expression of vpr is induced by 1 μM muristerone A (Invitrogen) as previously described (38).

Drug Treatment—OA, a potent PP2A inhibitor, was purchased from Sigma. OA also inhibits other phosphatases (PPases) such as PP1, PP2B, and PP2C at high concentration (39). The range of OA concentrations (10–25 nM) used here should specifically inhibit PP2A (40). To maintain proper final concentrations of OA during experiments, the same concentration of OA was added again 8 h after the first treatment. Cell treatment with HU or nocodazole (NOC) have been described previously (17). Briefly, cells were incubated with 10 mM HU for 2 h or 100 ng/ml NOC for 24 h before harvest.

Antibodies—Rabbit polyclonal anti-phospho-Cdk1-Tyr¹⁵, rabbit monoclonal anti-phospho-Chk1-Ser³⁴⁵ (133D3), and mouse monoclonal anti-PP2A(A) (4G7) antibodies were purchased from Cell Signaling Technology, Inc. (Danvers, MA). Mouse monoclonal anti-Chk1 (G-4) and rabbit polyclonal anti-
PP2A(C) (FL-309) antibodies were purchased from Santa Cruz Biotechnology, Inc. Mouse monoclonal anti-Cdk1 (Ab-2) and rabbit polyclonal anti-ATR (Ab-2) antibodies were from Calbiochem. Mouse monoclonal anti-β-actin (AC-15) antibody was from Sigma-Aldrich. Rabbit polyclonal anti-Claspin (BL73) antibodies were from Bethyl Laboratories Inc. (Montgomery, TX), and mouse monoclonal anti-phosphohistone γ-H2AX (Ser139) antibody was from Upstate, Inc. (Charlottesville, VA). Texas Red® goat anti-mouse IgG (H + L) secondary antibody was purchased from Molecular Probes, Inc (Eugene, OR). Goat anti-mouse IgG (H + L) horseradish peroxidase (HRP) conjugate and goat anti-rabbit IgG (H + L) HRP conjugate secondary antibodies were from Bio-Rad. Rabbit polyclonal anti-Vpr serum was custom generated through the Proteintech Group Inc. (Chicago, IL).

**Immunoﬂuorescence Staining**—Cells were ﬁxed with 2% paraformaldehyde in PBS for 35 min at 4 °C on Labtek II slides 48 h post-transfection. After washing 3 times for 5 min in PBS, cells were blocked and permeabilized for 20 min in blocking buffer (3% bovine serum albumin, 0.2% Triton X-100, 0.01% skim milk in PBS). Cells were then incubated with primary antibody with proper dilutions in the incubation buffer (1% bovine serum albumin and 0.02% Triton X-100 in PBS) for 45 min. After washing, cells were incubated with secondary antibody at the suggested dilutions in the incubation buffer for 35 min. After washing, cells were mounted with FluorSave reagent and visualized on a Leica DM4500B microscope (Leica Microsystems) with Openlab software (Improvision, Lexington, MA).

**Western Blotting**—Cells were lysed with lysis buffer (50 mM Tris, pH 7.5, 150 mM NaCl, 2 mM EDTA, 1% Triton X-100) on ice for 30 min, and the debris was removed by centrifugation at 13,000 rpm for 1 min. The protein concentrations of supernatants were measured by BCA protein assay kit (Pierce). After boiling, 50 μg of protein was loaded on Criterion Precast Gels (Bio-Rad) for electrophoretic separation. Proteins were transferred to the Trans-blot® nitrocellulose membranes and blocked with 5% skim milk in TBST buffer (10 mM Tris, pH 8.0, 150 mM NaCl, 0.1% Tween 20) for 1 h at room temperature. Primary antibodies were then applied overnight at 4 °C. After washing 3 times in TBST for 10 min each time, the membranes were incubated with secondary antibody for 1 h at room temperature. Membranes were washed again, and proteins were detected with Supersignal® West Pico chemiluminescent substrate (Pierce).

**siRNA Transfection**—SMARTpool™ combination of specific siRNAs, which were pre-designed commercially for PP2A(Aα) (PPPR21A, #M-010259), Claspin (#M-005288), and ATR-2 pub. siRNA Duplex (#P-002090), were purchased from Dharmacon (Chicago, IL). Pre-designed PP2A(Cα) siRNA mix (PP2PCA, #16704) was purchased from Ambion (Lafayette, CO), PP2A(Cβ) (#RI-4682, RI-4683, and RI-4684) were purchased from Bio-Rad. Rabbit polyclonal anti-Vpr antibodies were from Bio-Rad. Rabbit polyclonal anti-Claspin (BL73) were incubated with secondary antibody for 1 h at room temperature. Primary antibodies were then applied overnight at 4 °C. After washing 3 times in TBST for 10 min each time, the membranes were incubated with secondary antibody for 1 h at room temperature. Membranes were washed again, and proteins were detected with Supersignal® West Pico chemiluminescent substrate (Pierce).

**RESULTS**

**Inhibition of PP2A Enzymatic Activity Reduces Vpr-induced G 2 Arrest**—OA, a specific and potent inhibitor of PP2A, was first used to determine whether PP2A is involved in Vpr-induced G 2 arrest. A vpr-inducible system in the HEK293 cell line (38) was used to test the effect of Vpr in the presence or absence of PP2A. The expression of vpr was induced by 1 μM muristerone A, and increasing concentrations of OA in a range that specifically inhibits PP2A were added to the culture medium of vpr-expressing or vpr-repressing cells. Sixty-four hours after gene induction, cells were analyzed by flow cytometry for DNA content. The cell cycle profiles are shown in Fig. 1A, and the percentage of cells in G 2/M are shown in Fig. 1B. As expected, vpr-expressing HEK293 cells showed a significant accumulation (62.1%) of cells in G 2/M phase, whereas without vpr expression only 12.5% of cells were in G 2/M phase (Fig. 1A). No significant differences were seen in cells with or without vpr expression.
expression treated with 10 nM OA. However, Vpr-induced G₂ accumulation was significantly reduced when the concentrations of OA reached 17.5 and 25 nM. Although treatment with these higher concentrations of OA on cells without vpr expression caused small increases of cells in G₂ to 12.5–21.3%, the percentage of the G₂ cells decreased from 62.1 to 37.1 and 37.3% in the vpr-expressing cells treated with these higher concentrations of OA (Fig. 1B). These results suggest that the activity of PP2A is required for Vpr-induced G₂ arrest.

