Normal Cell Cycle and Checkpoint Responses in Mice and Cells Lacking Cdc25B and Cdc25C Protein Phosphatases

Angela M. Ferguson,1 Lynn S. White,2 Peter J. Donovan,3,4 and Helen Piwnica-Worms1,2,5*

Department of Cell Biology and Physiology,1 Howard Hughes Medical Institute,2 and Department of Internal Medicine,3 Washington University School of Medicine, St. Louis, Missouri, and Department of Obstetrics and Gynecology3 and Institute for Cell Engineering.4 Johns Hopkins University School of Medicine, Baltimore, Maryland

Received 1 October 2004/Returned for modification 10 December 2004/ Accepted 7 January 2005

The Cdc25 family of protein phosphatases positively regulates cell division by activating cyclin-dependent protein kinases (CDKs). In humans and rodents, there are three Cdc25 family members—denoted Cdc25A, Cdc25B, and Cdc25C—that can be distinguished based on their subcellular compartmentalization, their abundances and/or activities throughout the cell cycle, the CDKs that they target for activation, and whether they are overexpressed in human cancers. In addition, murine forms of Cdc25 exhibit distinct patterns of expression throughout development and in adult tissues. These properties suggest that individual Cdc25 family members contribute distinct biological functions in embryonic and adult cell cycles of mammals. Interestingly, mice with Cdc25 disrupted are healthy, and cells derived from these mice exhibit normal cell cycles and checkpoint responses. Cdc25B−/− mice are also generally normal (although females are sterile), and cells derived from Cdc25B−/− mice have normal cell cycles. Here we report that mice lacking both Cdc25B and Cdc25C are obtained at the expected Mendelian ratios, indicating that Cdc25B and Cdc25C are not required for mouse development or mitotic entry. Furthermore, cell cycles, DNA damage responses, and Cdc25A regulation are normal in cells lacking Cdc25B and Cdc25C. These findings indicate that Cdc25A, or possibly other phosphatases, is able to functionally compensate for the loss of Cdc25B and Cdc25C in mice.
bound form, and the overproduction of a mutant of Cdc25C that cannot bind to 14-3-3 proteins causes a partial bypass of both the DNA replication and G2 DNA damage checkpoints (49). Cdc25A stability is regulated as a function of the cell cycle, and Cdc25A is rapidly degraded in a proteasome-dependent manner in cells exposed to UV light or ionizing radiation (IR) (4, 6, 12, 16, 21, 24, 30, 41, 42, 44, 66). Chk1 phosphorylates Cdc25A to target it for proteolysis during an unperturbed cell cycle, and the integrity of the Chk1/Cdc25A pathway is required for cells to delay in the S and G2 phases of the cell cycle following checkpoint activation (57, 63, 66). In addition, the overexpression of Cdc25A bypasses the G1 DNA damage checkpoint and the intra-S-phase checkpoint, resulting in enhanced DNA damage and decreased cell survival (42, 44).

In mice, Cdc25A, Cdc25B, and Cdc25C exhibit overlapping but distinct patterns of expression during development and are expressed in tissue-specific patterns in adult mice (32, 46, 61, 62). This fact suggests that these genes have distinct biological functions in embryonic and adult mice. Mice lacking Cdc25C are viable, develop normally, and do not display any obvious abnormalities (7). Furthermore, the phosphorylation status of Cdk1, the timing of entry into mitosis, and the cellular responses to DNA damage are unperturbed in mouse embryo fibroblasts (MEFs) lacking Cdc25C (7). Mice with Cdc25B disrupted are also viable and healthy, although females are sterile due to a meiotic defect during oogenesis (40). One explanation for the lack of a cell cycle phenotype in mice with either Cdc25B or Cdc25C disrupted is compensation by the other family members and/or other phosphatases. To address this possibility, we generated mice lacking both Cdc25B and Cdc25C. Surprisingly, these mice are obtained at the expected Mendelian ratios and are healthy, demonstrating that Cdc25B and Cdc25C are dispensable for murine embryonic development and for mitotic entry.

