The promyelocytic leukemia zinc finger (PLZF) protein binds DNA in a high molecular weight complex associated with cdc2 kinase

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ABSTRACT

A binding site selection from a CpG island library for the promyelocytic leukemia zinc finger protein (PLZF) identified two high affinity PLZF binding sites. These sequences also bound RARα/PLZF, a fusion protein formed in chromosomal translocation t(11;17)(q23;q21) associated with acute promyelocytic leukemia. PLZF bound DNA as a slowly migrating complex with an estimated mol. wt of 600 kDa whose formation was dependent on the POZ/dimerization domain of PLZF. The PLZF–DNA complex was unable to form in the presence of cdc2 antibodies. A PLZF–cdc2 interaction was further demonstrated by co-immunoprecipitation and a biotin–streptavidin pull-down assay. PLZF is a phosphoprotein and immunoprecipitates with a cdc2-like kinase activity. The PLZF–DNA complex was abolished with the addition of a phosphatase. These studies suggest that the activity of PLZF, a regulator of the cell cycle, may be modulated by cell cycle proteins. RARα/PLZF did not complex with cdc2, this potentially contributing to its aberrant transcriptional properties and potential role in leukemogenesis.

INTRODUCTION

Acute promyelocytic leukemia (APL) is generally associated with a reciprocal chromosomal translocation t(15;17)(q21;q11) fusing the gene encoding the PML protein to retinoic acid receptor α (RARα) (1), yielding a dominant negative mutant that blocks RAR signaling (2–7). A form of APL resistant to all-trans retinoic acid (ATRA) therapy is associated with t(11;17)(q23;q21) and fuses the gene for the promyelocytic leukemia zinc finger protein (PLZF) to the RARα gene resulting in the expression of PLZF/RARα and RARα/PLZF fusion proteins (8,9). PLZF/RARα is a potent dominant negative retinoic acid receptor due to its ability to interact with transcriptional co-repressors such as NCoR and SMRT through PLZF sequences and failure to release such factors even in the presence of retinoic acid (10–15).

PLZF is expressed during the development of the central nervous system and limb buds and in the perinatal kidney, liver and heart (16). In the hematopoietic system, PLZF is expressed in CD34+ progenitor cells and undifferentiated hematopoietic cells (17). Overexpression of PLZF is growth suppressive in the murine 32Dcl3 cell line. This was associated with cell cycle arrest, repression of cyclin A expression, secretion of an autocrine growth inhibitory factor (18,19) and reduced expression of the interleukin-3 receptor α (IL-3Rα) subunit in this IL-3-dependent cell line (R.Shaknovich and J.D.Licht, unpublished results). The PLZF gene encodes a 673 amino acid transcription factor with nine Kruppel-like C2H2 zinc fingers located in the C-terminus of the protein (8). The N-terminal 118 amino acids comprise a POZ (pox virus and zinc finger) domain mediating dimerization and transcriptional repression (12–15).

Our group isolated a DNA binding site for PLZF using a glutathione S-transferase [GST–PLZF(9ZF)] fusion to select sequences from a pool of random oligonucleotides (23). Another PLZF binding site was fortuitously discovered as the lex operator during a yeast hybrid screening (24). The RARα/PLZF protein contains the last seven fingers of PLZF protein and this is sufficient to interact with a PLZF DNA binding site (23,24). PLZF modestly represses transcription through these sites while RARα/PLZF is a weak transcriptional activator which could antagonize PLZF effects on gene expression and contribute to ATRA-resistant APL (19,23).

The published PLZF binding sites differ significantly, so to clarify the nature of the PLZF binding site, we repeated the binding site selection using a modified whole genome PCR approach (25) with a human CpG island library as the initial source of DNA targets (26). Utilizing these novel binding sites, we found that PLZF bound DNA in a high molecular weight complex in association with cdc2 protein. PLZF DNA binding activity was phosphatase sensitive and PLZF could be phosphorylated in vitro by cyclin B1/cdc2, suggesting that PLZF activity might be regulated by cdc2 and other kinases.