The Aα and Cβ Subunits of PP2A, but Not Aα, Are Required for the Vpr-induced Cell Cycle G₂ Arrest—Even though OA has been shown to be a potent PP2A inhibitor at the concentrations used (40), we cannot rule out the possibility that other phosphatases might also be inhibited by OA. To further confirm the involvement of PP2A in Vpr-induced G₂ arrest, we used specific siRNA to deplete PP2A. Because siRNA technology is highly specific, different subunits of PP2A can be knocked down to allow detailed dissection of the role of PP2A in Vpr-induced G₂ arrest. Here we tested both isoforms of the catalytic subunit of PP2A(C), i.e. PP2A(Aα) and PP2A(Cβ), and the predominant regulatory PP2A(Aα) subunit. HeLa cells were transfected with specific siRNAs for PP2A(Aα), PP2A(Cβ), PP2A(Aα), and control siRNA. Twenty-four hours post-transfection, the cells were either mock-infected or infected with Adv or Adv-Vpr, and 48 h post-infection (p.i.), the cellular DNA content was analyzed by flow cytometry. All of the mock or Adv infected cells displayed near normal cell cycle profiles regardless of the siRNA treatment, indicating those siRNAs had no significant effect on the cell cycle (Fig. 2A). Consistent with the idea that Vpr induces cell cycle G₂ arrest, cells infected with Adv-Vpr and either untreated or pretreated with control siRNA had a marked accumulation of cells with G₂/M DNA content. The percentage of G₂/M cells increased from 8.0 to 8.4% without Vpr to 71.9–87.3% with Vpr. However, Vpr-induced accumulation of G₂/M cells was markedly reduced when either PP2A(Cβ) or PP2A(Aα) was depleted by siRNA (Fig. 2A, bottom panels). Only 28.4% of the cells had G₂/M DNA content after PP2A(Cβ) depletion and only 32.9% for PP2A(Aα) depletion. In contrast, no significant reduction of cells in G₂/M was observed in Adv-Vpr-infected cells when PP2A(Aα) was depleted (Fig. 2A, middle panel).

The extent of depletion by the siRNAs was examined by immunoblotting and, for the catalytic subunit, by a semiquantitative RT-PCR assay. Consistent with PP2A(Aα) being the predominant form of PP2A(A), an immunoblot showed that the PP2A(A) protein was nearly eliminated by PP2A(Aα) knockdown (Fig. 2Ca). Similarly, for the catalytic subunit C, PP2A(Cα)-knockdown cells had a stronger reduction (66%) in PP2A(C) protein levels compared with PP2A(Cβ)-depleted cells (22%) (Fig. 2Cb), consistent with PP2A(Cα) being more abundant than PP2A(Cβ) (44). However, the antibodies used in the PP2A(C) immunoblots react equally with the Cα and Cβ subunits so a semiquantitative RT-PCR assay was used to evaluate the specificity of the siRNAs for the catalytic subunits (Fig. 2Cd). The RT-PCR assay used two primers described under “Experimental Procedures” that are perfectly homologous to the cDNA from the mRNA for both Cα and Cβ and which generated a 161-bp PCR product (Fig. 2Cd, row a). When this PCR product was digested with the TaqI restriction enzyme, only the Cα product gave a 101-bp band, whereas only the Cβ product gave a 136-bp band. Consistent with the greater abundance of the Cα subunit, the 101-bp band was stronger in untreated or control siRNA-treated cell samples (Fig. 2Cd, row b, lanes 1 and 2). With siRNA against Cα, the 101-bp band characteristic of Cα disappeared, leaving only the 136-bp band characteristic of Cβ (Fig. 2Cd, row b, lane 3), and with siRNA against Cβ, only the 101-bp band characteristic of Cα is seen (Fig. 2Cd, row b, lane 4). Thus, the siRNAs used show good specificity for the Cα and Cβ subunits, and Vpr-induced G₂ arrest requires Cβ but not Cα.

As further evidence that PP2A has a specific role in Vpr-induced G₂ arrest as opposed to a nonspecific effect, we tested the same siRNA PP2A knockdowns on HeLa cells treated with NOC, a M-phase-arresting drug (45). Although treatment with NOC caused an accumulation of cells in the G₂/M phase similar to Vpr, none of the siRNA treatments was able to suppress NOC-induced G₂/M arrest (Fig. 2A). Together, these data suggest that PP2A(Aα/Cβ) is specifically required for Vpr-induced G₂ arrest.

Depletion of ATR and PP2A(Aα/Cβ) Alone or in Combination Reduces Vpr-induced G₂ Arrest to Similar Extents—Because ATR has been shown to be required for Vpr-induced G₂ arrest (10), we were interested in determining the relationship between PP2A(Aα/Cβ) and ATR during Vpr-induced G₂ arrest. HeLa cells were transfected with siRNAs against PP2A(Cβ) and ATR either individually or in combination (Fig. 2D).
PP2A(Av/Cβ) and ATR Activation

FIGURE 2. Depletion of Av or Cβ subunits of PP2A reduces Vpr-induced G2 arrest in HeLa cells to the same extent as depletion of ATR. A, the indicated subunits of PP2A were first depleted in HeLa cells with specific siRNAs. PP2A depleted or control HeLa cells were then mock-infected (mock), infected with Adv, or adenovirus carrying Vpr (Adv-Vpr). Cell cycle profiles were analyzed 48 h.p.i. NOC, a mitosis-arrest inducing agent, was used as a control where the cell cycle arrest is not affected by PP2A. The cell cycle profiles were analyzed 48 h.p.i. NOC, a mitosis-arrest inducing agent, was used as a control where the cell cycle arrest is not affected by PP2A.