**Materials and Methods**

**Generation and genotyping of mice with Cdc25B and Cdc25C disrupted.** Mice with both Cdc25B and Cdc25C disrupted were generated by first mating Cdc25B−/− (40) males with Cdc25C−/− (7) females. F2 double-heterozygous littersmates of the strain background C57BL/6J:129S1/SvJ were then interbred to generate Cdc25B−/−Cdc25C−/− double knockout mice. PCR analysis was carried out on genomic tail DNA of wild-type (WT) mice, Cdc25B−/− knockout mice, Cdc25C−/− knockout mice, and the Cdc25B−/−Cdc25C−/− double knockout mice (BCKO) by using specific primers to confirm the genotypes of the mice. PCR analysis for Cdc25B was achieved by using a three-primer PCR with primer 5′-GTTCTGGTACATTGCTTGAG and primer 5′-CACCTCTCAGTCCTGTGTA amplified a region between exon 2 and exon 3. Primer 5′-CCTTCATGCTTGAGCGC amplified the 1.2-kb BglII/SacII restriction fragment of the murine Cdc25B cDNA, which was detected with Gibco-BRL. Cdk1 was detected with a mouse monoclonal antibody (Zymed) or horseradish peroxidase-conjugated rat anti-rabbit antibody (Jackson ImmunoResearch, Inc.), and proteins were visualized by chemiluminescence with the ECL reagent (Amersham Pharmacia Biotech). Cdk1 was detected with a mouse monoclonal antibody (Zymed) or horseradish peroxidase-conjugated rat anti-rabbit antibody (Jackson ImmunoResearch, Inc.), and proteins were visualized by chemiluminescence with the ECL reagent (Amersham Pharmacia Biotech).

**Northern blotting.** RNA was isolated from cells by using the QuickPrep total RNA extraction kit (Amersham Pharmacia Biotech) according to the manufacturer’s suggestions. RNA was resolved on a 1.2% agarose gel and then transferred to a GeneScreen Plus membrane (NEN). The probes used for screening mouse tissues and cells include a 440-bp HincII/DraIII restriction fragment of the murine Cdc25C (mCdc25C) cDNA, a 427-bp XmnI/PvuI restriction fragment of the murine Cdc25A cDNA, a 666-bp BglII/SacII restriction fragment of the mCdc25A cDNA, a 600-bp HindIII/EcoRI restriction fragment of GAPDH, and an 1.2-kb XbaI/HindIII restriction fragment of the neomycin phosphotransferase gene. Probes were labeled with dCTP (α-32P; NEN) by using the Megaprime DNA labeling system (Amersham). Blots were prehybridized with ExpressHyb solution (Clontech) containing 100 μg of sonicated salmon sperm DNA/ml for 2 to 3 h at 68°C with shaking. Labeled probe was added to 10^6 cpm/ml, and the blot was hybridized in ExpressHyb solution for 2 h at 68°C. Blots were washed with 2× SSC (1× SSC is 0.15 M NaCl plus 0.01 M sodium citrate)–0.5% sodium dodecyl sulfate (SDS) at room temperature twice and then washed once with 2× SSC–0.5% SDS for 15 min with shaking at 50°C.

**Generation of mouse embryo fibroblasts.** Mouse embryonic fibroblasts were derived from 13.5-day-old embryos. Following the removal of the head and organs, each embryo was rinsed with PBS, minced, and digested with trypsin-EDTA (0.5% trypsin, 0.5 mM EDTA) for 10 min at 37°C, using 1 ml per embryo. Trypsin was inactivated by the addition of Dulbecco’s modified Eagle’s media (DMEM; Gibco-BRL) containing 10% fetal bovine serum (FBS), 2 mM l-glutamine, a 0.1 mM concentration of nonessential amino acids, 140 mM 2-mercaptoethanol, 100 U of penicillin G/ml, and 100 μg of streptomycin/ml. Cells from single embryos were plated into one 100-mm-diameter tissue culture dish and incubated at 37°C in a 10% CO2-humidified chamber for 3 days and then trypsinized. Each trypsinization and replating represented one passage, and the split ratio of each passage was 1:3. Early-passage cells (P2 to P4, prior to crisis) were analyzed for cell cycle progression and for cellular responses to DNA damage.

