Akt-mediated phosphorylation of CDK2 regulates its dual role in cell cycle progression and apoptosis

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Summary
Here, we show that CDK2, an S-phase cyclin-dependent kinase, is a novel target for Akt during cell cycle progression and apoptosis. Akt phosphorylates CDK2 at threonine 39 residue both in vitro and in vivo. Although CDK2 threonine 39 phosphorylation mediated by Akt enhances cyclin-A binding, it is dispensable for its basal binding and the kinase activity. Additionally, for the first time, we report a transient cytoplasmic shuttling of Akt during specific stages of the cell cycle, in particular during the late S and G2 phases. The Akt that is re-localized to the nucleus phosphorylates CDK2 and causes the temporary cytoplasmic localization of the CDK2–cyclin-A complex. The CDK2 cytoplasmic redistribution is required for cell progression from S to G2-M phase, because the CDK2 T39A mutant, which lacks the phosphorylation site and is defective in cytoplasmic localization, severely affects cell cycle progression at the transition from S to G2-M. Interestingly, we also show that the Akt/CDK2 pathway is constitutively activated by some anticancer drugs, such as methotrexate and docetaxel, and under these conditions it promotes, rather than represses, cell death. Thus, the constitutive activation of the Akt/CDK2 pathway and changed subcellular localization promotes apoptosis. By contrast, the transient physiological Akt/CDK2 activation is necessary for cell cycle progression.

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Key words: Akt, Apoptosis, Anti-cancer drugs, CdK2, Cell cycle

Introduction
The phosphatidylinositol 3-kinases (PI3K) constitute a lipid kinase family characterized by their ability to phosphorylate phosphatidylinositol (4,5)-biphosphate [PtdIns(4,5)P2] and convert it to phosphatidylinositol (3,4,5)-trisphosphate [PtdIns(3,4,5)P3] (Furman et al., 1998). The activation of PI3K by various growth factors or insulin is classically mediated by upstream receptor tyrosine kinase dimerization and its subsequent autophosphorylation, but it is also activated in non-canonical pathways, including activation by various (proto-)oncogenes such as Ras, Her2/neu, cKIT and also other intracellular adaptor proteins (reviewed by Vivanco and Sawyers, 2002). Akt is an important effector molecule of the PI3K pathway. It is activated by interaction with the PtdIns(3,4,5)P3 via its pleckstrin homology domain and subsequent phosphorylation at S473 and T308 sites mediated by PDK1 and PDK2. Activated Akt phosphorylates various substrates, either in the cytoplasm or in the nucleus at serine/threonine residues of its RXRXXS/T consensus motif, thus modulating their function in cell survival, cell cycle progression, growth and translation (Vanhaesebroeck and Alessi, 2000). An important function of activated PI3K/Akt in cells is maintaining cell survival via inhibition of apoptosis by targeting BAD, NF-κB and FKHR1 (Cantley, 2002), etc. Although many studies support the role of the PI3K/Akt pathway in cell proliferation and cell survival, there are some exceptions, in which the PI3K pathway is also reported to be involved during promotion of cell death (Aki et al., 2003; Lu et al., 2006; Nimbalkar et al., 2003; Shack et al., 2003). Other than cell survival, Akt regulates cell cycle progression at G1-S and G2-M phases by either phosphorylating directly or regulating the expression indirectly of various substrates, including, but not limited to, p21\textsuperscript{waf1}, p27\textsuperscript{kip1}, cyclin D, c-Myc, GSK-3β, cyclin D1 and FKHR1 (reviewed by Liang and Slingerland, 2003). However, the role of Akt in regulating the progression of S-to-G2 phase during the cell cycle has not been reported so far.

CDK2, a member of the serine/threonine kinase family of cyclin-dependent kinases, is a key regulator of G1-S cell cycle progression (Rosenblatt et al., 1992). By binding to its partners, cyclin E and cyclin A, and the subsequent phosphorylation at threonine 160 (T160) residue by CAK (CDK-activating kinase), CDK2 gets activated during G1 and S phases of the cell cycle. Apart from its role in cell cycle progression, CDK2 has been well documented to have a role in promoting apoptosis in various studies, although the precise mechanism of its dual role in cell cycle progression and apoptosis is not yet known. The subcellular distribution of CDK2 has been shown to be very important for its distinguished role in proliferation and apoptosis (Hiromura et al., 2002).
Here we report that CDK2 is a novel target for Akt. Furthermore, Akt-mediated CDK2 phosphorylation and their altered subcellular localizations are important for cell cycle progression at the G2-M phase. Interestingly, in addition to its role in cell cycle progression, we also report the role of the Akt/CDK2 pathway in promoting apoptosis upon triggering with selected death-inducing stimuli. The constitutive activation of Akt, and altered subcellular localization, promotes apoptosis, whereas transient, cell-cycle-regulated activation and shuttling between the nucleus and the cytoplasm is characteristic for normal activation by pro-survival pathways during cell cycle progression.