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MATERIALS AND METHODS

Cell lines

The cell lines used for transient transfections were 293T (ATCC CRL1573), CV-1 (ATCC CCL70) and COS-7 (ATCC CRL1651) cells. 293T, CV-1 and COS-7 cells were cultured in DMEM with penicillin and streptomycin and 10% fetal bovine serum (293T) or 10% calf serum (CV-1 and COS-7). The human erythroleukemia (HEL) cell line (ATCC TIB180) was grown in RPMI with 10% heat-inactivated fetal bovine serum.

Either 2 x 10^6 293T cells or 1 x 10^6 COS-7 cells were transfected with 20 μg of DNA using the CaPO_4 method (27). Nuclear extracts were prepared 48 h after transfection (28) and, for immunoprecipitations, cells were lysed in the buffer described below. For reporter gene assays, 2 x 10^5 293T cells/ well of a 12-well dish were transfected with 1 μg of DNA and 5 μl of Superfect (Qiagen, Valencia, CA). Luciferase levels were measured 48 h after transfection using a Dual Luciferase kit (Promega, Madison, WI).

Plasmids

Plasmids encoding the DNA binding domain of PLZF and full-length PLZF fused to glutathione S-transferase (GST–PLZF(9ZF)) and plasmids encoding PLZF–FLAG, PLZF, RARα/PLZF and (ΔPOZ)PLZF in the pSG5 expression vector were previously described (20,23,29). Two copies of a double-stranded oligonucleotide containing two copies of the PLZF binding site described below. For reporter gene assays, 2 x 10^6 293T cells or 10^5 CV-1 and COS-7 cells were transfected with 20 μg of DNA using the CaPO_4 method (27).

Binding site selection

GST and GST–PLZF(9ZF) were expressed and purified as previously described (23). Human CpG island DNA library constructed in the pGEM5F vector was obtained from the Human Genome Resource Centre (Cambridge, UK). One microgram of library DNA was incubated with 10 μg of GST or GST–PLZF(9ZF) immobilized on glutathione–agarose beads in 20 mM HEPES, pH 7.5, 50 mM KCl, 5 mM MgCl_2, 10 μM ZnCl_2, 4% glycerol, 100 μg/ml BSA, 50 ng sonicated salmon sperm DNA and was incubated on ice for 30 min. According to the experiment either unlabeled oligonucleotide competitors, potato acid phosphatase (Roche Molecular Biochemicals, Indianapolis, IN) or antibodies (Santa Cruz Biotechnology, Santa Cruz, CA) were added at least 20 min before the addition of 10 fmol of labeled duplex. After a further incubation of 20 min, the protein–DNA complexes were separated by electrophoresis on a 4% non-denaturing polyacrylamide (30:1 acrylamide:bis-acrylamide) gel in 0.5× TBE buffer at 300 V and 4°C and dried for autoradiography.

Electrophoretic mobility shift assays (EMSA)

Synthetic duplexes were end-labeled using the large Klenow fragment of Escherichia coli DNA polymerase and [α-32P]dCTP (3000 Ci/mmol), and purified by spin column chromatography. Each binding reaction (20 μl) contained ~2 μg nuclear extract protein in 20 mM HEPES, pH 7.5, 1 mM MgCl_2, 10 μM ZnCl_2, 4% glycerol, 100 μg/ml BSA, 50 ng sonicated salmon sperm DNA and was incubated on ice for 30 min. According to the experiment either unlabeled oligonucleotide competitors, potato acid phosphatase (Roche Molecular Biochemicals, Indianapolis, IN) or antibodies (Santa Cruz Biotechnology, Santa Cruz, CA) were added at least 20 min before the addition of 10 fmol of labeled duplex. After a further incubation of 20 min, the protein–DNA complexes were separated by electrophoresis on a 4% non-denaturing polyacrylamide (30:1 acrylamide:bis-acrylamide) gel in 0.5× TBE buffer at 300 V and 4°C and dried for autoradiography.