**Antibodies.** Fluorescein isothiocyanate (FITC)-conjugated mouse anti-brdU monoclonal antibody was purchased from BD Biosciences. Mouse monoclonal antibodies specific for Cdc25A were raised against baculovirus-produced glutathione S-transferase (GST)-Cdc25A (1:1000 dilution) or Cdc25C (1:1000 dilution) and were detected with a sheep polyclonal antibody (Sigma Immunogenetics) against mouse Cdc25B. A rabbit polyclonal antibody (48). β-Catenin mouse monoclonal antibodies were purchased from BD Transduction Laboratories. Phosphohistone H3 antibodies were purchased from Upstate Biotechnology (Lake Placid, N.Y.) and then visualized with FITC-conjugated goat anti-rabbit antibody (Jackson Immunoresearch, Inc.). Bound primary antibodies were detected with horseradish peroxidase-conjugated rat anti-rabbit antibody (Zymed) or horseradish peroxidase-conjugated goat anti-mouse immunoglobulin G (IgG) plus IgM antibody (Jackson ImmunoResearch, Inc.), and proteins were visualized by chemiluminescence with the ECL reagent (Amersham Pharmacia Biotech).

**S-to-G1-phase progression in absence and presence of IR.** A total of 10^5 MEFs were seeded onto 100-mm-diameter tissue culture dishes 36 h prior to bromodeoxyuridine (BrdU) labeling. Cells were incubated in 5 ml of culture medium (DMEM, 10% FBS, 2 mM l-glutamine, a 0.1 mM concentration of nonessential amino acids, 140 mM 2-mercaptoethanol, 100 U of penicillin G/ml, and 100 μg of streptomycin/ml) containing 20 mM bromodeoxyuridine (Amersham Pharmacia Biotech) at 37°C for 1 h. Medium was removed and replaced with 10 ml of culture medium, and cells were incubated for the indicated times. Cells were harvested by trypsinization and collected by centrifugation. After the removal of the supernatant by aspiration, cells were washed once in PBS and were then suspended in 0.5 ml of PBS. Cells were fixed by the addition of 5 ml of 70% ethanol at 4°C in the dark. Pelleted cells were resuspended in 1 ml of 0.4 μg of pepsin (Sigma Chemical Co.)/ml in 0.1 N HCl and incubated with rocking for 30 min. Nuclear pellets were suspended in 1 ml of 2 N HCl–0.5% NP-40, and incubated with rocking for 1 h. After neutralization by incubation with 1 ml of 0.1 M sodium borate (pH 8.5) for 5 min, nuclear pellets were suspended in 100 μl of PBS-TB (PBS, 0.5% Tween 20, 1% bovine serum albumin [BSA]) and stained with 5 μl of FITC-conjugated anti-BrdU monoclonal antibody for 1 h in the dark. Nuclei were washed once with 1 ml of PBS-TB and then incubated with 1 ml of PBS-TB containing 30 μg of propidium iodide (PI)/ml and 250 μg of RNase A/ml for 30 min in the dark. Cells were analyzed for DNA content by flow cytometry using a FACSCalibur instrument (Becton Dickinson Instruments). The data were analyzed using CELLQUEST analysis software (Becton Dickinson).

**G0-to-S-phase progression.** A total of 10^5 MEFs were seeded onto 100-mm-diameter tissue culture dishes. The following day, cells were incubated in 10 ml of medium (DMEM, 2 mM l-glutamine, a 0.1 mM concentration of nonessential amino acids, 100 U of penicillin G/ml, and 100 μg of streptomycin/ml) containing 0.1% FBS for 96 h. Cells were resuspended at 10^6 in medium containing 10% FBS.

**Histology.** Tissues were fixed in 10% neutral buffered formalin, rinsed in phosphate-buffered saline (PBS), and stored in 70% ethanol. Fixed tissues were embedded in paraffin by using standard procedures. Blocks were sectioned (5 mm) and stained with hematoxylin and eosin.
and pulsed with 20 μM BrdU for 1 h prior to harvest. Harvesting, fixing, staining, and analysis of cells were carried out as described above.

IR-induced G₁-phase checkpoint in MEFs. Cells were plated and serum starved as for G₁ analysis with the following changes. After serum starvation, 2 × 10⁶ cells were seeded onto 100-mm-diameter tissue culture dishes and either mock irradiated or exposed to 20 Gy of IR from a 60Co source. Medium containing 10% FBS and 20 μM BrdU was added, and cells were harvested 24 h later. Cells were fixed, stained, and analyzed by flow cytometry as described above.

