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Tumorigenesis and Neoplastic Progression

Limited Redundancy in Phosphorylation of Retinoblastoma Tumor Suppressor Protein by Cyclin-Dependent Kinases in Acute Lymphoblastic Leukemia

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Cyclin-dependent kinases (CDKs) successively phosphorylate the retinoblastoma protein (RB) at the restriction point in G_1 phase. Hyperphosphorylation results in functional inactivation of RB, activation of the E2F transcriptional program, and entry of cells into S phase. RB unphosphorylated at serine 608 has growth suppressive activity. Phosphorylation of serines 608/612 inhibits its binding of E2F-1 to RB. In Nalm-6 acute lymphoblastic leukemia extracts, serine 608 is phosphorylated by CDK4/6 complexes but not by CDK2. We reasoned that phosphorylation of serines 608/612 by redundant CDKs could accelerate phospho group formation and determined which G_1 CDK contributes to serine 612 phosphorylation. Here, we report that CDK4 complexes from Nalm-6 extracts phosphorylated *in vitro* the CDK2-preferred serine 612, which was inhibited by $p16^{INK4a}$, and fascaplysin. In contrast, serine 780 and serine 795 were efficiently phosphorylated by CDK4 but not by CDK2. The data suggest that the redundancy in phosphorylation of RB by CDK2 and CDK4 in Nalm-6 extracts is limited. Serine 612 phosphorylation by CDK4 also occurred in extracts of childhood acute lymphoblastic leukemia cells but not in extracts of mobilized $CD34^+$ hemopoietic progenitor cells. This phenomenon could contribute to the commitment of childhood acute lymphocytic leukemia cells to proliferate and explain their refractoriness to differentiation-inducing agents. ***(Am J Pathol 2006, 169:1074–1079; DOI: 10.2353/ajpath.2006.051137)***

Leukemias exhibit loss of normal proliferation and differentiation controls. Understanding how restriction point control could be restored should lead to new therapeutic strategies.[1–4] In mammalian cells, phosphorylation of the retinoblastoma tumor suppressor protein (RB) in late G_1 phase regulates passage of cells through the restriction point.[5] Underphosphorylated RB represses E2F activity through recruitment of a class I histone deacetylase.[6,7] Phosphorylation of RB is associated with functional inactivation[8] and initiation of the E2F transcriptional program to enter S phase.[9–12] Hyperphosphorylation of RB requires the sequential modification by at least two distinct cyclin-dependent kinase (CDK) complexes.[13–16] There is functional redundancy among CDK complexes, so neither CDK2, CDK4, CDK6, nor cyclin E- or D-type cyclin-dependent kinases are essential *in vivo*.[17–23] CDK1 may compensate the loss of CDK2 function.[24] Cyclin D/CDK4,6 complexes partially phosphorylate RB,[13,15] which retains the ability to repress E2F but has lost tight tethering to some nuclear proteins.[13,25] Regulation of E2F-1 binding to RB is a crucial function of the phosphorylation sites serine (Ser) 608/Ser-612.[26]

RB is not phosphorylated at Ser-608 in mobilized $CD34^+$ hemopoietic progenitor cells, in contrast to childhood acute lymphoblastic leukemias.[27] RB, which is unphosphorylated at Ser-608, is exclusively tethered to nuclear structures,[28,29] suggesting that it has growth-suppressive activity. In Nalm-6 cells, Ser-608 is phosphorylated by CDK4/6 complexes but not by CDK2.[29] Ser-608/Ser-612 are located close each other, and we reasoned that their phosphorylation by redundant ki-