Electrophoresis

Pore exclusion limit analysis was performed to determine the size of the protein–DNA complexes (31). The binding reactions were separated through either a 4–12 or 4–20% polyacrylamide gradient gel along with native protein molecular weight markers (31) (Amersham Pharmacia Biotech, Piscataway, NJ). Gradient gels were electrophoresed at 180 V for 18 h at 4°C. Molecular weight markers were visualized by staining the gel with 2% Coomassie blue in 10% methanol, 10% acetic acid for 1 h and destaining in 10% methanol, 10% acetic acid for 6 h before drying and autoradiography.

Immunoprecipitations

One 10 cm dish of transfected 293T cells or 2 x 10^5 HEL cells were lysed in 1 ml of 1% NP-40, 50 mM NaCl, 50 mM Tris, pH 8.0, 1 mM MgCl_2, 10 μM ZnCl_2, 4% glycerol and Complete protease inhibitors (Roche Molecular Biochemicals). One microgram of anti-cdc2, anti-Gal4p (Santa Cruz Biotechnology) or anti-PLZF monoclonal antibody was added to the lysate. After a 3 h incubation at 4°C, 30 μl of protein A–agarose (Roche Molecular Biochemicals) was added and the incubation continued overnight. The precipitates were washed four times with 1 ml of the lysis buffer, separated through a 12% SDS–PAGE gel and transferred to Immobilon PVDF membrane (Millipore, Bedford, MA) overnight at 10 V at 4°C. The membranes were
blocked in 5% milk dissolved in TBS-T (150 mM NaCl, 10 mM Tris, pH 8.0, 0.05% Tween-20) for 2 h and then washed three times in TBS-T. The membranes were incubated with 1 ng/ml primary antibody (rabbit anti-PLZF or anti-cdc2) in 0.5% milk/TBS-T for 2 h then washed three times. A 1:7500 dilution of anti-rabbit horse-radish peroxidase antibody (Roche Molecular Biochemicals) was incubated with the membrane for 1 h followed by four washes. The blot was visualized by chemiluminescence according to the manufacturer’s instructions (Amersham Pharmacia Biotech).

**Biotin–streptavidin pull-down assay**

Duplex oligonucleotides were labeled using the large Klenow fragment of *E. coli* DNA polymerase and biotin-dCTP (Gibco BRL, Gaithersburg, MD), and purified on a spin column (Amersham Pharmacia Biotech). One microgram of biotinylated oligonucleotide was incubated with 200 µg nuclear extract protein in 20 mM HEPES, pH 7.5, 1 mM MgCl₂, 10 µM ZnCl₂, 4% glycerol, 100 µg/ml BSA, 2.5 µg/ml salmon sperm DNA and Complete protease inhibitors for 1 h on ice. Thirty microliters of streptavidin–agarose beads (Gibco BRL), pretreated with 100 µg/ml BSA and 2.5 µg/ml salmon sperm DNA, were added to the reaction and incubation continued at 4°C with mixing for 2 h. Next, the beads were washed five times with 1 ml of the binding buffer and the bound proteins released by boiling in SDS loading buffer were analyzed by 12% SDS–PAGE and western blotting as described previously.