IR-induced G₂-phase checkpoint in thymocytes. Eight- to 10-week-old mice were irradiated with 10 Gy of IR, and injected intraperitoneally with 1 ml of 10 mM BrdU–1 mM fluorodeoxyuridine (Amersham Pharmacia Biotech) at 2 h postirradiation (3). Mice were sacrificed 1 h after the injection, and thymocytes were isolated by crushing thymi between glass slides. The prepared thymocytes were fixed, stained, and analyzed by flow cytometry as outlined above.

IR-induced S-phase checkpoint. MEFs were labeled with 10 nCi of [³H]-thymidine (NEN Life Science Products)/ml for 24 h to control for total DNA content between samples. Cells were then washed and incubated in culture medium for 24 h. Cells were either mock irradiated or exposed to 5, 10, or 20 Gy of IR, incubated for the indicated times, and then pulse labeled with 2.5 μCi of [³H]-thymidine (NEN Life Science Products)/ml for 15 min. Cells were harvested, washed twice in PBS, and then incubated in 100% methanol for 5 min, followed by incubation in 10% trichloroacetic acid for 5 min and then 0.3 N NaOH. After neutralization with HCl, radioactivity was determined by liquid scintillation counting. The resulting ratios of [³H] counts per minute to [¹³C] counts per minute, corrected for counts per minute that resulted from channel crossover, were measures of DNA synthesis.

IR-induced G₁-phase checkpoint. MEFs were either mock irradiated or exposed to 6 Gy of IR. After 40 min of incubation, 100 ng of nocodazole/ml was added to trap cells in mitosis, and samples were harvested by trypsinization 1 h 20 min later. A total of 5 × 10⁵ cells were fixed by the addition of 5 ml of 70% ethanol at 4°C for as long as 24 h. After fixation, cells were washed twice with PBS, resuspended in 1 ml of 0.25% Triton X-100 in PBS, and incubated at 4°C with rocking for 15 min. After centrifugation, cell pellets were suspended in 100 μl of PBS containing 1% BSA and 0.75 μg of anti-histone H3 antibody and incubated for 1.5 h at room temperature. Cells were rinsed with PBS containing 1% BSA and incubated with FITC-conjugated goat anti-rabbit IgG antibody diluted at a ratio of 1:1,200 in PBS containing 1% BSA. After a 30-min incubation at room temperature in the dark, cells were washed again and incubated with PBS with 1% BSA containing 30 μg of PI/ml and 250 μg of RNase A/ml for 30 min, also in the dark. Cells were analyzed by flow cytometry as described above.

Synchronization of MEFs and analysis of Cdk1 phosphorylation. A total of 5 × 10⁵ MEFs were seeded onto 100-mm-diameter tissue culture dishes. The following day, cells were incubated in 10 ml of culture medium containing 0.1% FBS for 48 h. Cells were then incubated in culture medium containing 15% FBS and 1 μg of aphidicolin/ml (Calbiochem) for 20 h. Cells were released from the aphidicolin block by being washed twice with 10 ml of PBS and then being incubated in culture medium containing 15% FBS. Cells were harvested prior to release (time zero) or at 3, 6, 9, or 12 h after release by trypsinization and collected by centrifugation. After the removal of the supernatant by aspiration, cells were washed once in PBS and then were suspended in 0.5 ml of PBS. Cells were fixed by the addition of 5 ml of 70% ethanol at 4°C. Cells were washed once with 1 ml of PBS–1% FBS and then incubated with 1 ml of PBS–1% FBS containing 30 μg of PI/ml and 250 μg of RNase A/ml for 30 min. Cells were analyzed for DNA content by flow cytometry using a FACSCalibur instrument (Becton Dickinson). The data were analyzed by using CELLQUEST analysis software (Becton Dickinson). For monitoring Cdk1 phosphorylation, cells were lysed in MCLB (50 mM Tris-HCl, pH 8.0, 2 mM dithiothreitol, 5 mM EDTA, 0.5% Nonidet P-40, 100 mM NaCl, 1 μM sodium orthovanadate, phosphatase inhibitor cocktail [containing 25 μM [γ-³P]-bromotetramisole oxalate, 5 mM cantharidin, and 5 mM microcystin LR; Calbiochem] and protease inhibitor cocktail [containing 1,04 mM 4-(2-aminoethyl) benzene sulfonyl fluoride (AEBSF), 15 μM pepstatin A, 14 μM E-64, 40 μM bestatin, 20 μM leupeptin, and 800 nM aprotinin; Sigma] for 15 min. at 4°C. Cell lysates containing 2 mg of total cellular protein were incubated with 50 μl of packed p13suc1 agarose (Upstate Biotechnology). After incubation at 4°C for 3 h, precipitates were washed four times with 1 ml of MCLB. Proteins were resolved by SDS-polyacrylamide gel electrophoresis on a 12% SDS gel. Cdk1 phosphorylation status was monitored by immunoblotting.