Results

Akt phosphorylates CDK2 both in vitro and in vivo

To determine whether Akt phosphorylates CDK2 in vitro, we used a recombinant bacteria-expressed GST-CDK2 protein as a substrate in the kinase assay. Recombinant active Akt, but not its inactive form, is able to phosphorylate CDK2 (Fig. 1A). Further, the measurement of stoichiometry of CDK2 phosphorylation using γ-32P ATP revealed a high level of phosphorylation in the presence of active Akt but not catalytically inactive Akt (supplementary material Fig. S1A). These results were also confirmed by an in vitro kinase assay using a constitutively active form of Akt (Akt-CA), or a catalytically inactive Akt (Akt-K179M) immunoprecipitated from 293T cells (Fig. 1A, lower panel). A preliminary analysis of CDK2 protein for the putative Akt phosphorylation sites revealed two partial Akt consensus sites at threonine 39 (T39) and T160 residues. To determine if these residues are the possible Akt phosphorylation sites, the mutants replaced with a non-phosphorylatable alanine instead of the T39, T160 or both residues were used in the kinase assay. Both, active recombinant Akt or immunoprecipitated Akt phosphorylated CDK2-T160A mutant to the same level as the wild-type protein. No phosphorylation is seen with T39A and T39A/T160A mutants (Fig. 1B and supplementary material Fig. S1B), indicating that the T39 residue is the only Akt target on CDK2.

To determine whether Akt phosphorylates CDK2 in vivo, within cultured cells, we constructed HA-tagged CDK2 wild type, T39A and T160A vectors and transiently transfected them into 293T cells. In the presence of serum, the phosphorylation of CDK2 revealed that both CDK2 and CDK2 T160A mutants were phosphorylated, but T39A and T39A/T160A mutants were not phosphorylated in vivo (Fig. 1C). This was checked using anti-phospho-Akt substrate antibody after immunoprecipitation of CDK2. Further, to determine if Akt is the kinase phosphorylating CDK2 at the T39 site in vivo, either CDK2 wild type or CDK2 T39A vector was transiently expressed in transformed mouse 3T3 fibroblasts. The serum starvation of cells for 30 hours, which inactivated Akt, completely abolished the phosphorylation of CDK2. The activation of Akt, stimulated by either EGF or by reintroduction of 10% serum to the cells restored the CDK2 phosphorylation, which is inhibited in the presence of wortmannin, a selective PI3K/Akt pathway inhibitor (Fig. 1D and supplementary material Fig. S1B). As expected, no Akt-mediated phosphorylation was observed with the CDK2 T39A mutant. This indicates that CDK2 phosphorylation at T39 residue occurs in vivo and is mediated by the PI3K/Akt pathway. In addition, we also tested for the Akt-mediated T39 phosphorylation of endogenous CDK2. Addition of EGF or serum enhances the T39 phosphorylation of endogenous CDK2, which is inhibited by wortmannin (Fig. 1E). To further support the role of Akt as a direct kinase phosphorylating CDK2 at T39 residue, we used an Akt-specific inhibitor (Barnett et al., 2005) in the presence of EGF. Pretreatment of cells with an Akt-specific inhibitor abolished the EGF-induced CDK2 phosphorylation (Fig. 1F), but no effect was seen with pretreatment with rapamycin, an inhibitor of S6 kinase (Akt downstream molecule). Further, we performed a timecourse for T39 phosphorylation of CDK2 and GSK-3β, a known substrate for Akt. The phosphorylation of GSK-3β was spontaneous and was seen as early as 15 minutes after EGF stimulation, whereas CDK2 phosphorylation occurred around 6 hours after EGF stimulation (Fig. 1G), indicating that CDK2 phosphorylation by Akt is a late event and differs from other early Akt substrate phosphorylations.