**Phosphorylation of PLZF**

PLZF fused to a Flag epitope was transfected into CV-1 cells. The transfected cells were grown in phosphate-free medium for 3 h followed by a 3 h incubation in medium containing 2 mCi [³²P]orthophosphate. The cells were washed three times and lysed in 150 mM NaCl, 50 mM Tris, pH 8.0, 1% NP-40, 1 µM sodium orthovanadate and protease inhibitors. A 90 kDa protein corresponding to PLZF–Flag was immunoprecipitated with M2 monoclonal anti-Flag antibody (Kodak, Rochester, NY). The labeled protein was excised from a polyacrylamide gel, eluted and subjected to trypsin digestion and acid hydrolysis (32). The mixture of single amino acids and incompletely digested peptides was electrophoretically separated on a thin layer chromatography plate in two dimensions at pH 1.9 and pH 3.5, respectively, and compared to standards. GST–PLZF, immobilized on glutathione–agarose beads, was resuspended in 20 mM MOPS, pH 7.2, 25 mM β-glycerol phosphate, 5 mM EGTA, 1 mM sodium orthovanadate, 1 mM dithiothreitol, 37 mM MgCl₂, 250 µM ATP and 10 µCi [γ-³²P]ATP (3000 Ci/mmol). The beads were incubated at 30°C for 30 min followed by four washes with PBS. The proteins were separated by 12% SDS–PAGE followed by autoradiography. The amount of kinase activity immunoprecipitated was quantified using a cdc2 kinase assay kit (Upstate Biotechnology, Lake Placid, NY).

**RESULTS**

**Binding site selection**

Binding site selections from the CpG island library were performed with GST–PLZF(9ZF) purified from bacteria and with PLZF protein in nuclear extracts from transfected 293T cells. A high affinity binding site was mapped in a clone from each selection, with site A selected by binding to GST–PLZF(9ZF) and site B selected by binding to full-length PLZF expressed in 293T cells (Fig. 1A). The affinity of PLZF for these sites and other published PLZF binding sites was compared by EMSA using PLZF protein from transfected 293T cells (Fig. 1B). Site B was strikingly similar to a site identified in the lex operator and these two sites had the highest affinity for PLZF. The affinity of PLZF for site A was intermediate between site B/lex and a previously published PLZF binding site. An oligonucleotide containing a 1 bp mutation of site A had no affinity for PLZF. In general, the sites showed similar competition patterns for RARα/PLZF and endogenous PLZF (data not shown). To determine whether sequences from the PLZF binding site selection resemble a physiological PLZF binding site, we identified a sequence similar to site B in the promoter of the IL-3Rα subunit, a protein down-regulated by PLZF expression (R.Shaknovich and J.D.Licht, unpublished results). As well as binding PLZF (Fig. 3B), this sequence could mediate repression by PLZF when multimerized in front of a luciferase reporter gene (Fig. 1C). Repression was modest but highly reproducible and enhanced by co-transfection of the SMRT co-repressor (Fig. 1D).

**Size of protein–DNA complexes**

PLZF formed a very slowly migrating protein–DNA complex in the EMSA. Using pore exclusion limit analysis, the size of this complex was determined to be 600 kDa (Fig. 2A). In comparison, PLZF migrates with a molecular weight of 80–90 kDa on an SDS–polyacrylamide gel (18,29). The use of PLZF protein deleted for the POZ domain reduced the size of the complex to 180 kDa. While the PLZF and (ΔPOZ)/PLZF complexes migrated significantly slower than predicted by their molecular weights, the RARα/PLZF–DNA complex migrated at ~40 kDa (Fig. 2B), which is slightly larger than the native fusion protein (~35 kDa). This suggests that the N-terminus of the PLZF protein allows the formation of a multiprotein complex on DNA.

**PLZF and cdc2 interact on DNA**

PLZF antibodies can immunoprecipitate PLZF from cells metabolically labeled with [³⁵S]methionine along with several other proteins. One co-immunoprecipitated protein was 35 kDa in size, which is approximately the size of many cyclin-dependant kinases (data not shown). In view of PLZF’s effects on the cell cycle (18) and its regulation of cyclin A
levels (19), we investigated whether PLZF interacted with cyclin-dependant kinases. By several methods we demonstrated that PLZF interacts with cdc2. Adding cdc2 antibodies to an electrophoretic mobility shift reaction prevented formation of the PLZF–site A complex (Fig. 3A). A CDK2 antiserum also had this effect; however, we believe this was due to cross-reactivity with cdc2, as the sera recognized in vitro translated cdc2 protein. The antibodies had the same effect on the PLZF–DNA complex formed with endogenous PLZF from the HEL cell line (Fig. 3B). The addition of cdc2 antibodies had no effect on the RARα/PLZF–DNA complex (Fig. 3C).