Monitoring Cdc25A levels. Asynchronously growing populations of MEFs were lysed in MCLB. Lysates were resolved by SDS-polyacrylamide gel electrophoresis on an 8% SDS gel, transferred to nitrocellulose, and incubated with mouse ascites specific for mCdc25A. For synchronization studies, 6 × 10⁶ MEFs were seeded onto 100-mm-diameter tissue culture dishes and serum starved in the same manner as for the Cdk1 studies. Cells were harvested prior to release (time zero) or at 3, 6, 9, or 12 h after release, analyzed for DNA content by flow cytometry, and processed to monitor Cdc25A levels by Western blotting.

RESULTS AND DISCUSSION

Mice heterozygous for both Cdc25B (40) and Cdc25C (7) were bred to generate mice lacking both phosphatases. PCR analysis was carried out on genomic tail DNA by using specific primers to confirm the genotypes of all mice (Fig. 1A). The cumulative genotyping of heterozygous crosses resulted in the expected ratio of each genotype (Table 1). Given that mice lacking both Cdc25B and Cdc25C were obtained at the expected Mendelian ratios, this outcome indicates that Cdc25B and Cdc25C are not required for mouse development or mitotic entry. All major organs from BCKO mice were examined histologically after hematoxylin-eosin staining and appeared normal. Microscopy revealed that histology of the major organs was indistinguishable from that of WT and homozygous Cdc25C−/− mice. This result indicates that Cdc25B and Cdc25C are not required for normal mouse development. All mice were normal in size, structure, and behavior. No differences were evident in the offspring of heterozygous Cdc25B−/− mice compared to the offspring of WT mice. This result indicates that Cdc25B is not required for normal mouse development.
The weights of individual mice from birth to 3 months of age were also determined (Fig. 1B and C). As previously reported, males and females were 8 to 14% and 6 to 10% smaller than their WT littermates, respectively, beginning at 4 weeks of age. The basis for the differences in weights is unknown at this time.

Wild-type and BCKO MEFs were also analyzed for their ability to traverse from G0 into S phase. MEFs were synchronized by serum starvation, and at various times after the addition of complete media, cells were harvested and processed for analysis by flow cytometry. By gating on BrdU-positive cells, the timing of S-phase entry from G0 into S phase can be monitored. As seen in Fig. 2A, similar percentages of WT and Cdc25B/−/− mice were indistinguishable (data not shown) (7). In contrast, Cdc25B/−/+ and BCKO mice were 8 to 14% and 6 to 10% smaller than their WT littermates, respectively, beginning at 4 weeks of age. The basis for the differences in weights is unknown at this time.

**Cell cycle progression is normal in cells lacking Cdc25B and Cdc25C.** Fibroblasts derived from wild-type and knockout mouse embryos were analyzed for their ability to traverse from G0 into S phase. MEFs were synchronized by serum starvation, and at various times after the addition of complete media, cells were incubated with BrdU for 1 h to specifically label cells that were undergoing DNA replication. Cells were stained for DNA content with PI and for replicative DNA synthesis with anti-BrdU antibody. As expected, a loss of the slower electrophoretic form of Cdk1 was observed as cells entered mitosis (lane 1), as well as cells in the S (lane 2) and G2 (lane 3) phases of the cell cycle. As expected, a loss of the slower electrophoretic form of Cdk1 with a concomitant increase in the fastest electrophoretic form of Cdk1 was observed as cells entered mitosis (lane 4) and moved into G1 (lane 5). The slight delay in the dephosphorylation of Cdk1 seen in BCKO cells at the 9-h time point is a consistent finding. However, the cell cycle analysis did not reveal significant differences between wild-type and BCKO cells in their abilities to traverse through the cell cycle after release from the G1/S-phase arrest. These findings demonstrate that sufficient quantities of active Cdk1 were generated in BCKO cells to drive cells into mitosis.