Akt-mediated CDK2 phosphorylation regulates its cellular localization

To test the functional significance of CDK2 phosphorylation by Akt, we checked the CDK2 kinase activity by using histone H1 as its substrate. The co-transfection of Akt CA, a constitutively active form of Akt along with CDK2 wild type, significantly enhanced the CDK2 kinase activity (Fig. 2A). However, the expression of dominant-negative Akt restored the kinase activity to normal levels, similar to transfection with an empty vector. The expression of active Akt has no effect on total CDK2 protein levels. Interestingly, the kinase activity assessment with the wild-type CDK2 along with the non-phosphorylatable T39A mutant and T39E phosphomimetic mutants revealed that both the mutants were able to phosphorylate histone H1 as effectively as the wild-type protein (Fig. 2A). This indicates that although Akt enhances the CDK2 kinase activity, the T39 phosphorylation has no significant role during basal CDK2 kinase activity. The increase in CDK2 activity during the presence of active Akt might be an indirect effect, as Akt is also known to target other regulators of CDK2 such as p27kip1 and p21^Cip1^ (Liang and Slingerland, 2003). As expected, the other CDK2 mutants lacking the phosphorylation site T160 have no kinase activity. Furthermore, to determine the effect of T39 phosphorylation on CDK2, we analyzed the association of cyclin A and cyclin E with CDK2 under different conditions. The expression of active Akt significantly enhanced the binding of cyclin A to CDK2, which was reduced to normal levels with the expression of dominant-negative Akt (Fig. 2B). By contrast, the activation of Akt had no effect on the binding of cyclin E to CDK2. Interestingly, although the CDK2 T39E phosphomimetic mutant showed slightly enhanced cyclin A binding compared with wild-type CDK2 (Fig. 2C), the T39A mutation on CDK2 again had no effect on the binding of either cyclin A or cyclin E, thus indicating that T39 phosphorylation might not be required for cyclin A or cyclin E binding.

We next tested whether the activation of Akt and CDK2 T39 phosphorylation affects the intracellular localization of CDK2. As shown in Fig. 2D, the wild-type CDK2 was predominantly localized in the cytoplasm in the presence of Akt-CA, but was relocated to the nucleus when dominant-negative Akt was co-expressed. The mutation of CDK2 T39 to a non-phosphorylatable alanine abolished the cytoplasmic localization even in the presence of Akt-CA. CDK2 T39A was predominantly nuclear in the presence of either Akt-CA or Akt-DN. The CDK2 T39E mutant, which mimics the phosphorylation status, was predominantly localized in the cytoplasm irrespective of the activation status of the Akt pathway. As the expression of different forms of Akt might perturb the cell cycle distribution and thus might affect the localization of CDK2 mutants, we further confirmed that the CDK2 localization is dependent on Akt-mediated T39 phosphorylation by checking the localization of T39A mutant in cells synchronized in different phases.
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of the cell cycle. The CDK2 T39A mutant is nuclear irrespective of the cell cycle phase (supplementary material Fig. S2). Thus, these results indicate that the T39 site is important for the regulation of CDK2 intracellular localization and the phosphorylation of this residue by Akt mediated phosphorylation in CDK2 cytoplasmic localization. The phosphorylation-dependent cytoplasmic localization of CDK2 is similar to the effect of Akt-mediated phosphorylation of its substrates Foxo3a and Bad. The Akt-dependent phosphorylation of Foxo3a and Bad leads to their cytoplasmic export from the nucleus via 14-3-3 interactions. Thus, we tested the possibility of phosphorylation-dependent interaction of CDK2 with 14-3-3 by transfecting the cells with either pSer-CDK2 wild-type vector or the mutant proteins all tagged with HA epitope, immunoprecipitated using anti-HA antibodies after 48 hours and blotted with anti-phospho-Akt substrate antibody. The expression of CDK2 wild-type and mutant proteins was detected by anti-HA antibodies. (D) Murine 3T3 fibroblasts were transiently transfected with a wild-type CDK2 or CDK2-T39A mutant and serum-starved for 30 hours, then incubated with 20 ng/ml EGF, in combination with EGF and wortmannin (5 nM) or 10% fetal calf serum. The phosphorylation of CDK2 was detected as in A. Total CDK2 expression, phosphorylated Akt and total Akt were detected by the respective antibodies. (E) 3T3 fibroblasts were serum-starved for 30 hours, then treated exactly as in D. The phosphorylation of endogenous CDK2 was detected by immunoprecipitating with CDK2 antibodies and then immunoblotting with phospho-Akt substrate-specific antibody. Total CDK2 expression was detected by anti-CDK2 antibody. The phosphorylation of CDK2 was detected by immunoprecipitating with CDK2 antibodies and then immunoblotting with phospho-Akt substrate-specific antibody. Total CDK2 expression was detected by anti-CDK2 antibody. (F) Murine 3T3 fibroblasts were transfected with HA-tagged CDK2 wild-type plasmid and then serum-starved for 30 hours. Then they were incubated with a combination of EGF (20 ng/ml) and specific Akt inhibitor (210 nM, Calbiochem) or EGF alone or in combination with EGF and rapamycin. The phosphorylation of CDK2 was detected by immunoprecipitating with CDK2 antibodies and then immunoblotting with phospho-Akt substrate-specific antibody. Total CDK2 expression was detected by anti-CDK2 antibody. (G) 3T3 fibroblasts were transfected with HA-tagged CDK2 wild-type plasmid and then serum-starved for 30 hours. The phosphorylation of CDK2 was detected by immunoprecipitating with CDK2 antibodies and then immunoblotting with phospho-Akt substrate-specific antibody after stimulating the cells with EGF at different indicated time points. For detection of phosphorylated GSK3, cell lysates at indicated time points were immunoblotted with phospho-GSK3 antibody (cell signaling). Total GSK3 and CDK2 were detected using respective antibodies.