cdc2 kinase was co-immunoprecipitated with PLZF antibody from 293T cells transfected with PLZF or ($\Delta$POZ)/PLZF, but not from vector transfected cells (Fig. 4A). PLZF was also co-immunoprecipitated with cdc2 antibody (Fig. 4B). In a biotin-streptavidin affinity chromatography assay, cdc2 was purified from cell lysates only in the presence of PLZF and an oligonucleotide with a wild-type binding site sequence (Fig. 4C). If no oligonucleotide or mutant oligonucleotide (containing a 1 bp mutation that abolishes PLZF binding) was present, cdc2 was not co-purified with PLZF. The two proteins could also be co-immunoprecipitated from HEL cells, which naturally express PLZF (Fig. 4D). Together these data indicate that cdc2 can complex with PLZF even on a DNA binding site.

**PLZF associates with a cdc2-like kinase activity**

Proteins precipitated from transfected 293T cells by either PLZF or cdc2 antibodies were assayed for cdc2 kinase activity in a reaction containing histone H1 as a substrate and several inhibitors for other kinases. A cdc2-like kinase activity could be immunoprecipitated with PLZF from the PLZF transfected cells but very little kinase activity was isolated from the vector transfected cells (Fig. 5A). PLZF expression did not alter cdc2 activity in whole cell lysates (data not shown). Addition of [γ-32P]ATP to PLZF immunoprecipitates yielded the phosphorylation of a 90 kDa protein (Fig. 5B). As this phosphorylated protein could only be immunoprecipitated...
from the PLZF transfected cells, it was likely to be the PLZF protein.

**PLZF is a phosphoprotein**

When expressed in CV-1 cells, PLZF was phosphorylated on serine and threonine residues, but tyrosine phosphorylation was not detected (Fig. 6A). PLZF, expressed and purified from *E. coli* as a GST fusion protein, could be phosphorylated in vitro by cyclin B/cdc2 kinase (Fig. 6B). As PLZF is a phosphoprotein and can bind DNA in a complex with cdc2, we investigated whether phosphorylation affected DNA binding by PLZF. Incubating the PLZF–DNA or (ΔPOZ)PLZF complexes with increasing amounts of potato acid phosphatase abolished the complexes (Fig. 6C). In contrast, the RARα/PLZF–DNA complex was much less sensitive to the enzyme. The phosphatase did not alter the abundance of the PLZF or (ΔPOZ)PLZF protein in the extracts as determined by immunoblotting (Fig. 6C).

**DISCUSSION**

A binding site selection yielded PLZF recognition sequences with a greater affinity than those we previously identified (23). Competition analysis suggests that the lex/site B sequence is optimal for PLZF binding, although site A also has significant affinity for PLZF. A PLZF binding site, previously identified by our laboratory (23), showed lower affinity for PLZF binding. This site bound PLZF from transfected 293T cells weakly under the conditions tested, although it interacts with GST–PLZF(9ZF) strongly (data not shown) and can repress transcription through this site (19,23). Selection with GST–PLZF(9ZF) resulted in a complex library of selected sequences while the library produced using full-length PLZF from transfected cells contained very few sequences. This indicates that bacterially expressed GST–PLZF(9ZF) binds to a much wider variety of sequences than the full-length protein. The specificity of DNA binding may be regulated by sequences upstream of the zinc fingers as well as post-translational modifications specific to eukaryotic cells, such as phosphorylation. The new selection also suggested a novel target gene for PLZF, namely the IL3-Rα subunit. The IL-3Rα subunit is also down-regulated by PLZF expression (R.Shaknovich and J.D.Licht, unpublished results) and we have identified a PLZF binding site, similar to site B, in its promoter (H.Ball and J.D.Licht, manuscript in preparation). PLZF modestly repressed transcription through the isolated IL-3Rα site and this could be significantly enhanced by the presence of the SMRT co-repressor, in accordance with previous results (14).