**Cells lacking Cdc25B and Cdc25C respond normally to ionizing radiation.** Ionizing radiation activates the IR-induced DNA damage checkpoint to induce cell cycle delays. This checkpoint monitors the integrity of the genome and arrests cells in G1 before DNA replication (termed the IR-induced G1-phase checkpoint), in S phase (the IR-induced S-phase checkpoint), or in G2 before mitosis (the IR-induced G2-phase checkpoint). Checkpoints target the Cdc25A and Cdc25C regulatory pathways to elicit cell cycle delays following ionizing radiation exposure (15, 37, 41, 42, 44, 49, 66). To monitor the IR-induced G1-phase checkpoint in wild-type and BCKO cells, early passage MEFs were synchronized by serum starvation, released into complete medium containing BrdU, and immediately mock irradiated or gamma irradiated. Cells harvested 24 h after release were stained for DNA content with PI and for replicative DNA synthesis with anti-BrdU antibody. As seen in Fig. 3A, wild-type and BCKO cells showed 57 and 48% reductions, respectively, in the numbers of S-phase cells relative to untreated controls. These findings indicate that the time required for cells to progress from S to G1 phases of the cell cycle is unaltered in murine cells lacking Cdc25B and Cdc25C.

Next, the phosphorylation status of Cdk1 was monitored in early passage fibroblasts derived from wild-type and BCKO mouse embryos. Cdk1 is proposed to be a key target of Cdc25B and Cdc25C in vivo. The phosphorylation of Cdk1 varies as a function of the cell cycle, and changes in the electrophoretic mobility of Cdk1 can be used as a specific indicator of Cdk1 phosphorylation status and cell cycle position (2, 56). Wild-type and BCKO MEFs were arrested in early S phase, by first being cultured in medium containing low serum and then being released into complete medium containing aphidicolin. Cells released from G1/S arrest were then analyzed for DNA content by flow cytometry and were processed to monitor Cdk1 phosphorylation status (Fig. 2C). Wild-type and BCKO cells were in S phase by 3 h after release, in G2 phase by 6 h after release, and in the M and G1 phases of the cell cycle by 9 and 12 h after release, respectively. As seen in Fig. 2C, two electrophoretic forms of Cdk1 were present in cells arrested at the G1/S border (lane 1), as well as cells in the S (lane 2) and G2 (lane 3) phases of the cell cycle. As expected, a loss of the slower electrophoretic form of Cdk1 with a concomitant increase in the fastest electrophoretic form of Cdk1 was observed as cells entered mitosis (lane 4) and moved into G1 (lane 5). The slight delay in the dephosphorylation of Cdk1 seen in BCKO cells at the 9-h time point is a consistent finding. However, the cell cycle analysis did not reveal significant differences between wild-type and BCKO cells in their abilities to traverse through the cell cycle after release from the G1/S-phase arrest. These findings demonstrate that sufficient quantities of active Cdk1 were generated in BCKO cells to drive cells into mitosis.