B, depletion of PP2A(Av) or PP2A(Cβ), ATR, or both have equivalent effects on Vpr-induced G2 arrest. PP2A subunit Cβ and/or ATR were first depleted in HeLa cells with specific siRNAs. Depleted and control HeLa cells were then mock-infected (Mock) or infected with Adv or Adv-Vpr. Cell cycle profiles were analyzed 48 h.p.i.

C, depletion by siRNAs and specificity for Av and Cβ. Immunoblots of HeLa cell lysates 48 h.p.i. for mock-transfected (None) or transfected with control siRNA (Control) or specific siRNA were probed with antibodies against the PP2A A subunit (Av), the PP2A C subunit (Cβ), or ATR (ATR). Note that because PP2A(Av) is more abundant than PP2A(Cβ) (44), PP2A(Av) is reduced more in the PP2A(Cβ) knockdown cells than in the PP2A(Av) knockdown cells. β-Actin was used as the protein loading control. The specificity of the Av and Cβ siRNAs is shown in d. As described under "Experimental Procedures," RT-PCR gives a 161-bp product for mRNA from both subunits (row a). After digestion with the Taq restriction enzyme, the Co product gave a band of 101 bp, and the Cβ product gave a 136-bp band. In the untreated and control samples, the Co band is stronger than the Cβ band, consistent with the greater abundance of Co. However, after treatment with Co siRNA, only the 136-bp Cβ band was observed, whereas only the 101-bp Co band was observed after treatment with Cβ siRNA (row b).

These cells were then infected with Adv-Vpr as described above. Similar to the results shown in Fig. 2A, no significant changes in the cell cycle profiles were observed in cells either mock-infected or infected with Adv, and pretreatment with any siRNAs alone had no obvious effect on the cell cycle profile (Fig. 2B). As expected, Adv-Vpr-infected cells showed strong accumulation (89.1%) of cells with G2/M DNA content, indicating cell cycle G2 arrest. Consistent with the results shown in Fig. 2A, depletion of PP2A(Cβ) reduced cells in G2/M from 89.1 to 32.7%. A similar reduction (31.8%) of cells in G2/M was also observed when ATR was depleted with specific siRNA against ATR (Fig. 2B; Ref. 10). When PP2A(Cβ) and ATR were simultaneously depleted, 31.8% of cells were in the G2/M phase, indicating that no additional reduction of Vpr-induced G2 arrest occurred with the combined depletion compared with PP2A(Cβ) and ATR single depletions (Fig. 2B). These similar amounts of reduction suggest that PP2A and ATR are on the same regulatory pathway for Vpr-induced G2 arrest.

PP2A Is Necessary for the Hyperphosphorylation of Cdk1-Tyr15 Induced by Vpr—Because Vpr induces cell cycle G2 arrest through the inhibitory Cdk1-Tyr15 phosphorylation (11–13), we next tested whether PP2A is also involved in Vpr-induced Cdk1-Tyr15 phosphorylation. Expression of vpr was reduced in HEK293 cells and cells treated with OA as described for Fig. 1A. Immunoblots of cell extracts were probed with anti-phospho-Cdk1-Tyr15 antibody. Although cells without vpr induction had no detectable Cdk1-Tyr15 phosphorylation (Fig. 3A, lanes 1, 3, 5, and 7), as expected, cells with induction of vpr expression had strong hyperphosphorylation of Cdk1-Tyr15 (Fig. 3A, lane 2). Cells treated with 10 nM OA showed no decrease in Cdk1-Tyr15 phosphorylation (Fig. 3A, lane 4), but vpr-expressing cells treated with 17.5 and 25 nM OA nearly reduced Tyr15 phosphorylation of Cdk1 to undetectable background levels (Fig. 3A, lanes 6 and 8).

The role of PP2A(Av/Cβ) in Vpr-induced phosphorylation of Cdk1-Tyr15 was also evaluated in HeLa cells with the specific siRNAs against the PP2A subunits. The untreated cells (NT) showed a low background level of Cdk1-Tyr15 phosphorylation (Fig. 3B, lane 1). As a positive control, cells were treated with HU, which induced strong hyperphosphorylation of Cdk1-Tyr15 (Fig. 3B, lane 2; Ref. 46). NOC-treated cells were used as a negative control because it does not induce Cdk1-Tyr15 phosphorylation (Fig. 3B, lane 3; Ref. 47). Adenoviral infection alone did not trigger a significant increase in phosphorylated Cdk1-Tyr15 (Fig. 3B, lane 4). However, Adv-Vpr infection caused a
strong hyperphosphorylation of Cdk1-Tyr15 to levels similar or higher than the HU-treated cells (Fig. 3B, lane 5). The same level of Cdk1-Tyr15 phosphorylation was also seen in Adv-Vpr-transduced cells transfected with control siRNA, indicating that siRNA transfection by itself had no effect on phosphorylation of Cdk1-Tyr15 (Fig. 3B, lane 6). Significant reduction of Cdk1-Tyr15 phosphorylation was, however, observed in the same Vpr-producing HeLa cells when they were transfected with siRNA either against PP2A(C) or PP2A(Aα) (Fig. 3B, lanes 7 and 8). The levels of Cdk1-Tyr15 phosphorylation in these two cell lysates were slightly higher but comparable with that seen with Adv infection, suggesting depletion of PP2A(Cβ) or PP2A(Aα) reduced Cdk1-Tyr15 phosphorylation to near the background levels. Therefore, PP2A is required for most of the hyperphosphorylation of Cdk1-Tyr15 induced by Vpr.