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nases could quickly generate a large phospho group in the RB spacer domain. We determined which G_1 CDK from Nalm-6 cells contributes to Ser-612 phosphorylation by *in vitro* kinase assays. We used antibodies to phosphorylated forms of RB for Western blot analysis and found that CDK4 protein kinase from Nalm-6 extracts phosphorylated the CDK2-preferred Ser-612, which was inhibited by $p16^{INK4a}$, and fascaplysin. In contrast, CDK2 immunoprecipitates did not phosphorylate the CDK4 substrates Ser-780 and Ser-795. The data suggest that the redundancy in phosphorylation of RB by CDK2 and CDK4 in Nalm-6 extracts is limited. Ser-608/Ser-612 and Ser-807/Ser-811 in RB are surrounded by amino acids that share sequence homology, implying that CDK4 complexes from acute lymphoblastic leukemia cells could phosphorylate all four residues, thereby disturbing sequential phosphorylation of RB by CDK4 and CDK2 at these sites. Ser-612 phosphorylation by CDK4 also occurred in different childhood acute lymphocytic leukemia (ALL) extracts but not in $CD34^+$ cell extracts. This phenomenon could contribute to the commitment of childhood ALL cells to proliferate and explain their refractoriness to differentiation-inducing agents.

Materials and Methods

Cell Lines and Patient Samples

Nalm-6 cells were derived from a human precursor B cell acute lymphoblastic leukemia and were maintained in RPMI 1640 medium with 10% fetal calf serum (Sigma, St. Louis, MO), in 5% CO_2 at 37°C. Each ALL sample examined here was bone marrow-derived from untreated children with T cell or precursor B cell ALL. After Ficoll (Nycomed Pharma, Oslo, Norway) density gradient centrifugation, smears contained over 90% leukemia cells as assessed by morphology. Hemopoietic progenitor cells were collected by leukapheresis after combined mobilization treatment with chemotherapy and granulocyte colony-stimulating factor.[27] NIH3T3 fibroblasts (ATCC: CCL 92), which were contact–inhibited, were incubated one week in DMEM medium containing 2% fetal calf serum at 37°C in 5% CO_2 to achieve quiescence (NIH3T3/c). This study was approved by the Institutional Review Boards of the Department of Pediatrics, University Hospitals Bern, and of the Faculty of Medicine, University of Bern, Switzerland.

In Vitro *Kinase Assays*

Ten million cells were resuspended in lysis buffer (1% nondidet P-40, 20 mmol/L Na_2HPO_4, pH 7.4, 250 mmol/L NaCl, 5 mmol/L ethylenediamine tetraacetic acid, 5 mmol/L dithiothreitol [DTT], 25 mmol/L β-glycerophosphate, 0.1 mmol/L sodium orthovanadate, 2 μg/ml leupeptin, 1.5 μg/ml aprotinin, and 1 mmol/L phenylmethylsulfonyl fluoride [PMSF]). Lysates were centrifuged for 15 minutes at 13,000 rpm at 4°C and were immune precipitated with 6-μg antibody for 1 hour on ice. Immobilized immune complexes were washed four times with lysis buffer, once with buffer containing 100 mmol/L Tris, pH 7.5, 0.5 mol/L LiCl, 1 mmol/L PMSF, and once with kinase buffer (20 mmol/L Tris, pH 7.5, 7.5 mmol/L $MgCl_2$, 1 mmol/L DTT, 0.5 mmol/L ethyleneglycol-bis [β-aminoethyl]-*N*,*N*,*N*′,*N*′-tetraacetic acid [EGTA], 25 mmol/L β-glycerophosphate, 0.5 mmol/L sodium orthovanadate, 1 mmol/L PMSF, 2 μg/ml leupeptin, and 1.5 μg/ml aprotinin). Immune complexes were resuspended in 30 μl of kinase buffer, and 10 μl was added to kinase buffer containing 10 μCi [γ-^{33}P]ATP (Amersham, Buckinghamshire, UK) and 2.5 μg of full-length biologically active recombinant RB (QED-Bioscience, San Diego, CA). Fascaplysin, which was purchased from Dr. Gordon Gribble (Dartmouth College, Hanover, NH), was added to immobilized CDK immunoprecipitates. The vehicle for fascaplysin was methanol. Labeling was tested for 30 minutes at 37°C. Proteins were boiled 3 minutes in sample buffer. After sodium dodecyl sulfate polyacrylamide gel electrophoresis and blotting, Biomax Scientific Imaging Film (Eastman Kodak Co., Rochester, NY) was used for autoradiography. Autoradiographies were scanned with PSC2200 (Hewlett-Packard, Palo Alto, CA) and imported to Coral Draw 11.0 (Corel, Ottawa, ON, Canada).