Fig. 1. Akt phosphorylates CDK2 both in vitro and in vivo. (A) A non-radioactive in vitro kinase assay was performed with GST-CDK2 alone, using a recombinant active Akt or inactive Akt, and the phosphorylation of CDK2 was detected by immunoblotting with an anti-phospho substrate Akt antibody (upper panel). The levels of GST-CDK2 were detected by immunoblotting with CDK2 antibodies. Constitutively active Akt (Akt-CA) or catalytically inactive Akt (Akt-K179M) immunoprecipitated from 293T cells were used in a similar kinase assay using GST-CDK2 as a substrate, and the phosphorylation of CDK2 was detected as above (lower panel). (B) CDK2 wild-type (WT) proteins or mutated ones at T39, T160 or both were used in a kinase assay with a recombinant active Akt (Rec. Akt) or immunoprecipitated active Akt, and the CDK2 phosphorylation was detected by immunoblotting as in A. The expression of GST-CDK2 wild-type and mutant proteins was monitored by immunoblotting with CDK2 antibody. (C) The CDK2 phosphorylation in vivo was monitored by transfecting 293T cells with either pSer-CDK2 wild-type vector or the mutant proteins all tagged with HA epitope, immunoprecipitated using anti-HA antibodies after 48 hours and blotted with anti-phospho-Akt substrate antibody. The expression of CDK2 wild-type and mutant proteins was detected by anti-HA antibodies. (D) Murine 3T3 fibroblasts were transiently transfected with a wild-type CDK2 or CDK2-T39A mutant and serum-starved for 30 hours, then incubated with 20 ng/ml EGF, in combination with EGF and wortmannin (5 nM) or 10% fetal calf serum. The phosphorylation of CDK2 was detected as in A. Total CDK2 expression, phosphorylated Akt and total Akt were detected by the respective antibodies. (E) 3T3 fibroblasts were serum-starved for 30 hours, then treated exactly as in D. The phosphorylation of endogenous CDK2 was detected by immunoprecipitating with CDK2 antibodies and then immunoblotting with phospho-Akt substrate-specific antibody. Total CDK2 expression was detected by anti-CDK2 antibody. (F) Murine 3T3 fibroblasts were transfected with HA-tagged CDK2 wild-type plasmid and then serum-starved for 30 hours. Then they were incubated with a combination of EGF (20 ng/ml) and specific Akt inhibitor (210 nM, Calbiochem) or EGF alone or in combination with EGF and rapamycin. The phosphorylation of CDK2 was detected by immunoprecipitating with CDK2 antibodies and then immunoblotting with phospho-Akt substrate-specific antibody. Total CDK2 expression was detected by anti-CDK2 antibody. (G) 3T3 fibroblasts were transfected with HA-tagged CDK2 wild-type plasmid and then serum-starved for 30 hours. The phosphorylation of CDK2 was detected by immunoprecipitating with CDK2 antibodies and then immunoblotting with phospho-Akt substrate-specific antibody after stimulating the cells with EGF at different indicated time points. For detection of phosphorylated GSK3, cell lysates at indicated time points were immunoblotted with phospho-GSK3 antibody (cell signaling). Total GSK3 and CDK2 were detected using respective antibodies.
arrested cells had a lower Akt activity. The arrest in different phases of the cell cycle had no effect on the total Akt levels. Furthermore, we tested the Akt-mediated phosphorylation of CDK2 at T39 by immunoprecipitating CDK2 and blotting with anti-phospho-Akt substrate antibody at different phases of the cell cycle. CDK2 was heavily phosphorylated at T39 residue in G2-phase arrested cells, whereas weaker phosphorylation was seen during S phase of the cell cycle (Fig. 3A, lower panel). No phosphorylation was seen during other phases.