The size of the PLZF–DNA complex was determined to be 600 kDa by pore exclusion limit analysis. As the POZ domain mediates dimerization of PLZF (22), it is probable that the complex contains at least two molecules of 90 kDa PLZF protein. Recent results with the *Drosophila* GAGA protein indicates that GAGA can dimerize and then multimerize and cooperatively bind DNA through the action of the POZ domain.
PLZF antibodies and immunoblotted with a cdc2 antibody. Proteins were immunoprecipitated from vector, PLZF- and (ΔPOZ)PLZF transfected 293T cells with PLZF antibody, followed by western analysis with cdc2 antibody. Lanes 1–3, cdc2 expression in the lysates. (B) PLZF co-immunoprecipitates with cdc2 antibody. Proteins were immunoprecipitated from vector and PLZF transfected 293T cells with cdc2 antibody and subjected to western analysis with a PLZF antibody. Lanes 1–2, lysate from the vector and PLZF transfected cells. (C) cdc2 is purified on a PLZF binding site. Site A or an oligonucleotide containing a single base pair mutation that abolishes PLZF binding was labeled with biotin, immobilized on streptavidin beads and incubated with nuclear extract from vector or PLZF transfected cells. The bound proteins were immunoblotted with a cdc2 antibody. (D) Endogenous PLZF and cdc2 interact. Proteins from HEL cells were immunoprecipitated with either gal4 or PLZF antibodies and immunoblotted with a cdc2 antibody.

Figure 4. PLZF and cdc2 interact in vivo. (A) cdc2 co-immunoprecipitates with PLZF antibody. Proteins were immunoprecipitated from vector, PLZF- and (ΔPOZ)PLZF transfected 293T cells with PLZF antibody, followed by western analysis with cdc2 antibody. Lanes 1–3, cdc2 expression in the lysates. (B) PLZF co-immunoprecipitates with cdc2 antibody. Proteins were immunoprecipitated from vector and PLZF transfected 293T cells with cdc2 antibody and subjected to western analysis with a PLZF antibody. Lanes 1–2, lysate from the vector and PLZF transfected cells. (C) cdc2 is purified on a PLZF binding site. Site A or an oligonucleotide containing a single base pair mutation that abolishes PLZF binding was labeled with biotin, immobilized on streptavidin beads and incubated with nuclear extract from vector or PLZF transfected cells. The bound proteins were immunoblotted with a cdc2 antibody. (D) Endogenous PLZF and cdc2 interact. Proteins from HEL cells were immunoprecipitated with either gal4 or PLZF antibodies and immunoblotted with a cdc2 antibody.

(33). Given that deletion of the PLZF POZ domain reduces the apparent molecular weight of the PLZF–DNA complex from 600 to ~180 kDa (Fig. 2) the high molecular weight complex may represent a multimer of PLZF consisting of four individual molecules. PLZF interacts with NcoR, SMRT and sin3a through its POZ domain (12–15); however, the inclusion of NcoR, SMRT and sin3a antibodies in EMSA reactions had no effect on the PLZF–DNA complex (data not shown). However, this does not preclude these proteins from being present in the complex. Antibodies for other reported PLZF-interacting proteins such as retinoblastoma protein and PML protein (34,35) also had no effect. The (ΔPOZ)PLZF–DNA complex was also significantly larger (180 kDa) than the (ΔPOZ)PLZF protein, suggesting that other proteins bind, along with PLZF, to its cognate DNA binding site. A monoclonal antibody for cdc2 abolished the PLZF–DNA and (ΔPOZ)PLZF–DNA complexes, indicating that cdc2 is a component of the PLZF–DNA complex. A PLZF–cdc2 interaction was confirmed by co-immunoprecipitation and biotin–streptavidin pull-down assay in transfected cells and co-immunoprecipitation from a human erythroleukemia cell line. The PLZF–cdc2 interaction did not require the conserved POZ domain of PLZF. Together these results indicate that cdc2 is involved in DNA binding by PLZF.

