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The retroviral cyclin of walleye dermal sarcoma virus binds cyclin-dependent kinases 3 and 8

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ABSTRACT

Walleye dermal sarcoma virus encodes a retroviral cyclin (rv-cyclin) with a cyclin box fold and transcription activation domain (AD). Co-immune precipitation (co-IP) identified an association of rv-cyclin with cyclin-dependent kinase 8 (cdk8). Cdk8 is dependent upon cyclin C and regulates transcription with the Mediator complex, a co-activator of transcription. Mutation of cyclin residues, required for cdk binding, disrupts rv-cyclin–cdk8 co-IP. Mutation or removal of the AD has no effect on cdk8 interaction. Direct rv-cyclin–cdk8 binding is demonstrated by pulldown of active cdk8 and by GST-rv-cyclin binding to recombinant cdk8. Cdk3 is also activated by cyclin C and phosphorylates retinoblastoma protein to initiate entry into the cell division cycle. Co-IP and pulldowns demonstrate direct rv-cyclin binding to cdk3 as well. The rv-cyclin functions as a structural ortholog of cyclin C in spite of its limited amino acid sequence identity with C cyclins or with any known cyclins.

Introduction

Walleye dermal sarcoma virus (WDSV) is a complex retrovirus that encodes three known accessory proteins in addition to the structural and enzymatic proteins encoded by the *gag*, *pro*, *pol*, and *env* genes (Holzschu et al., 1995; Martineau et al., 1992, 1991). Transcripts from two of these accessory genes, *orf a* and *orf b*, are the only WDSV transcripts present during the development of dermal sarcoma (Bowser et al., 1996; Quackenbush et al., 1997), implicating their protein products in tumor induction and progression. The protein product of the *orf a* transcript contains a predicted cyclin box fold and is referred to as the retroviral cyclin or rv-cyclin protein (LaPierre et al., 1998). The cyclin box fold is a protein-binding domain common to cyclins, transcription factor 2B (TFIIB), and retinoblastoma protein (Rb) (Noble et al., 1997). Each of these contains two copies of the domain, which is characterized by a similar alpha-helical structure, but with remote linear sequence identity. Alignments of the rv-cyclin with cyclins A, C, and D have been made based on combinations of sequence identity and proposed function (LaPierre et al., 1998; Rovnak and Quackenbush, 2002; Zhang and Martineau, 1999).

In addition to its cyclin box fold, the rv-cyclin has a functionally separable, transcription activation domain (AD) (Rovnak et al., 2005). The AD directly contacts TATA binding protein (TBP)-associated factor 9 (TAF9) in mammalian and piscine cells (Rovnak and Quackenbush, 2006). Mutation of valine to serine at position 260 (V260S) within the TAF9 binding motif interferes physically and functionally with TAF9 binding (Quackenbush et al., 2009; Rovnak and Quackenbush, 2006).

The rv-cyclin localizes in the nucleus and is concentrated in interchromatin granule clusters (IGCs or nuclear speckles) and perichromatin fibrils (Rovnak et al., 2001; Rovnak and Quackenbush, 2002). The rv-cyclin co-localizes and co-purifies with hyperphosphorylated forms of the large subunit of eukaryotic RNA polymerase II (RNAPII) and is co-precipitated with antibodies against RNAPII (Rovnak and Quackenbush, 2002). RNAPII is phosphorylated predominantly at serines 2 and 5 of the heptad repeat (YSPTSPS)52 in its C-terminal domain (CTD) (reviewed in Buratowski, 2009). Progressive phosphorylation and dephosphorylation of the CTD at these sites are associated with transcription initiation and elongation. Serine 5 is highly phosphorylated during transcription initiation and gradually declines during elongation when serine 2 phosphorylation increases (Komarnitsky et al., 2000). Cdks 7 and 8 phosphorylate serine 5 of the

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heptad repeat (Ramanathan et al., 2001; Rickert et al., 1999). Cdk9 phosphorylates serines 2 and 5, but primarily serine 2 (Price, 2000; Ramanathan et al., 2001).