With Vpr Activation, PP2A Is Required for ATR Phosphorylation of Chk1—Activated ATR phosphorylates Chk1, and this phosphorylation is thought to activate Chk1, leading ultimately to inhibitory phosphorylation of Cdk1 to generate an ATR-dependent cell cycle arrest (7). Given the role of ATR in Vpr-induced G2 arrest (Fig. 2B) and the strong effect of PP2A on Cdk1-Tyr15 phosphorylation (Fig. 3), it seemed likely that PP2A is required for ATR phosphorylation of Chk1. This possibility was examined in HeLa cells pretreated with control, PP2A(Cβ), PP2A(Aα), or ATR siRNA (Fig. 4A). These cells were then infected with Adv-Vpr. The cell lysates were subject to electrophoresis and blotted with anti-phospho-Chk1-Ser345 or anti-Chk1 antibodies. Untreated cells (NT) showed almost no detectable phosphorylation of Chk1-Ser345 (Fig. 4A, lane 1). As a positive control, cells treated with HU showed very strong phosphorylation of Chk1-Ser345 (Fig. 4A, lane 2; Ref. 48). NOC treatment induced weak phosphorylation of Chk1-Ser345 (Fig. 4A, lane 3; Ref. 48). Adenoviral infection alone also triggered a low level phosphorylation of Chk1-Ser345 similar to that of NOC-treated cells (Fig. 4A, lane 4). In contrast, Adv-Vpr infection caused relatively strong phosphorylation of Chk1-Ser345 as previously described (Fig. 4A, lane 5; Ref. 10). Similar levels of Chk1-Ser345 phosphorylation were seen in
Adv-Vpr-transduced cells containing control siRNA, indicating that siRNA by itself had little effect on phosphorylation of Chk1-Ser345 (Fig. 4A, lane 6). However, a significant reduction in Chk1-Ser345 phosphorylation was seen in the same Vpr-producing HeLa cells when they were transfected with siRNA against either PP2A(Cβ) or PP2A(Aα) (Fig. 4A, lanes 7 and 8). Levels of Chk1-Ser345 phosphorylation in these two cell lysates were slightly higher but comparable with that of Adv-infected or NOC-treated cells (Fig. 4A, lanes 7 and 8, compared with lanes 3 and 4), suggesting that depletion of PP2A(Cβ) or PP2A(Aα) reduced Chk1-Ser345 phosphorylation almost to background levels. Noticeably, the phosphorylation of Chk1-Ser345 was completely eliminated in the ATR-knockdown cells (Fig. 4A, lane 9). The elimination of Chk1-Ser345 phosphorylation by ATR depletion compared with a residual signal with PP2A depletion suggests that ATR is responsible for Chk1 phosphorylation induced by both Adv-Vpr and Adv infection and that PP2A is required for most of the Chk1 phosphorylation induced by vpr expression.

With HU or UV Activation, PP2A Has No Effect on ATR Phosphorylation of Chk1—There have been no reports that ATR-mediated phosphorylation of Chk1 after HU or UV treatment is dependent on PP2A. To see if the ATR-mediated phosphorylation of Chk1 after HU or UV treatment is dependent on PP2A, the effects of siRNA knockdown were done with activation of ATR by HU or UV (Fig. 4B). As above, HeLa cells were pretreated with control, PP2A(Cβ), PP2A(Aα), or ATR siRNA. Cells were then treated with HU or UV for the measurement of Chk1-Ser345 phosphorylation. Little phosphorylation was seen in the untreated cells (Fig. 4B, NT, lane 1), but treatment with HU (lane 2) or UV (lane 7) gave a large increase in Chk1-Ser345 phosphorylation. Pretreatment with control siRNA had little effect on the level of phosphorylation, and pretreatment with PP2A(Cβ) or PP2A(Aα) siRNA did not cause any significant change in Chk1-Ser345 phosphorylation compared with the control siRNA for either HU (lane 3 compared with lanes 4 and 5) or UV (lanes 8 compared with lanes 9 and 10). Pretreatment with ATR siRNA reduced Chk1-Ser345 phosphorylation for both HU (lane 6) or UV (lane 11), demonstrating as expected that this phosphorylation is ATR-dependent (7). Thus, under the same experimental conditions, knock down of PP2A(Aα/Cβ) eliminates most of the Chk1-Ser345 phosphorylation induced by Vpr but has no significant effect on the phosphorylation induced by HU and UV even though all these phosphorylations are ATR-dependent.

PP2A Is Not Required for Vpr-induced γH2AX Phosphorylation or Foci Formation, and H2AX Depletion Has Little Effect on Vpr-induced Chk1 Phosphorylation and G2 Arrest—Previous studies showed that the expression of vpr induces γH2AX foci formation, and this process is ATR-dependent (17). H2AX is a variant of the H2A histone protein, which is quickly phosphorylated at the site of DNA damage to form a nuclear structure known as γH2AX foci (20, 49). We were interested in determining whether PP2A, like ATR, is also required for Vpr-mediated γH2AX foci formation. HeLa cells, transfected with siRNAs against PP2A(Cβ), PP2A(Aα), or ATR, were infected with Adv or Adv-Vpr. Forty-eight hours p.i., γH2AX foci formation was visualized by immunofluorescence (Fig. 5A), and the percentage of cells with foci was determined (Fig. 5B). Most of the Adv-Vpr-infected cells that were pretreated with or without the control siRNA (89.8% ± 1.7%; 90.1% ± 2.0%) formed γH2AX foci with a background level of ~11.8 ± 1.5% as shown in the control Adv-infected cells (Fig. 5, A and B). In agreement with a previous report (17), pretreatment of Adv-Vpr-infected cells with ATR siRNA eliminated most of the γH2AX foci (25.5 ± 2.6%). In contrast, pretreatment with PP2A(Cβ) or PP2A(Aα) siRNA had no effect on the γH2AX foci formation in vpr-expressing cells as more than 90% of the cells were found to be positive in both experiments (91.6 ± 1.5%; 92.0 ± 1.6%) (Fig. 5B). We further measured the effect of PP2A down-regulation on the amount of γH2AX-Ser139 phosphorylation. As shown in (Fig. 5C, lanes 7 and 8), depletion of PP2A(Cβ) or PP2A(Aα) actually seems to increase the amount of γH2AX phosphorylation after vpr expression. Therefore, ATR is required, but PP2A is not required for Vpr-mediated γH2AX foci formation.