Antibodies and Recombinant Proteins

The following antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA): anti-CDK2 (sc-163), anti-CDK4 (sc-260), anti-CDK6 (sc-177), anti-LexA (nonspecific IgGs, sc-1725), and anti-total RB (sc-7905). Anti-phosphorylated (pp) Ser-780, and anti-ppSer-795 RB antibodies were purchased from New England Biolabs, Beverly, MA. Recombinant $p16^{INK4a}$ was purchased from AmProx (Mountain View, CA) and was added to Nalm-6 extracts.

Western Blotting

After separation in 12% acrylamide, proteins were transferred to polyvinylidene difluoride (PVDF) membrane (Roche, Basel, Switzerland) in a Mini Trans Blot Cell (Bio-Rad, Hercules, IL) for 45 minutes at 60 V. Blots were saturated for 30 minutes at room temperature in 0.25% gelatin and Tris-buffered saline, pH 7.1.[30] Blots were washed twice for 5 minutes in TBST buffer (Tris-buffered saline, 0.02% gelatin, 0.12% Triton X-100, pH 7.1) and incubated with 0.5 μg/ml antibody in TBST buffer over night at room temperature. Blots were washed 5 minutes with TBST buffer and incubated with 0.15 μg/ml protein G-horseradish peroxidase (Zymed, South San Francisco, CA) in TBST buffer 30 minutes at room temperature. Blots were washed four times in TBST buffer 15 minutes at room temperature. Proteins were visualized with Lumi-Light Plus substrate and the Lumi-Imager F1 workstation using the Lumianalyst software (Roche). Prints were scanned, and images were imported to Corel Draw 11.0.

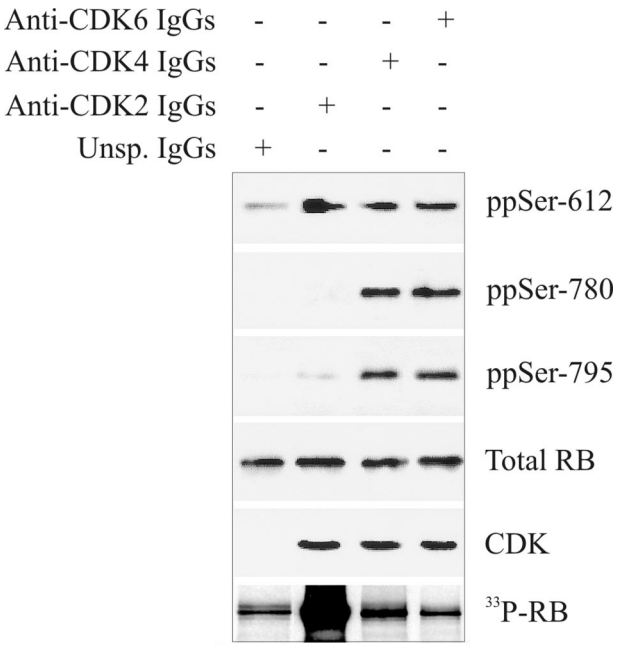

Figure 1. Phosphorylation of Ser-612 by G_1 cyclin-dependent kinases. CDK2, CDK4, CDK6, or nonspecific (IgG) immunoprecipitates from Nalm-6 extracts were subjected to *in vitro* kinase assays, SDS-PAGE, autoradiography (^{33}P-RB), and Western blotting with antibodies to phosphorylated (pp) Ser-612, ppSer-780, ppSer-795, or to total RB. Immune-precipitated CDKs were revealed during immunoblotting.

Sequence Analysis

The alignment was obtained by BLAST protein database searches. This BLAST program[31] is available from *http://www.ncbi.nlm.nih.gov*.