Figure 5. PLZF associates with an active kinase. (A) The amount of histone H1 kinase activity in the PLZF immunoprecipitates was quantitated using a cdc2 kinase assay, compared in immunoprecipitates from vector (lane 1) and PLZF (lane 2) transfected cells using PLZF antibody. The amount of kinase activity precipitated with cdc2 antibody is shown (lane 3). (B) PLZF is phosphorylated by its associated kinase. Proteins immunoprecipitated from vector (lane 1) and PLZF (lane 2) transfected 293T cells with a PLZF monoclonal antibody were resuspended in a reaction containing [γ-32P]ATP. Phosphorylated proteins were detected by separation on a 12% SDS–polyacrylamide gel followed by autoradiography.

PLZF expressed in 293T cells co-immunoprecipitated with an active kinase. The immunoprecipitated PLZF complex was able to phosphorylate histone H1, a substrate for cdc2 kinase as well as a number of other kinases. We included inhibitors for protein kinase A, protein kinase C and Ca2+/calmodulin-dependent protein kinase in the assay, and histone H1 was still phosphorylated by the PLZF complex; however, this does not rule out the presence of other kinases such as CDK2 and MAP kinases. Bcl-6, another POZ domain transcription factor, was shown to be a substrate for MAP kinase, with phosphorylation causing degradation of protein (36).

PLZF is phosphorylated on serine and threonine residues, and contains two potential cdc2 phosphorylation consensus sequences (37) found in the region between the POZ domain and the zinc fingers of PLZF (amino acids 197–200 and 282–285), which might also represent the binding sites for cdc2. A kinase assay performed on PLZF immunoprecipitates from PLZF transfected cells resulted in the phosphorylation of a 90 kDa protein, presumably PLZF. An in vitro assay using purified cyclin B/cdc2 kinase and bacterially expressed PLZF indicated that PLZF is a substrate for the enzyme. The DNA binding activity of many transcription factors, including c-Jun and GATA-1, is regulated by phosphorylation (38,39). Incubating the PLZF reactions with potato acid phosphatase abolished the PLZF–DNA complex, suggesting that phosphorylation may play a role in DNA binding by PLZF. The monoclonal cdc2 antibody used in this study can immunoprecipitate cdc2 kinase activity, so it is unlikely that the antibody blocks the ability of the enzyme to phosphorylate its substrates. It is more likely that the cdc2 antibody sterically hinders the ability of PLZF, possibly in combination with other proteins, to bind DNA.
There is a precedent of a transcription factor forming a complex with a kinase on its DNA binding site in the E2F transcription factor. The major E2F DNA binding complex contains the p130 protein (40). However, E2F can also form a complex with p107, cyclin A and cdk2 (41–43) that has DNA binding activity and kinase activity (44). More recently it was shown that cdc2 was part of a multiprotein complex which regulates the transcription of histone genes at the G1/S boundary (45). While generally associated with mitotic events at G2, cdc2 is up-regulated and forms a complex with cyclin A at the G1/S transition in hematopoietic cells and cdc2 activity has been associated with S phase progression (46–49). Hence cdc2 could play a role in the ability of PLZF to inhibit the G1/S transition and progression through S phase previously described (18,19). However, we have yet to identify a cyclin in the PLZF–DNA complex, raising the alternative possibility that cdc2 protein may play a role in which its kinase activity is not required. In an analogous manner a novel role for cyclin D in recruiting cofactors to the estrogen receptor was shown to be independent of kinase interaction (50,51). We are currently investigating whether other kinases, such as CDK2, interact with PLZF and which cyclins may be involved.