PP2A has been reported to interact with γH2AX, and this interaction is necessary for PP2A to form nuclear foci after DNA damage (22). To see if the PP2A-γH2AX interaction is important for the role that PP2A plays in Vpr-induced G2 arrest, H2AX was depleted by siRNA. Surprisingly, even when H2AX is greatly reduced by siRNA (Fig. 6B), there is little effect on Vpr-induced G2 arrest, whereas a control experiment with depletion of ATR shows the expected reduction in Vpr-induced G2 arrest (Fig. 6A). Similarly, H2AX depletion has no significant effect on the Vpr-induced Chk1-Ser345 phosphorylation by ATR (Fig. 6C). Thus, even though Vpr induces γH2AX foci (Ref. 17, Fig. 5A), unless the small amounts remaining after siRNA depletion (Fig. 6B) are sufficient, the γH2AX foci do not play an important role in Vpr-induced G2 arrest or Chk1 phosphorylation. This in turn means that PP2A does not need to interact with γH2AX to fulfill its role in Vpr-induced Chk1 phosphorylation and G2 arrest.

Claspin Is a Potential Target for PP2A during Activation of ATR by Vpr—A number of cellular factors, such as Rad17-RFC complex, Rad9/Hus1/Rad1 (9-1-1) complex and Claspin, are required for Chk1 activation by ATR in response to DNA damage/replication stresses, and many of these required proteins are activated or inactivated by phosphorylation (5, 50–53). PP2A is likely to fulfill its role in the Vpr-activated ATR pathway by dephosphorylating one or more of these proteins. The properties of Claspin make it a particularly good candidate to be a phosphoprotein with an important role in the PP2A-dependent Vpr pathway. Claspin is phosphorylated by at least three kinases, ATR, Chk1, and Plk1 (54–58), and Plk1 phosphorylation is at a phosphodegron sequence of Claspin. If Claspin has similar roles after ATR is activated, the phosphodegron sequence of Claspin...
Claspin is required for phosphorylation of Chk1 when ATR is activated by Vpr. Depletion of Claspin by siRNA reduced Chk1 phosphorylation after infection with Adv-Vpr to about 10% that of the level seen with control siRNA. In the positive control, siRNA against ATR essentially eliminated Chk1 phosphorylation (Fig. 7B, lane 4). In contrast, there is no detectable effect of PP2A(Aa/Cβ) depletion on the phosphorylation of Chk1 when ATR is activated by HU or UV (Fig. 4B). In considering the possible roles of PP2A(Aa/Cβ) and ATR in the induction of G2 arrest by Vpr, it is important to note that depletion of Vpr still induces phosphorylation of Claspin and suggesting that ATR phosphorylation of Claspin is independent of PP2A(Aa/Cβ). The major effect of PP2A(Aa/Cβ) depletion is to reduce the amount of Claspin (Fig. 7B, compare lanes 4 and 5 to lanes 2 and 3). This is the expected result if PP2A activity reduces phosphorylation of the phosphodegron sequence (55–57) so that Claspin is stabilized, but other interpretations are possible (see “Discussion”).

**DISCUSSION**

In this study we demonstrated that Vpr requires PP2A(Aa/Cβ) and ATR to induce cell cycle G2 arrest in mammalian cells. PP2A(Aa/Cβ) and ATR are likely to be part of the same regulatory pathway since depletion of both PP2A(Aa/Cβ) and ATR decreases G2 arrest to about the same extent as depletion of either one alone (Fig. 2B). The phosphorylation of Chk1 by activated ATR is thought to be an essential step in the induction of G2 arrest by Vpr, and this Vpr-induced Chk1-Ser345 phosphorylation is nearly eliminated when PP2A(Aa/Cβ) are depleted (Fig. 4A). In contrast, there is no detectable effect of PP2A(Aa/Cβ) depletion on the phosphorylation of Chk1 when ATR is activated by HU or UV (Fig. 4B). In considering the possible roles of PP2A(Aa/Cβ) in Vpr-induced phosphorylation of Chk1, it is important to note that depletion of PP2A(Aa/Cβ) has no effect on the formation of γH2AX foci after vpr is expressed (Fig. 5B), and the amount of γH2AX actually increases after depletion of PP2A(Aa/Cβ) (Fig. 5C). These H2AX results indicate that at least some parts of the
ATR activation by Vpr do not require PP2A and make it unlikely that PP2A is required exclusively for the initial interaction of Vpr with the cellular machinery, which leads to activation of ATR.

Based on these results, PP2A is likely to be involved in some later step of the ATR pathway induced by Vpr. Phosphorylation of H2AX, Claspin, and Chk1 are all dependent on ATR, and they are thought to be directly phosphorylated by activated ATR (2, 7, 19, 58). Therefore, Vpr does activate ATR with respect to phosphorylation of the H2AX and Claspin substrates without PP2A(Aα/Cβ) (Fig. 5, 7), but PP2A(Aα/Cβ) is required for Vpr-induced phosphorylation of the Chk1-Ser345 substrate by ATR (Fig. 4A). There is no evidence that ATR is regulated by phosphorylation (7), which is another reason that it is unlikely that PP2A regulates the pathway by directly dephosphorylating ATR.