Results

To examine Ser-612 phosphorylation, we tested RB labeling with CDK immunoprecipitates by *in vitro* kinase assays and by Western blotting with phosphorylation state-specific antibodies. When RB was labeled with CDK2, CDK4, or CDK6 immunoprecipitates, Ser-612 was phosphorylated, in contrast to labeling with nonspecific immune precipitate (Figure 1). There is evidence that CDK4/6 complexes phosphorylate Ser-780, and Ser-795 of RB.[32,33] During control labeling, Ser-780 and Ser-795 were phosphorylated to the same extent with CDK4 and CDK6 immunoprecipitates from Nalm-6 extracts but not with CDK2 complexes (Figure 1). When CDK4 and CDK6 were blocked with the specific inhibitor p16^{INK4a}[34] in immunoprecipitates^{INK4a}, inhibition of catalytic activity and Ser-612 labeling occurred (Figure 2, lanes 3, 4, 6, and 7), compared to labeling without antibody (Figure 2, lane 1) and in contrast to labeling without p16^{INK4a} (Figure 2, lane 2). When the catalytic activity in CDK4 immunoprecipitates was inhibited with increasing concentrations of fascaplysin, a natural product that specifically inhibits CDK4 protein kinase *in vitro*,[35] Ser-612 kinase activity was abrogated in a dose-dependent manner (Figure 3, lanes 6–9), in contrast to addition of vehicle alone

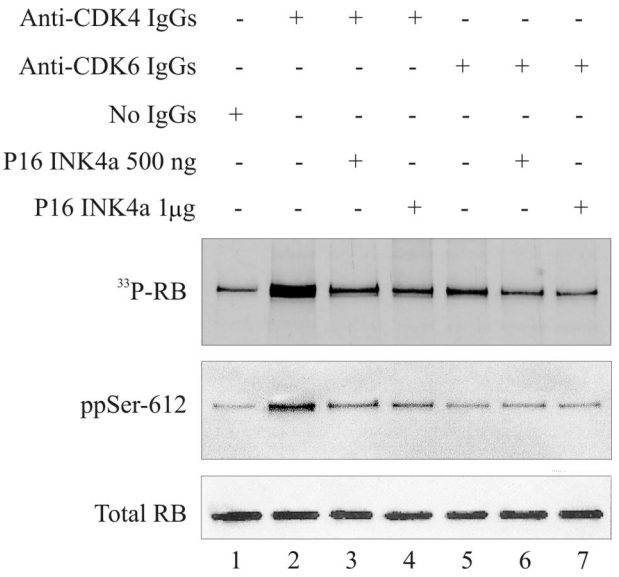

Figure 2. P16^{INK4a} inhibited Ser-612 phosphorylation. CDK immunoprecipitates from Nalm-6 extracts were subjected to *in vitro* kinase assays,[55] SDS-PAGE, and autoradiography ([^{33}P]-RB). The phosphorylation status of RB at Ser-612 was determined by immunoblotting with a phosphorylation site-specific antibody. Total RB was detected by Western blotting.

(Figure 3, lanes 2–5) or Ser-612 labeling without vehicle and fascaplysin (Figure 3, lane 1).

Ser 612 labeling was subsequently tested with CDK immunoprecipitates from extracts of 10 childhood acute lymphoblastic leukemia cell samples. We also used extracts from reference cells, mobilized CD34$^+$ hemopoietic progenitor cells that reside mainly in early G_1 phase[27] and quiescent NIH3T3 cells, because their proliferation rate is different to that of ALL cells. When ALL extracts were tested, CDK2 precipitates labeled Ser-612 in every extract. CDK4 precipitates efficiently labeled Ser-612 in five ALL extracts and poorly in four extracts. In