### Table 1. Actual and expected genotypes of double-heterozygous mating

<table>
<thead>
<tr>
<th>Parameter (n)</th>
<th>W/W</th>
<th>W/H</th>
<th>W/K</th>
<th>H/W</th>
<th>H/H</th>
<th>H/K</th>
<th>K/W</th>
<th>K/H</th>
<th>K/K</th>
</tr>
</thead>
<tbody>
<tr>
<td>Expected ratio</td>
<td>1 (6.25)</td>
<td>2 (13)</td>
<td>1 (6.25)</td>
<td>2 (13)</td>
<td>4 (26)</td>
<td>2 (13)</td>
<td>1 (6.25)</td>
<td>2 (13)</td>
<td>1 (6.25)</td>
</tr>
<tr>
<td>No. of mice</td>
<td>Males (398)</td>
<td>32 (8.04)</td>
<td>47 (11.81)</td>
<td>29 (7.29)</td>
<td>46 (11.56)</td>
<td>93 (23.37)</td>
<td>48 (12.06)</td>
<td>25 (6.28)</td>
<td>53 (13.32)</td>
</tr>
<tr>
<td></td>
<td>Females (412)</td>
<td>25 (6.07)</td>
<td>68 (16.50)</td>
<td>33 (8.01)</td>
<td>51 (12.38)</td>
<td>95 (23.06)</td>
<td>44 (10.68)</td>
<td>25 (6.07)</td>
<td>40 (9.71)</td>
</tr>
<tr>
<td>No. of MEFs (236)</td>
<td>19 (8.05)</td>
<td>31 (13.14)</td>
<td>14 (5.93)</td>
<td>24 (10.17)</td>
<td>80 (33.89)</td>
<td>22 (9.32)</td>
<td>7 (2.97)</td>
<td>24 (10.17)</td>
<td>15 (6.36)</td>
</tr>
<tr>
<td>Total no. of mice and MEFs (1,046)</td>
<td>76 (7.26)</td>
<td>146 (13.96)</td>
<td>76 (7.27)</td>
<td>121 (11.57)</td>
<td>268 (25.62)</td>
<td>114 (10.89)</td>
<td>57 (5.45)</td>
<td>117 (11.19)</td>
<td>71 (6.79)</td>
</tr>
</tbody>
</table>

* The first genotype represents Cdc25B, and the second genotype represents Cdc25C. W, wild type; H, heterozygous; K, knockout.

normal (data not shown). Fluorescence-activated cell-sorting (FACS) analysis using the T-cell markers CD4 and CD8 revealed the expected percentages of single-negative (CD4−CD8− and CD4−CD8+) and double-negative (CD4−CD8+) T cells in the peripheral blood of BCKO animals (data not shown).

The weights of individual mice from birth to 3 months of age were also determined (Fig. 1B and C). As previously reported, weights of WT and Cdc25C/−/− mice were indistinguishable (data not shown) (7). In contrast, Cdc25B/−/− and BCKO mice were 8 to 14% and 6 to 10% smaller than their WT littermates, respectively, beginning at 4 weeks of age. The basis for the differences in weights is unknown at this time.

**Cell cycle progression is normal in cells lacking Cdc25B and Cdc25C.** Fibroblasts derived from wild-type and knockout mouse embryos were analyzed for their ability to traverse from G0 into S phase. MEFs were synchronized by serum starvation, and at various times after the addition of complete media, cells were incubated with BrdU for 1 h to specifically label cells that were undergoing DNA replication. Cells were stained for DNA content with PI and for replicative DNA synthesis with anti-BrdU antibody. As expected, a loss of the slower electrophoretic form of Cdk1 was observed as cells entered mitosis (lane 1), as well as cells in the S (lane 2) and G2 (lane 3) phases of the cell cycle. As expected, a loss of the slower electrophoretic form of Cdk1 with a concomitant increase in the fastest electrophoretic form of Cdk1 was observed as cells entered mitosis (lane 4) and moved into G1 (lane 5). The slight delay in the dephosphorylation of Cdk1 seen in BCKO cells at the 9-h time point is a consistent finding. However, the cell cycle analysis did not reveal significant differences between wild-type and BCKO cells in their abilities to traverse through the cell cycle after release from the G1/S-phase arrest. These findings demonstrate that sufficient quantities of active Cdk1 were generated in BCKO cells to drive cells into mitosis.

**Cells lacking Cdc25B and Cdc25C respond normally to ionizing radiation.** Ionizing radiation activates the IR-induced DNA damage checkpoint to induce cell cycle delays. This checkpoint monitors the integrity of the genome and arrests cells in G1 before DNA replication (termed the IR-induced G1-phase checkpoint), in S phase (the IR-induced S-phase checkpoint), or in G2 before mitosis (the IR-induced G2-phase checkpoint). Checkpoints target the Cdc25A and Cdc25C regulatory pathways to elicit cell cycle delays following ionizing radiation exposure (15, 37, 41, 42, 44, 49, 66). To monitor the IR-induced G1-phase checkpoint in wild-type and BCKO cells, early passage MEFs were synchronized by serum starvation, released into complete medium containing BrdU, and immediately mock irradiated or gamma irradiated. Cells harvested 24 h after release were stained for DNA content with PI and for replicative DNA synthesis with anti-BrdU antibody. As seen in Fig. 3A, wild-type and BCKO cells showed 57 and 48% reductions, respectively, in the numbers of S-phase cells rela-
tive to the numbers of mock-irradiated control cells at the 24 h time point. The difference was not statistically significant ($P = 0.2909$), indicating that the ability of cells to delay entry into S phase is intact in cells lacking Cdc25B and Cdc25C.