Most models for activation of ATR postulate that formation of multiprotein complexes, often observed as nuclear foci, is essential (7), although it has recently been proposed that binding of TopBP1 is the initial step in ATR activation (9). One model for the PP2A requirement in Vpr-induced G2 arrest is that the correct assembly and/or signaling by these multiprotein complexes depends on PP2A. PP2A could conceivably be required since numerous phosphorylation events occur during and after ATR activation with more than 10 ATR substrates having been identified thus far (1). The requirement for PP2A would be explained if the appropriate sequence of phosphorylation and dephosphorylation of at least one of these substrates depends on PP2A and is required for complete assembly of a fully functional complex.

The specific ATR substrate requiring PP2A might be RPA based on an analogy to the role of PP5 in ATR signaling (8). Although there have been no previous reports of a positive regulatory role for PP2A in ATR signaling, Zhang et al. (8) showed that the PP5 phosphatase is required for ATR phosphorylation of Chk1 after treatment with UV or HU. They found that ATR formed nuclear foci independently of PP5 after UV or HU treatment, but that formation of RPA foci required PP5. It is known that phosphorylation of RPA plays a role in its assembly into foci (60), and PP5 may be required so that the phosphorylation levels of RPA are appropriate for its assembly into functional foci (8). RPA is present in foci induced by Vpr (18), and based on the analogy to PP5, it will be of interest to determine whether RPA no longer associates with Vpr-induced foci when PP2A is absent.

Claspin is another protein with an important role in the ATR pathway activated by Vpr, which may be targeted by PP2A(Aα/Cβ). When ATR is activated by other agents, Claspin is important for phosphorylation of Chk1 but not of H2AX (53, 59). Thus, regulation of Claspin has the potential to explain the similar pattern of PP2A-dependent phosphorylation seen after Vpr activation (Fig. 4A and 5). Indeed, Claspin is required for Chk1 phosphorylation (Fig. 7A), and depletion of PP2A(Aα/Cβ) does reduce the amount of Claspin after Vpr activation.
Claspin is required for Vpr-induced phosphorylation of Chk1, and PP2A affects the amount of Claspin after vpr expression. A, depletion of Claspin nearly eliminates Vpr-induced Chk1-Ser345 phosphorylation (p). HeLa cells were transfected with siRNA against Claspin or ATR along with control siRNA and 24 h later were mock-infected or infected with Adv or Adv-Vpr. The phosphorylation status of Chk1-Ser345 in cellular extracts was determined with anti-phospho-Chk1-Ser345 or anti-Chk1 antibodies. Cells in the ionizing radiation (IR) control were exposed to 10 Gray and harvested 2 h after radiation. In the bottom two panels, depletion of ATR or Claspin by siRNA was demonstrated by immunoblotting of cell lysates with antibodies against ATR or Claspin. B, depletion of PP2A(Av/CB) or PP2A(Au) does not affect Vpr-induced phosphorylation but reduces the Vpr-induced increase in Claspin expression. Control HeLa cells or cells transfected with the indicated siRNAs were treated with HU or infected with Adv or Adv-Vpr. Cellular extracts were then subjected to 5% SDS-PAGE followed by Western blotting analysis using anti-Claspin antibody. p-Claspin indicates hyperphosphorylated Claspin (52). NT, not treated.

Claspin is required for Vpr-induced G2 arrest (Fig. 7B). However, based on the known effects of Claspin phosphorylations, there are two opposite interpretations for this observed correlation between amounts of Claspin and Chk1 activation. The first interpretation is based on the finding that Plk1 phosphorylation of Ser30 and Ser34 in the phosphodegron sequence is destabilizing and leads to rapid proteolysis of Claspin (55–57). In this case, a model in which PP2A acts directly on Claspin to keep it in the stable, unphosphorylated form can explain the high levels of Claspin and the PP2A dependence of Chk1 phosphorylation. The second interpretation is based on the stabilization and phosphorylation of Claspin on Thr916 and probably other sites by Chk1 (54, 61). In this case it is the activated Chk1 that causes the high levels of Claspin after vpr expression. A model based on this interpretation proposes that the PP2A dependence of Chk1 activation results from PP2A acting directly on a phosphoprotein other than Claspin to give active Chk1 which then phosphorylates and stabilizes Claspin. Future studies with Claspin mutants that cannot be phosphorylated at these different sites will be required to clarify the contribution of Claspin to Vpr-induced, PP2A-dependent phosphorylation of Chk1. Interestingly, a slight increase of ATR protein level was also noted when vpr is expressed (Fig. 4A, lanes 6–8; Fig. 7A, lane 2). This increase appears to be inversely correlated with Claspin as depletion of Claspin further enhanced ATR protein level (Fig. 7A, lane 4). These data implicate a possible negative feedback regulation of Claspin on ATR when it is under the influence of Vpr. Further in depth studies are needed to substantiate this premise.

Assuming that assembly of fully functional complex by Vpr for the phosphorylation of Chk1 requires PP2A, why would ATR phosphorylation of Chk1 after activation by HU/UV not require PP2A? One possibility is that the detailed structure of the foci differs between Vpr and HU/UV activation. One known difference in the structure of the foci is Vpr itself since many of the H2AX foci contain Vpr (18). Vpr could recruit or prevent the recruitment of proteins so that the detailed structure of foci induced by Vpr might have additional differences from foci induced by UV/HU, and these foci unique to Vpr might require PP2A for signaling to the cell cycle, whereas foci induced by UV/HU do not.