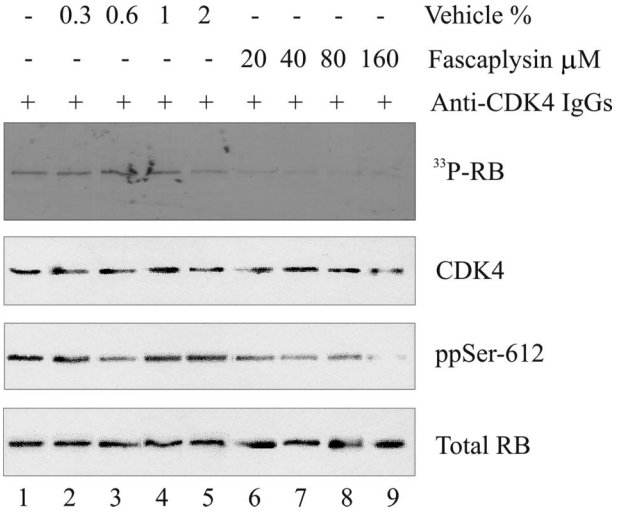

Figure 3. Dose-dependent inhibition of Ser-612 phosphorylation by fascaplysin. CDK4 immunoprecipitates (**lanes 1–9**) from Nalm-6 extracts were subjected to *in vitro* kinase assays, SDS-PAGE, and autoradiography (^{33}P-RB). Western blotting with activation state-specific antibody detected phosphorylated (pp) Ser-612 in RB. Protein levels of total RB or precipitated CDK4 protein kinase were detected by immunoblotting.

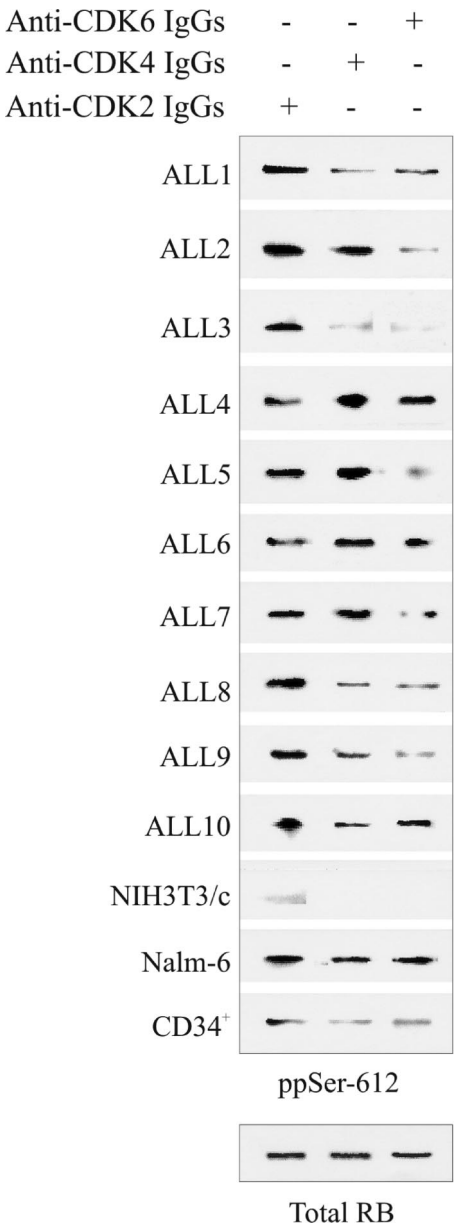

Figure 4. Ser-612 phosphorylation *in vitro* by CDK4 complexes from childhood ALL extracts. Western blotting with anti-phosphorylated (pp) Ser-612 RB antibody or anti-total RB antibody after *in vitro* kinase assays with CDK immunoprecipitates. The reference cells were quiescent NIH3T3 mouse fibroblasts (three independent experiments) and mobilized $CD34^+$ hemopoietic progenitor cells residing in early G_1 phase (four independent experiments).

contrast, Ser 612 was not labeled in one ALL extract (ALL3), in $CD34^+$ extracts, or in quiescent NIH3T3 extracts (Figure 4). CDK6 immunoprecipitates labeled Ser-612 efficiently in three ALL extracts and poorly in two extracts, whereas Ser-612 was not labeled in five extracts (Figure 4). Ser-612 was not labeled with CDK4/6 immunoprecipitates from extracts of mobilized $CD34^+$ samples, consistent with previous results.[27] In summary, Ser-612 was phosphorylated by CDK4 in nine of 10 ALL extracts but in none of four $CD34^+$ cell extracts tested.