The IR-induced G1-phase checkpoint in mice was also monitored (3). Animals were mock irradiated or irradiated with 10 Gy of IR and injected with BrdU 2 h postirradiation. At 1 h postinjection, mice were sacrificed and their thymi were dissected. Thymocytes were analyzed by flow cytometry. As seen in Fig. 3B, irradiated wild-type and BCKO mice exhibited 48 and 51% reductions, respectively, in the numbers of thymocytes undergoing DNA replication compared with the numbers of their mock-irradiated counterparts. The difference was not statistically significant ($P = 0.1638$), indicating that the ability of thymocytes to delay entry into S phase is intact in animals lacking Cdc25B and Cdc25C.

To determine whether the absence of Cdc25B and Cdc25C perturbed the IR-induced S-phase checkpoint, [3H]thymidine incorporation into DNA was monitored 1 h after exposure of cells to various doses of IR (Fig. 3C). Both wild-type and BCKO cells had an intact S-phase checkpoint, as indicated by...
Cdc25B and Cdc25A, mRNAs are due to the insertion of the neomycin phosphotransferase gene after homologous recombination. Importantly, Cdc25B and Cdc25C proteins are not produced in BCKO cells (7, 40). A mouse monoclonal antibody raised against recombinant GST-mCdc25A was used to examine the levels of Cdc25A protein in various primary MEF strains. To test the specificity of the monoclonal antibody, GST-tagged Cdc25A, Cdc25B, and Cdc25C were blotted with the monoclonal antibody and an antibody against GST (Fig. 4B). Only GST-mCdc25A was recognized by the monoclonal antibody, indicating that it does not cross-react with mCdc25B or mCdc25C. Differences in total cellular levels of Cdc25A proteins of various MEF strains were observed, but a consistent pattern of elevated levels of Cdc25A in BCKO-derived MEFs was not seen (Fig. 4C). Next, Cdc25A regulation as a function of the cell cycle was monitored because the abundance of Cdc25A is regulated in a cell cycle-specific manner by ubiquitin-mediated proteolysis (4, 6, 12, 26, 30, 41, 42, 44). As seen in Fig. 4D, Cdc25A levels rose as wild-type and BCKO MEFs progressed from S phase through G2 and into mitosis and then fell as cells exited mitosis and entered G1. A substantial increase in Cdc25A was observed in cells arrested with nocodazole in mitosis. Thus, the regulation of Cdc25A turnover during the cell cycle was normal in cells lacking Cdc25B and Cdc25C.

Concluding remarks. Mice lacking two members of the Cdc25 family of protein phosphatases, Cdc25B and Cdc25C, are viable, develop normally, and do not display any obvious abnormalities with the exception that females lacking both phosphatases are sterile, as are females lacking Cdc25B alone. Importantly, these findings challenge the paradigm that entry into mitosis requires Cdc25B and Cdc25C. It is unknown at this time whether Cdc25A, the remaining member of the Cdc25 family, functionally compensates for the lack of Cdc25B and Cdc25C or whether other compensatory pathways are involved. Importantly, Cdc25A has a docking site for Cdk1/cyclin B1 within its C terminus, indicating that Cdc25A is fully capable of activating Cdk1 to regulate mitotic entry (8). Mice that can have Cdc25A, Cdc25B, and Cdc25C conditionally deleted are currently being generated. In this way, Cdc25 phosphatases can be disrupted individually and in combination to determine if “adaptation” during development accounts for the lack of observed phenotype in the BCKO animals, as has been seen in other germ line knockout models (53).

ACKNOWLEDGMENTS

A.M.F. was supported by an award from the American Heart Association. This work was supported by a grant from the National Institutes of Health. H.P.-W. is an investigator of the Howard Hughes Medical Institute.

REFERENCES


Downloaded from http://mcb.asm.org/ on December 18, 2017 by guest