In addition to the differential Akt activation status, the localization of Akt in cells arrested in different phases of the cell cycle revealed Akt either in the cytosol or nucleus, depending on the phase of the cell cycle. Akt was mainly cytoplasmic during G0 and G1 phases, whereas during G2 phase it was exclusively seen in the nucleus. During S phase, Akt was dispersed both in the cytoplasm and the nucleus (Fig. 3B, upper panel). As Akt regulates the cellular localization of CDK2, in a separate experiment we also tested the localization of CDK2 in cells arrested in different phases of the cell cycle. CDK2 was mainly nuclear in G1 phase, whereas it was exclusively cytoplasmic during G2 phase of the cell cycle. During G0 and S phases, CDK2 was found both in the nucleus and the cytoplasm (Fig. 3B, lower panel). This correlates with the Akt nuclear localization at S and G2 phases of the cell cycle. These results indicate that activated Akt may translocate to the nucleus during the S phase, phosphorylate CDK2 and promote cytoplasmic CDK2 localization during late S and G2 phases of the cell cycle. To provide further conclusive evidence on the role of phosphorylation on the cellular localization of CDK2, we performed kinetics studies of Akt activation and CDK2 phosphorylation as the cells progressed through the cell cycle. Murine 3T3 fibroblasts arrested in G0 phase by 30-hour serum starvation, were released from G0, and Akt and CDK2 phosphorylation was assessed at different time points as the cells progressed through the cell cycle. Akt was activated immediately after G0 release as the cells entered G1, but was only activated transiently. As the cells progressed from G1 to S phase, the Akt was deactivated. Once S phase was initiated, as the cells prepared for S-to-G2 progression, a second peak of Akt activation was seen around 16-18 hours after G0 release in these cells (Fig. 3C). By contrast, CDK2 activation was not seen during G1 or early S phases but only during late S and G2 phases, as it was evident only after 16-18 hours of G0 release in correlation with a second peak of Akt activation (Fig. 3C, lower panel). As the cells
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went from G2 to M phase, both Akt and CDK2 phosphorylation was decreased (data not shown). The level of total Akt or total CDK2 remained constant for the duration of the experiment. CDK2 was mainly nuclear during G1 and early S phases, but upon Akt-mediated phosphorylation translocated to the cytoplasm during last S phase and G2 phase around 16-18 hours after G0 release (Fig. 3D) and re-localized to the nucleus during late phases of the cell cycle. To test further whether CDK2 alone or an active cyclin-A-bound CDK2 complex is exported to the cytoplasm during G2 phase, we isolated the nuclear and cytoplasmic fractions from the cells...
synchronized in either early S phase or G2 phase and assessed for the localization of CDK2, cyclin A and their activity. As shown in Fig. 3E, the nuclear S-phase-active cyclin-A bound CDK2 was exported to the cytoplasm during G2 phase in an active cyclin-A-bound complex. To further determine the effect of CDK2 phosphorylation on the progression of the cell cycle, we assessed both the G1-S and G2-M population under different conditions. The overexpression of a non-phosphorylatable mutant of CDK2, CDK2-T39A, or inactivation of Akt by overexpression of Akt-DN, resulted in an increase in G1-S cell population and a significant decrease of cells in G2-M phase of the cell cycle. However, the transfection of Akt-CA or wild-type CDK2 increased the G2-M population (Fig. 3F). This indicates that Akt activation and CDK2 phosphorylation are important for S to G2-M progression.