PLZF represses transcription (23), at least in part, by interactions with histone deacetylases (12–15). Trichostatin A, a histone deacetylase inhibitor, does not completely block transcriptional repression by PLZF (52), indicating that other mechanisms of transcriptional repression are also at work. The recruitment of a kinase activity to the promoters of PLZF target genes could contribute to repression by phosphorylation and inhibition of components of the basal transcription machinery. Transcription within the cell is repressed during mitosis (53), when cdc2 kinase is particularly active. Several studies have shown that activated cdc2 can repress transcription by phosphorylating members of the basal transcriptional machinery such as TBP (54,55). We co-transfected cdc2 kinase and PLZF along with a construct containing PLZF binding sites upstream of a luciferase reporter gene. cdc2 kinase had no effect on PLZF repression (data not shown); however, our immunoprecipitation studies indicated that only a fraction of cdc2 in cell lysates was associated with PLZF. This suggests that cdc2 is already in excess in cells and an in vitro transcription system may have to be utilized in order to see an effect of cdc2 kinase on PLZF activity.

In contrast to PLZF, an RARα/PLZF interaction with cdc2 could not be demonstrated. Antibodies to cdc2 had no effect on the RARα/PLZF–DNA complex and the size of the complex was determined to be ~40 kDa, indicating that cdc2 could not be in the RARα/PLZF complex. RARα/PLZF can bind to the same DNA sequences as PLZF but is a weak transcriptional activator. This is likely due to loss of the POZ domain and a second, poorly understood, repression domain within PLZF (23) and gain of the A activation domain of RARα. If the PLZF–cdc2 interaction is linked to transcriptional repression by PLZF, the aberrant transcriptional activity of the fusion protein may also be due to loss of the cdc2 interaction domain.

Figure 6. PLZF is a phosphoprotein. (A) PLZF–Flag was expressed in CV-1 cells and the cells were metabolically labeled with [32P]orthophosphate. A 90 kDa protein corresponding to PLZF–Flag was immunoprecipitated with M2 monoclonal anti-Flag antibody. The labeled protein was excised from a polyacrylamide gel and eluted, then subjected to trypsin digestion and acid hydrolysis. The mixture of single amino acids and incompletely digested peptides was electrophoretically separated on a thin layer chromatography plate in two dimensions at pH 1.9 and pH 3.5, respectively, then compared to standards. (B) PLZF is phosphorylated by cyclin B/cdc2. GST–PLZF was expressed in E.coli and purified on glutathione–agarose beads. The protein was incubated with [γ-32P]ATP with or without cyclin B/cdc2 kinase (2 U). The fusion protein was cleaved with Factor Xa and separated on a 12% SDS denaturing gel which was then dried for autoradiography. (C) The PLZF–DNA complex is more sensitive to the addition of phosphatase than the RARα/PLZF–DNA complex. In an EMSA, 0.001 and 0.003 U of potato acid phosphatase were added to the reaction mixtures and incubated on ice for 30 min before electrophoresis. In the second part of the figure, 0.003 U of potato acid phosphatase were added to extracts from vector, PLZF or (ΔPOZ)PLZF transfected 293T cells and incubated for 30 min on ice before electrophoresis on an SDS denaturing gel. Proteins were subjected to western blot analysis with a monoclonal PLZF antibody.
If the DNA binding activity of PLZF is regulated by cdc2 and the cell cycle, then DNA binding by RARα/PLZF may be less constrained, further potentiating its effects on PLZF activity.

In summary, PLZF is a phosphoprotein and can act as a substrate for cdc2 kinase. The function of cdc2 may be to modulate DNA binding or transcriptional repression by PLZF. The lack of an RARα/PLZF-cdc2 interaction further indicates that, although binding to the same DNA sequences, the fusion protein has altered protein interactions contributing to its aberrant function.

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