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Functional interaction of Purα with the Cdk2 moiety of cyclin A/Cdk2☆

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Abstract

Purα is a sequence-specific single-stranded nucleic acid-binding protein and a member of the highly conserved Pur family. Purα has been shown to colocalize with cyclin A/Cdk2 and to coimmunoprecipitate with cyclin A during S-phase. Here we show that this interaction is mediated by a specific affinity of Purα for Cdk2. In pull-down assays GST-Purα efficiently binds Cdk2 and Cdk1, binds Cdk4 less efficiently, and does not display binding to Cdk6. Purα stimulates several-fold the phosphorylation in vitro of histone H1 by cyclin A/Cdk2, produced from baculovirus constructs. Double chromatin immunoprecipitation using antibodies to Cdk2 and Purα reveals that both proteins colocalize in HeLa cells to DNA segments upstream of the *c-MYC* gene. Pur family member Purγ colocalizes with Cdk2 to a specific DNA segment in this region.

Keywords: Cell cycle; Origin of DNA replication; Cdk1; Cdk4; Cyclin D1; Cyclin B1; Cyclin E1; Cyclin H; p21; Purα; Purγ; Chromatin immunoprecipitation; c-*MYC* gene

Purα was first discovered and cloned through its affinity for the G-rich single strand of an element located upstream of the human *c-MYC* gene [1,2]. Purα is both a DNA- and RNA-binding protein, and it has now been implicated in processes as diverse as DNA replication, gene transcription, RNA transport, and mRNA translation (see [3,4] for review). Purα plays an enhancing role in the replication of two distinct viruses, HIV-1 [5] and JCV [6], in the brain. Purα has been reported to modulate transcription in concert with family member, Purβ [7,8]. Purα has been shown to form ternary complexes with single-stranded DNA and the Tat protein of HIV-1 [9] or the retinoblastoma protein, Rb [10]. The ability to thus recruit proteins to specific single-stranded regions of nucleic acids may be a functional feature of Pur family members conserved through phylogeny from bacteria through humans [3].

Interactions between Purα and cyclin/Cdk complexes have been observed since the initial finding that Purα coimmunoprecipitates with cyclin A and is colocalized with it at replication foci during S-phase of CV-1 cells [11]. Since then, colocalization with cyclin B1/Cdk1 during mitosis has been noted [12], as have interactions of Pur with cyclin T1/Cdk9 [13] and Cdk5-containing complexes [14]. Despite these intriguing findings, little is known regarding the mechanism or functional consequences of Purα association with these protein kinase complexes. Here we report that Purα binds several

☆ *Abbreviation:* Cdk, cyclin-dependent kinase.

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Cdk proteins. Deletion mutational analyses reveal that Purα has a very specific affinity for the Cdk2 moiety of cyclin A/Cdk2 and that it stimulates histone H1 phosphorylation in an in vitro reaction employing purified proteins.

Materials and methods

Purification of Purα and Purα deletion mutants. Purα fused at its N-terminus to glutathione-*S*-transferase (GST) was purified from transformed *Escherichia coli* BL21-LysS using glutathione–agarose beads as previously described [10]. The various Purα deletion mutants have also been previously described [10,12] and were purified in the same way.

Purification of the cyclin A/Cdk2 complex. Extracts containing high levels of both cyclin A and Cdk2 were prepared from insect Sf9 cells coinfected with baculovirus constructs to overexpress both proteins. Prominent bands for each protein could be seen in the total extracts and confirmed by immunoblotting. The Cdk2 protein was fused at its N-terminus to an HA epitope tag, allowing the cyclin A/Cdk2 complex to be purified by immunoaffinity chromatography essentially as described by Dynlacht et al. [15]. Briefly, mouse monoclonal antibody 12CA5 (Boehringer–Mannheim) was cross-linked to protein A–agarose beads (Sigma) using dimethylpimelimidate. The beads were then incubated with Sf9 cell extracts in binding buffer containing 140 mM NaCl, 0.01% NP-40, 0.5 mM dithiothreitol, 0.5 mM EDTA, protease inhibitors (10 μg/ml leupeptin, 10 μg/ml pepstatin, and 20 μg/ml aprotinin), and 20 mM Hepes, pH 7.6, for 4 h at 4 °C. Beads were then collected by centrifugation and washed three times in the same binding buffer containing 0.5 M NaCl. The cyclin A/Cdk2 complex was then eluted with HA peptide (1.0 mg/ml) in the binding buffer with Arg-insulin (0.2 mg/ml) as a carrier protein.

Extracts of NIH3T3 cells. Extracts of exponentially growing NIH3T3 cells were prepared as follows. Ten plates of 4×10^5 cells were rinsed twice with cold phosphate-buffered saline and scraped into 0.5 ml lysis buffer [0.1% NP-40, 50 mM Tris–Cl, pH 7.4, 5 mM EDTA, 0.25 M NaCl, 50 mM NaF, 1 mM of 1,2-epoxy-3-(*p*-nitro-phenoxy)propane, 1 mM phenylmethylsulfonyl fluoride, 1 mM dithiothreitol, 1 μg/ml leupeptin, and 1 μg/ml aprotinin]. Cells were homogenized with 20 strokes in a Dounce homogenizer with a tight-fitting pestle, and then diluted with an equal volume of lysis buffer lacking NaCl such that the final concentration of NaCl was 0.125 M. The diluted lysate was centrifuged at 13,000*g* at 4 °C for 5 min. The resulting supernatant was used immediately or frozen at −80 °C.