To determine the role of CDK2 T39 phosphorylation on the progression through the S- and G2 phases of the cell cycle, we examined the progression of S to G2 phase in the presence of both phosphorylation active and inactive CDK2 mutants. The 3T3 fibroblasts arrested in S phase were released and the percentage of cells entering G2 was assessed. After 3 hours of S-phase release, the presence of wild-type CDK2 and the CDK2-T160A mutant had no significant effect on the percentage of cells entering G2 phase compared to controls, whereas the CDK2-T39A transfection resulted in the reduction of cells entering from S to G2 (Fig. 3G). Although the CDK2-T39A/T160A has a non-phosphorylatable T39 site, it has no effect on the percentage of G2 cells, as it is inactive as CDK2 kinase. Thus, these results indicate that Akt-mediated transient phosphorylation of CDK2 at the T39 site and the resulting CDK2 cytoplasmic localization occurs in late S and G2 phases and is absolutely necessary for the cells to progress from S to G2-M phases.

**Selected anticancer drugs constitutively activate the PI3K/Akt pathway**

Although Akt and CDK2 are required for cell cycle progression, they have also been assigned an important role in regulating apoptosis, either positively or negatively upon different apoptotic stimuli (Deb-Basu et al., 2006; Shi et al., 1996). We thus tested whether the above-described PI3K/Akt-CDK2 pathway has a role in apoptosis induced by selected anticancer drugs. We screened for the activation of the PI3K-Akt pathway in MCF7 cells using several known anticancer drugs and found that methotrexate (Fig. 4A, upper panel), docetaxel (Fig. 4, lower panel) and doxorubicin (data not shown) were able to constitutively activate Akt. The activation of Akt by methotrexate occurred very rapidly after around 30 minutes, and the kinase remained constitutively active even upon 12 hours of treatment. Docetaxel-induced Akt activation occurred late, after around 6 hours of treatment, and it remained constitutively active even after 30 hours (data not shown). We further tested the localization of Akt in the presence of methotrexate and docetaxel. Akt is mainly cytoplasmic (Fig. 4B, upper panel), but the treatment of MCF7 cells with docetaxel for 6 hours resulted in the nuclear localization of Akt. Also, the treatment of cells with methotrexate for 3 hours induced Akt nuclear localization (Fig. 4B). The nuclear localization of Akt is dependent on the upstream activation of PI3K, as the inhibition of PI3K activity by wortmannin abrogated the nuclear localization of Akt even in the presence of the chemotherapeutics. To further
CDK2 is required for methotrexate- and docetaxel-induced cell death

As methotrexate and docetaxel activated Akt and induced nuclear Akt localization constitutively, we tested whether the Akt/CDK2 pathway is involved in the cell death induced by these drugs. The CDK2 phosphorylation detected by phospho-specific antibody after CDK2 immunoprecipitation revealed that CDK2 was phosphorylated at the T39 site upon treatment of methotrexate (Fig. 5A, upper panel) for 6 hours and docetaxel (Fig. 5A, lower panel) for 9 hours, respectively. The CDK2 phosphorylation induced by methotrexate and docetaxel is dependent on Akt activation, as pretreatment of the cells with wortmannin severely reduced the CDK2 phosphorylation even in the presence of the anticancer drugs. The CDK2 phosphorylation at T39 upon methotrexate and docetaxel treatment results in the constitutive translocation of CDK2 to the cytoplasm (Fig. 5B). To determine whether the CDK2 activity in the cytoplasm is required for cell death induced by these drugs, we used two different approaches. First, we inhibited the CDK2 activity using a pharmacologic CDK2 inhibitor, roscovatine, and treated the cells with anticancer drugs. Roscovatine treatment significantly inhibited cell death induced by both methotrexate and docetaxel (Fig. 5C). In the second approach, we compared the cell death induced by these anticancer drugs in immortalized CDK2 wild-type fibroblasts and the CDK2-deficient fibroblasts. Both methotrexate and docetaxel were able to induce cell death efficiently in wild-type fibroblasts, whereas the cell death is significantly inhibited in CDK2 knockout cells (Fig. 5D). Reconstitution of CDK2-deficient fibroblasts with CDK2 wild-type vector but not T39A mutant restores their sensitivity to cell death induced by methotrexate and docetaxel. These results indicate that Akt-mediated CDK2 phosphorylation at T39 contributes to the cell death induced by methotrexate and docetaxel.