Although there have been no previous reports of a positive role for PP2A in Chk1 phosphorylation by ATR, there have been three reports where PP2A antagonizes ATR activity by dephosphorylating ATR substrates. Petersen et al. (62), based on experiments with soluble Xenopus extracts, proposed that PP2A dephosphorylates a number of ATR substrates including Chk1 to shut off an ATR/ATM-dependent double-strand breaks (DSB) checkpoint after DNA repair has been completed (62). Leung-Pineda et al. (63) showed that PP2A does in fact dephosphorylate Chk1 and that this dephosphorylation is regulated by Chk1 during the normal cell cycle (63). In experiments concentrating on the recovery after the repair of DNA damage induced by camptothecin in HeLa cells, Chowdhury et al. (22) presented evidence that PP2A binds to and dephosphorylates γH2AX during the disassembly of nuclear foci (22). Although it originally seemed possible that the binding of PP2A to γH2AX played some role in Vpr-induced G2 arrest, our subsequent experiments with depletion of H2AX showed little effect on Vpr-induced G2 arrest (Fig. 6A), suggesting that binding of PP2A to γH2AX is not required for PP2A to fulfill its role in Vpr-induced G2 arrest.

Surprisingly, this work suggests that the less abundant CB catalytic subunit of PP2A is the major source of PP2A for Vpr-induced G2 arrest. The Cα and Cβ subunits of PP2A are quite similar with only 8 amino acid differences of 309 amino acid, and the biochemical properties of the Cα and Cβ appear to be identical (64, 65). It is noteworthy that mouse and human Cα and Cβ are nearly identical with Cβ being identical and only one amino acid difference between mouse and human Cα. This nearly complete conservation between mouse and human suggests that, despite their high degree of similarity, Cα and Cβ each have at least one exclusive function that cannot be carried out by the other subunit. The Cα subunit has in fact been shown to have a exclusive, required role in embryogenesis (66, 67). However, no exclusive function for the less abundant Cβ subunit has been identified until now with the demonstration here that the Cβ subunit has a required role in Vpr-induced G2 arrest, whereas the Cα subunit plays no detectable role (Fig. 2A).
Dephosphorylating γH2AX may be another functional difference between the Cα and Cβ subunits. Depletion of PP2A(ΔCβ) or PP2A(Δα) actually increases the amount of γH2AX formed after vpr expression (Fig. 5C, lanes 7 and 8, compared with lane 6). Chowdhury et al. (22) also found that the amount of γH2AX formed in response to DNA damage increased if PP2A was inhibited, and they proposed that PP2A directly dephosphorylates γH2AX. Chowdhury et al. (22) did not distinguish between the Cα and Cβ catalytic subunits, but the results in Fig. 5C raise the possibility that the Cβ subunit may be primarily responsible for dephosphorylating γH2AX since the siRNA against the less abundant Cβ subunit (Fig. 5C, lane 7), which specifically depletes Cβ (Fig. 2C), shows the increase in γH2AX. Given the greatly different roles for the PP2A catalytic subunits in Vpr-induced G2 arrest and their possible different roles in dephosphorylating γH2AX, studies on the role of PP2A in the response to DNA damage should in the future examine whether the Cα and Cβ subunits play differential roles in these responses.

Acknowledgments—We are grateful to Dr. Ling-Jun Zhao (St. Louis University, St. Louis, MO) for providing the Adv and Adv-Vpr vectors and Dr. Lee Ratner (Washington University, St. Louis, MO) for providing the Muristerone A-inducible HEK293VE-632 cell line.