Ser-612 is located in RB within amino acids 604–613 (Figure 5A) that share sequence homology with amino

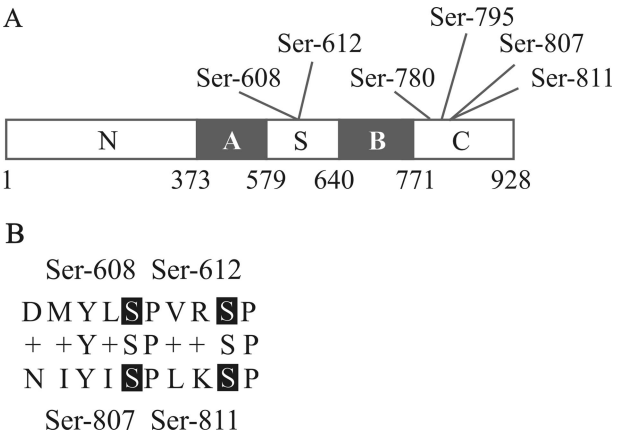

Figure 5. RB phosphorylation sites Ser-608/Ser-612 and Ser-807/Ser-811 are surrounded by amino acids that share sequence homology. **A:** Scheme of RB. A subset of CDK phosphorylation sites in RB are indicated on top. Amino acid numbers below indicate the location of pocket domains A and B. Ser-612 is situated in the RB nonconserved spacer domain (S). N terminus (N), COOH terminus (C). **B:** Alignment.

acids 803–812 (Figure 5B), suggesting that the phosphorylation sites Ser-608/Ser-612 and Ser-807/Ser-811 may regulate the same process, consistent with a collaboration of spacer domain sites, and C-terminal sites to regulate G_1 arrest.[14,26,36–38] The motif contains the epitope YLSPVR, carrying Ser-608, which is recognized by monoclonal antibodies that detect the growth suppressive underphosphorylated RB.[28] An RB mutant with alanine substitutions at Ser-807/Ser-811 has enhanced growth-suppressing activity.[39,40] The sequence homology also implied that CDK4/6 complexes from acute lymphoblastic leukemia cells could phosphorylate all four sites. Ser-807 and Ser-811 are efficiently phosphorylated by CDK4/6 complexes.[41–44] Ser-807/Ser-811 are also phosphorylated by cyclin C/CDK3, which is required for cells to exit G_0 efficiently.[45] In extracts of mobilized $CD34^+$ hemopoietic progenitor cells, which reside mainly in early G_1 phase, Ser-807/Ser-811 are phosphorylated, in contrast to Ser-608/Ser-612.[27,46]

Discussion

Based on our findings, CDK4 complexes from Nalm-6 extracts phosphorylated *in vitro* the CDK2-preferred Ser-612 residue in full-length RB. This redundancy in phosphorylation of RB in Nalm-6 extracts was limited. Ser-780 and Ser-795 were phosphorylated by CDK4/6 complexes as Ser-608[29] but not by CDK2 complexes, in keeping with the literature.[32,33,44]

Whereas the *in vitro* phosphorylation of Ser-780 and Ser-795 in RB by CDK4 complexes from extracts of childhood acute lymphoblastic leukemias remains to be investigated, Ser-612 phosphorylation by CDK4 also occurred in different childhood ALL extracts. In contrast, Ser-612 phosphorylation by CDK4 during *in vitro* kinase assays was not detectable in $CD34^+$ cell extracts. This is in accordance with our previous findings that Ser-608 and Ser-612 were not phosphorylated in $CD34^+$ cells.[27,46] The differences between ALL and $CD34^+$ cells are con-