Discussion

Akt phosphorylates several proteins during cell cycle progression, in particular at the boundary of G1-S phases (Liang and Slingerland, 2003). In this report, we demonstrate CDK2 as a novel target for Akt that has a role not only during cell cycle progression but also during apoptosis. The Akt-mediated CDK2 T39 phosphorylation is mainly important for regulating the subcellular distribution of CDK2. The T39 residue is highly conserved among different species, such as humans, mouse and Xenopus laevis. Also, the T39 phosphorylation site is highly conserved among other CDKs, such as p34cdc2. In fact, phosphorylation of p34cdc2 at serine 39 mediated by casein kinase II has previously been reported during G1 phase of the cell cycle (Russo et al., 1992). CDK2 has a very important role as an S-phase cyclin-dependent kinase, in particular during priming of DNA synthesis and its progression (Jiang et al., 1999; Krude et al., 1997; Nougarede et al., 2000). Oscillation of CDK2 activity during G1-S phase is required for proper DNA synthesis and prevention of DNA endo-reduplication (Dahmann et al., 1995). Thus, it is possible that CDK2 phosphorylation by Akt, and its transient cytoplasmic relocation during late S and G2 phases, may be essential for the prevention of DNA endo-reduplication and proper DNA segregation in the cell. Although the expression of active Akt enhances its kinase activity by increased cyclin A binding, we did not observe any defect in the kinase activity of the mutant lacking the T39 phosphorylation site, indicating that T39 phosphorylation is not an essential regulatory event for CDK2 kinase activity, but it may serve other purposes. The phosphorylation of CDK2 occurs at a specific stage of the cell cycle, during late S and G2 phases, and results in a transient relocation of
CDK2 to the cytoplasm, which is required for normal cell cycle progression from S to G2 phase. The experiment demonstrating the entry from S to G2 phase of the cell cycle in the presence of different CDK2 mutants clearly explains the importance of CDK2 phosphorylation, as the CDK2 T39A mutant, which is non-phosphorylatable but is still active as a CDK2 kinase, delays the entry of cells from S to G2-M phases. Thus, we emphasize the fact that the presence of active nuclear CDK2 during the transition to G2 phase inhibits the cell cycle progression. It has been previously reported that Akt should only be transiently active during the cell cycle, as prolonged activation of Akt resulted in G2-M cell cycle arrest (Alvarez et al., 2001). Here we observed a similar effect, that constitutive Akt expression results in G2-M cell cycle arrest. This might be partly due to the CDK2 phosphorylation. The expression of Akt-CA might result in constitutive CDK2 cytoplasmic relocation, and thus CDK2 no longer has access to its nuclear substrates, and to modulate the progression through the M and G1 phases of the cell cycle. In agreement with the previously demonstrated transient Akt activation during the cell cycle, we also report here that under physiologic conditions, not only is Akt activation transient but also the nuclear localization of Akt occurs transiently. Akt is found in the nucleus only during late S and G2 phases, thus acting on a specific set of substrates during these stages of the cell cycle. In addition to CDK2, Akt might also target other cyclin-dependent kinases for phosphorylation. We observed that CDK1 is phosphorylated by Akt in vitro (data not shown). Thus, finding additional targets for Akt during different phases of the cell cycle will provide a clearer picture of the role of Akt during cell cycle progression. Akt has no nuclear localization signal; thus it may require additional interaction partners or nuclear targeting proteins for its nuclear translocation during a particular phase of the cell cycle. Identifying these Akt partner proteins might give us a better understanding of the regulation of its selective nuclear localization.