REFERENCES
1. Shchter, D., Costanzo, V., and Gautier, J. (2004) DNA Repair 3, 901–908
2. Zhao, H., and Piwnica-Worms, H. (2001) Mol. Cell. Biol. 21, 4129–4139
3. Sanchez, Y., Wong, C., Thoma, R. S., Richman, R., Wu, Z., Piwnica-Worms, H., and Elledge, S. J. (1997) Science 277, 1497–1501
4. Boutrous, R., Dozier, C., and Ducommun, B. (2006) Curr. Opin. Cell Biol. 18, 185–191
5. Bartek, J., Lukas, C., and Lukas, J. (2004) Nat. Rev. Mol. Cell. Biol. 5, 792–804
6. Lou, L., and Elledge, S. J. (2003) Science 300, 1542–1548
7. Bakkenist, C. J., and Kastan, M. B. (2004) Nat. Rev. Mol. Cell Biol. 5, 15443–15451
8. Lai, M., Zimmerman, E. S., and Elledge, S. J. (2000) J. Biol. Chem. 275, 33276–33282
9. Morita, E., Tada, K., Chisaka, H., Asao, H., Sato, H., Yaegashi, N., and Okayama, H. (2000) J. Virol. 74, 2636–2646
10. Zhang, S., Feng, Y., Narayan, O., and Zhao, L. J. (2001) Gene (Amst.) 263, 131–140
11. Zhou, J., and Ratner, L. (2001) Virology 287, 133–142
12. Arino, J., Woon, C. W., Brautigan, D. L., Miller, T. B., Jr., and Johnson, G. L. (1988) Proc. Natl. Acad. Sci. U. S. A. 85, 4252–4256
13. Stone, S. R., Hofsteenge, J., and Hemmings, B. A. (1987) Biochemistry 26, 7215–7220
14. Green, D. S., Yang, S. L., and Mumber, M. C. (1987) Proc. Natl. Acad. Sci. U. S. A. 84, 4880–4884
15. Hemmings, B. A., Adams-Pearson, C., Maurer, F., Muller, P., Goris, J., Merlevede, W., Hofsteenge, J., and Stone, S. R. (1990) Biochemistry 29, 3166–3173
16. Hendrix, P., Turowski, P., Mayer-Jaekel, R. E., Goris, J., Hofsteenge, J., Merlevede, W., and Hemmings, B. A. (1993) J. Biol. Chem. 268, 7330–7337
17. Dezati, S., Yang, S. L., and Mumber, M. C. (1987) Proc. Natl. Acad. Sci. U. S. A. 84, 6705–6711
18. Doi, H., Swafford, L., Heaton, K. L., and Hemmings, B. A. (1992) Trends Biochem. Sci. 17, 159–162
19. Bialojan, C., and Takai, A. (1988) Biochem. J. 256, 283–290
20. Favre, B., Turowski, P., and Hemmings, B. A. (1997) J. Biol. Chem. 272, 13856–13863
21. Recher, C., Yasebaert, L., Beyer-Rauzy, O., Mansat-Des Mas, V., Ravidats, J. B., Cariven, P., Demur, C., Payrastre, B., Laurent, G., and Racaud-Sultan, C. (2004) Cancer Res. 64, 3191–3197
22. Zhao, L. J., Jhi, H., and Zhu, H. (2003) Gene (Amst.) 316, 137–141
23. Zhang, S., Feng, Y., Narayan, O., and Zhao, L. J. (2001) Gene (Amst.) 263, 131–140
24. Kewl-Goelland, Y., and Hemmings, B. A. (1988) FEBS Lett. 238, 265–268
25. Baumgartner, M., Tardieux, I., Ohayon, H., Gounon, P., and Langsley, G. (1999) Microbes Infect. 1, 1181–1188
26. Nishijima, H., Nishitani, H., Seki, T., and Nishimoto, T. (1997) J. Cell Biol. 138, 1105–1116
27. Furuta, T., Takemura, H., Liao, Z. Y., Aune, G. I., Redon, C., Sedelnikova, O. A., Plitch, D. R., Rogakou, E. P., Celeste, A., Chen, H. T., and Nussenzweig, A., Aladjem, M. I., Bonner, W. M., and Pommier, Y. (2003) J. Biol. Chem. 278, 20303–20312
28. Chowdhury, D., Keogh, M. C., Ishii, H., Peterson, C. L., Buratowski, S., and Lieberman, J. (2005) Mol. Cell 20, 801–809
29. Janssens, V., and Goris, J. (2001) Biochem. J. 353, 417–439
30. Zolnierowicz, S. (2000) Biochem. Pharmacol. 60, 1225–1235
31. Virshup, D. M. (2000) Curr. Opin. Cell Biol. 12, 180–185
32. Janssens, V., Goris, J., and Van Hoof, C. (2005) Curr. Opin. Genet. Dev. 15, 34–41
33. Kremmer, E., Ohst, K., Kiefer, J., Brewis, N., and Walter, G. (1997) Mol. Cell. Biol. 17, 1692–1701
34. Liu, Q., Guntuku, S., Cui, X. S., Matsuoka, S., Cortez, D., Tamai, K., Luo, Y., and Elledge, S. J. (2000) J. Virol. 74, 1950–1955
35. Mailand, N., Bekker-Jensen, S., Bartek, J., and Lukas, J. (2006) Nature Rev. Mol. Cell Biol. 7, 5821–5826
36. Mailand, N., Bixby, M., Aladjem, M. I., Bonner, W. M., and Pommier, Y. (2003) Curr. Opin. Genet. Dev. 13, 587–598
37. Zhou, J., Pham, H. T., Ruediger, R., and Walter, G. (2003) Biochem. J. 369, 387–398
38. McConnell, S., and Darnell, J. E. (1997) Cell 88, 185–191
39. Martin, P., and Chou, L. (2001) Mol. Cell. Biol. 21, 3698–3707
40. Patel, S., and Walker, R. (2000) J. Virol. 74, 6859–6864
41. Liu, Q., Guntuku, S., Yau, T., and Elledge, S. J. (2000) J. Virol. 74, 6859–6864
42. Alam, S., and Darnell, J. E. (2001) Cell 106, 563–572
**PP2A(Aα/Cβ) and ATR Activation**

57. Peschiaroli, A., Dorrello, N. V., Guardavaccaro, D., Venere, M., Halazoneto, T., Sherman, N. E., and Pagano, M. (2006) *Mol. Cell* **23**, 319–329
58. Yoo, H. Y., Jeong, S. Y., and Dunphy, W. G. (2006) *Genes Dev.* **20**, 772–783
59. Liu, S., Bekker-Jensen, S., Mailand, N., Lukas, C., Bartek, J., and Lukas, J. (2006) *Mol. Cell. Biol.* **26**, 6056–6064
60. Robison, J. G., Elliott, J., Dixon, K., and Oakley, G. G. (2004) *J. Biol. Chem.* **279**, 34802–34810
61. Chini, C. C., Wood, J., and Chen, J. (2006) *Oncogene* **25**, 4165–4171
62. Petersen, P., Chou, D. M., You, Z., Hunter, T., Walter, J. C., and Walter, G. (2006) *Mol. Cell. Biol.* **26**, 1997–2011
63. Leung-Pineda, V., Ryan, C. E., and Piwnica-Worms, H. (2006) *Mol. Cell. Biol.* **26**, 7529–7538
64. Ikehara, T., Shinjo, F., Ikehara, S., Imamura, S., and Yasumoto, T. (2006) *Protein Expression Purif.* **45**, 150–156
65. Zhou, J., Pham, H. T., and Walter, G. (2003) *J. Biol. Chem.* **278**, 8617–8622
66. Gotz, J., Probst, A., Ehler, E., Hemmings, B., and Kues, W. (1998) *Proc. Natl. Acad. Sci. U. S. A.* **95**, 12370–12375
67. Gotz, J., Probst, A., Mistl, C., Nitsch, R. M., and Ehler, E. (2000) *Mech. Dev.* **93**, 83–93