sistent with the refractoriness of ALL cells to differentiation-inducing agents. As most $CD34^+$ cells reside in early G_1 phase, they can undergo differentiation.[46] Cell cycle analysis of ALL cells indicates that most cells reside in G_1 phase, few cells are in G_0 phase, and the compartment of $S/G_2/M$ cells is small.[47,48] Despite the large G_1 compartment, ALL cells do not respond to differentiation-inducing agents. In contrast to $CD34^+$ cells, most ALL cells reside in late G_1 phase, and traversed the restriction point.[27,49] CDK4 phosphorylation of both Ser-608[27] and Ser-612 (this study) in full-length RB could abolish the binding of E2F-1 to RB in ALL cells, and activate E2F-1 transcription at the execution point of CDK4 in mid G_1 phase. This untimely phosphorylation of Ser-612 by CDK4 could render CDK2 phosphorylation of this site dispensable, implying that cell cycle progression from mid to late G_1 phase were advanced in ALL cells. This would explain why most childhood ALL cells traversed the restriction point and are refractory to differentiation-inducing signals.

Generation of free E2F-1 after Ser-608/Ser-612 phosphorylation by CDK4 could up-regulate the PI3k/Akt pathway,[51] which promotes blast survival in childhood ALL.[52] RB/E2F-1 also interacts with the licensing factor MCM4.[29] Hence, Ser-608/Ser-612 phosphorylation by CDK4 could facilitate DNA licensing in ALL cells.

E2F-1 activates genes whose products contribute to G_1 exit and S phase traversal.[50] The compartment of cells in $S+G_2/M$ phase is small in ALL cell populations, their proliferation rate is slow, most cells reside in G_1 phase,[47,48] and they contain little nuclear tethered RB. In contrast, rapidly proliferating Nalm-6 cells, with 44% cells in S phase,[49] do not contain nuclear tethered RB.[29] The nuclear tethered RB in ALL cells could be associated with E2F-1.

As RB phosphorylation by CDKs can increase E2F activity, we determined the level of CDK4 catalytic activity in extracts of ALL samples by *in vitro* kinase assays. As shown previously, CDK2 catalytic activity was increased in ALL extracts compared to $CD34^+$ cell extracts,[49] whereas the level of CDK4 catalytic activity in most ALL and $CD34^+$ extracts was similar (N.M.R.S., unpublished results). These findings implied that increased RB phosphorylation in ALL cells by CDK4 were not associated with increased CDK4 activity and that phosphorylations at Ser-608/Ser-612 would occur because the substrate specificities of the CDK4 complexes were deregulated in ALL cells.

In contrast to Ser-608/Ser-612, many studies indicate that Ser-807/Ser-811 phosphorylation does not abolish the regulation of E2F binding to RB[53,54] but is required to disrupt c-Abl binding.[53] The sequence homology implied that CDK4 complexes from acute lymphoblastic leukemia cell extracts could phosphorylate all four sites, which would disturb the sequential phosphorylation of RB by CDK4 and CDK2 at these sites.

In conclusion, the data suggest that the redundancy in phosphorylation of RB by CDK2 and CDK4 in Nalm-6 extracts is limited. The CDK2-preferred Ser-612 residue in full-length RB was phosphorylated *in vitro* by CDK4 complexes from extracts of Nalm-6 cells. In contrast, Ser-780 and Ser-795 were phosphorylated by CDK4 complexes, but not by CDK2, from Nalm-6 extracts. The amino acids surrounding Ser-608/Ser-612 and Ser-807/Ser-811 share sequence homology, implying that CDK4 complexes from acute lymphoblastic leukemia cell extracts could phosphorylate all four residues, which would disturb sequential RB phosphorylation by CDKs at these sites. Whereas the *in vitro* phosphorylation of Ser-780 and Ser-795 in RB by CDK4 complexes from extracts of childhood acute lymphoblastic leukemias remains to be investigated, Ser-612 phosphorylation by CDK4 also occurred in different childhood ALL extracts. In contrast, Ser-612 phosphorylation by CDK4 during *in vitro* kinase assays was not detectable in $CD34^+$ cell extracts. This phenomenon could contribute to the commitment of childhood ALL cells to proliferate and explain their refractoriness to differentiation-inducing agents.

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