In addition to the role of Akt/CDK2 in the progression of the cell cycle at the transition from S to G2-M, we have also demonstrated that it has a very important role during apoptosis induced by some anticancer drugs. Here we report that Akt is activated and constitutively translocated to the nucleus during cell-death-inducing stimuli and that this is required for their cell-death-inducing activity. Intriguingly in this context, Akt inhibitors have proved to be only moderately successful in experimental cancer therapy (Stein, 2001; Workman, 2004); furthermore, prolonged Akt activation leads to apoptosis (van Gorp et al., 2006). As shown in Fig. 3B,C, Akt is transiently activated and is localized in the nucleus only during late S and G2 phases of the cell cycle, but then it becomes cytoplasmic as the cell cycle progresses. However, in the presence of anticancer drugs, Akt is constitutively active and is constitutively localized in the nucleus irrespective of the phase of the cell cycle. Thus, the consecutive nuclear localization of Akt contributes to its pro-apoptotic role, instead of normal cell cycle progression.

In addition to the pro-apoptotic role of the nuclear Akt, the phosphorylation and cytoplasmic translocation of CDK2 is also important for the cell-death-inducing activity of both tested drugs methotrexate and docetaxel. The downstream targets for cytoplasmic CDK2 during cell death are not clear. Depending on the localization, CDK2 either promotes cell cycle progression or cell death (Fig. 3B, Fig. 5B). p53 is reported to be one of the targets for CDK2 (Hakem et al., 1999), and we have recently shown that Bcl2 is also phosphorylated by CDK2 in the cytoplasm, and targeted for degradation (S.M. and M.L., unpublished). Identification of additional nuclear and cytoplasmic CDK2 targets might provide clues about the differential roles of CDK2 in the cell cycle and apoptosis. Taken together, the differential activation of Akt/CDK2 (transient vs. constitutive) and localization (nuclear vs. cytoplasmic) might contribute to the different roles of these pathways, and their participation in either cell cycle progression or in apoptosis (summarized in Fig. 6). Thus, our data provide an array of new opportunities to target Akt, CDK2 and possibly other molecules involved in the newly discovered pathway. We envisage the development of a new class of anticancer drugs that influence cellular localization of Akt, and/or CDK2, rather than their activity. Designing molecules that target these pathways might contribute to the development of novel anticancer therapies.

**Materials and Methods**

**Cell culture and reagents**

293-T, MCF7, PC-3 cells, 3T3 fibroblasts, CDK2 wild-type and CDK2<sup>2−/−</sup> immortalized fibroblasts were grown in RPMI-1640 medium supplemented with 10% FBS (HyClone), 100 μg/ml penicillin and 0.1 μg/ml streptomycin (Gibco BRL). The cells were grown at 37°C with 5% CO<sub>2</sub> in a humidified incubator. The primary CDK2<sup>2−/−</sup> fibroblasts were obtained from Dr Kaldis (Berthet et al., 2003) and immortalized with the use of a temperature-sensitive adenoviral T-large antigen, as described previously (Maddika et al., 2005). The following antibodies were used: rabbit anti-Akt, rabbit anti-phospho-Akt, rabbit anti-phospho-specific Akt-substrate antibody (all from Cell Signaling), anti-CDK2, anti-cyclin A, anti-cyclin E (all from Santa Cruz Biotechnologies), rabbit anti-HA, goat-anti-rabbit Cy-3-conjugated secondary antibody (Sigma), anti-phospho-histone H1, anti-rabbit and anti-mouse HRP-conjugated secondary antibodies (Upstate Solutions).

**Plasmids, site-directed mutagenesis and transfections**

The following plasmids were used: Akt-CA and Akt-K179M vectors were described before (Gibson et al., 2002). Adenoviral Akt-DN (a gift from Dr K. Walsh) and adenoviral nuclear Akt (a kind gift from Dr Marek Sussman, San Diego) infections were performed as described before (Fujio et al., 1999). p5R-HA CDK2 T160A mutant...
Determination of stoichiometry of CDK2 phosphorylation
The stoichiometry of CDK2 phosphorylation was determined as described previously (Nunoki et al., 1989). Briefly, the kinase assay was carried out as described above, using γ-32P ATP to a final concentration of 200 μM. Samples were analyzed by SDS-PAGE and silver stained. The bands corresponding to phosphorylated CDK2 were cut out, de-stained in 40% isopropanol at 56°C and dissolved in 2 ml of 50% H2O2 (v/v). The amount of incorporated 32P was counted and the stoichiometry of phosphorylation was calculated from the counts along with the specific activity of 32P and the protein concentration applied to the gel.

Cell fractionation
The separation of nuclear and cytoplasmic fractions was performed as exactly described (Maddika et al., 2005).

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