Binding of proteins to GST-Purα. GST-Purα fusion proteins were expressed and bound to glutathione–agarose beads as described previously [10]. For each reaction, the amount of fusion protein or GST, the volume of eukaryotic cell lysate, and the total reaction volume were equal. After washing and resuspension in SDS loading buffer, an equal volume of each sample was loaded in wells of a SDS–polyacrylamide gel. Gel bands were blotted to Immobilon P membranes (Millipore) and probed with antibodies as previously described [10]. All antibodies for immunoblots were obtained from Santa Cruz Biotechnology. Detection was carried out with the Pierce Supersignal chemiluminescence kit.

Protein kinase assays. Assays of phosphorylation catalyzed by cyclin A/Cdk2 were performed in reactions (20 μl) containing purified cyclin A/Cdk2 complex, unless otherwise indicated, 0.2 μg of purified histone H1 (this substrate generously provided by Dr. Thomas M. Fasy), 5.0 mM NaF, 0.5 mM EGTA, BSA (0.2 mg/ml), 10 mM $MgCl_2$, and 50 μM ATP containing 5 μCi [γ-^{32}P]ATP (6000 Ci/mmol). The reaction was initiated after a 15 min preincubation period at 30 °C by the addition of $MgCl_2$ and ATP. After further incubation for 10 min, the reaction was stopped by addition of SDS gel sample buffer and a final concentration of 20 mM EDTA. After gel electrophoresis, radiolabeled bands were analyzed using a phosphorimager (Molecular Diagnostics). Known quantities of radiolabeled H1 were employed as standards. Background levels of phosphorylation, which were minimal, were subtracted for all points plotted. Levels of phosphate incorporation into histone were calculated from the specific activity of the ATP.

Double chromatin immunoprecipitation (double ChIP) of Cdk2 with other proteins on DNA segments upstream of the c-MYC gene. ChIP was performed exactly as described previously for MCM proteins bound to DNA upstream of the c-*MYC* gene [16]. HeLa cells were incubated for 15 min in medium containing 1% formaldehyde at room temperature, and cross-linking was quenched by adding glycine to 125 mM. ChIP was performed on each sample of cell lysate by overnight incubation at 4 °C with 4 μg of primary antibody followed by incubation with protein G PLUS–Agarose (Santa Cruz Biotechnology) for 2 h. Antibodies against Cdk2 (rabbit polyclonal) and p21 (mouse monoclonal) were obtained from Santa Cruz Biotechnology. Antibodies against Purγ (rabbit polyclonal) and Purα (mouse monoclonal) were prepared at this institution. For single ChIP samples were washed four times and eluted in SDS-containing buffer. For re-ChIP, re-ChIP elution buffer (10 mM EDTA, 50 mM Tris–HCl, pH 8.0, and 0.7 M NaCl) was used to elute. The elution buffer was diluted to 1/5, and re-ChIP was performed using a second primary antibody as for the first ChIP. Reversal of cross-linking, isolation of immunoprecipitated DNA, and PCR were as described [16]. Primers used, as indicated schematically in the figure legend, amplify two segments of DNA located in a zone of initiation of DNA replication upstream of the c-*MYC* gene.

Results

Binding of Purα to different cyclin/Cdk complexes

Interaction of Purα with cyclin A/Cdk2 and with p35/Cdk5 has been well documented [11,12,14]. We have sought to determine whether Purα is capable of interaction with other cyclin/Cdk complexes. For this study mouse NIH-3T3 cell lysates were used. It should be noted that although we have employed human Purα as GST-Purα, human and mouse Purα differ only by two conservative changes in amino acids [17]. Similarly, human and mouse Cdk2 differ only slightly. As controls we have employed GST alone linked to glutathione–agarose beads and beads alone. We present samples of lysates after pre-clearing with beads alone. After incubation of the beads with the cell lysate, recovery of the beads, and washing, the beads are suspended in SDS gel sample buffer and loaded directly onto SDS–polyacrylamide gels. Immunoblotting of the gels with various antibodies allows detection of proteins pulled down by Purα. It can be seen in Fig. 1 that Purα very effectively retrieves from cell lysates cyclin A, cyclin B1, cyclin D1, and cyclin E1 but less so cyclin H. Note that cyclins D1 and E1 are present at very low levels in the lysates used and are effectively concentrated to detectable levels by affinity purification with GST-Purα. Purα also retrieves Cdk1, the partner of cyclin B1, and, to a lesser extent Cdk4, a partner of D-type cyclins, but does not retrieve significant amounts of Cdk6, another