In co-immune precipitations (co-IP) with antibodies reactive to seven different cdks, rv-cyclin was co-precipitated only with cdk8 (Rovnak and Quackenbush, 2002). Cdk8, its partner, cyclin C, and proteins, Med12 and Med13, are components of the cdk8 submodule of the Mediator complex. Mediator is targeted by activators and inhibitors of transcription and functions via its close association with RNAPII (reviewed in (Taatjes, 2010)). After transcription initiation, cdk8 phosphorylation of the CTD enhances the processivity of elongation (Donner et al., 2010, 2007). Cdk8-Mediator interacts with positive transcription elongation factor b (pTEFb) and affects the recruitment of pTEFb and bromodomain protein, Brd4 (Donner et al., 2010). Cdk8 also phosphorylates transcription factor, E2F-1, repressing its inhibition of β-catenin/T-cell factor-dependent transcription (Morris et al., 2008), and serine 10 of histone H3, which leads to GCN5L acetylation of lysine 14, a mark of active transcription (Meyer et al., 2008).

The dysregulation of either cyclin C or cdk8 has been implicated in cancer. In cases of osteosarcoma and in osteosarcoma cell lines, there is frequent allelic loss of the *CCNC* gene encoding cyclin C, and overexpression of exogenous cyclin C inhibits the continued growth of these cells (Ohata et al., 2006). In contrast, the gene encoding cdk8 resides in a region of the human genome that is often amplified in colon cancers and overexpression of exogenous cdk8 leads to cell transformation of NIH3T3 cells (Firestein et al., 2008).

Although not included in the original screen of rv-cyclin–cdk interaction, cdk3 has since been identified as an alternative partner of cyclin C (Ren and Rollins, 2004). Cyclin C/cdk3 promotes the transition of quiescent cells into the cell division cycle. This transition from G0 to G1 and subsequently to S phase is dependent upon phosphorylation of Rb by cyclin C/cdk3, cyclin D/cdk4/6, and cyclin E/cdk2 complexes as well as by cdk3 complexes with cyclins A and E (Connell-Crowley et al., 1997; Harper et al., 1995). These cyclin/cdk pairs phosphorylate Rb at residues S807 and S811 causing its disassociation from E2F transcription factors to allow E2F-dependent transcription.

The common interaction of the cyclin box of the rv-cyclin with the two cyclin-dependent kinases that pair with cyclin C suggests its role as a functional ortholog of this host cyclin, even though their sequence homology is low (Rovnak and Quackenbush, 2002). In spite of their potent gene regulatory and cell division functions, to our knowledge, viral proteins that target cyclin C or its cdk partners have not been identified previously. The results presented here confirm the direct interaction of rv-cyclin with cdk8 and with cdk3 and define it as the first viral ortholog of cyclin C. The outcome of this function includes enhanced cell proliferation and induction of host gene expression.

Results

The rv-cyclin–cdk8 interaction is dependent upon the cyclin box fold

Using the protein homology/analogy recognition engine (Phyre) (Kelley and Sternberg, 2009), rv-cyclin was found to align with 1000 different cyclins. Cyclin A from Drosophila (*Drosophila melanogaster*) aligned with rv-cyclin with the highest probability (*E*-value = $4e^{-43}$). Fig. 1A shows an alignment of the amino acid sequence of rv-cyclin with sequences of *Drosophila* cyclin A and human cyclin C, which have 19.2% and 14.9% sequence identity with the rv-cyclin, respectively. Cyclin C is included because previous studies showed a physical association of the rv-cyclin with cdk8 and cyclin C is the partner of cdk8 (Rovnak and Quackenbush, 2002).

In order to assess the significance of a cyclin from a walleye virus associating with cdk8 from different species, walleye cdk8 mRNA was cloned and its amino acid sequence was found to be 98% identical to that of human cdk8 (9 amino acid variations out of 464; GenBank accession no.1383538). The walleye cdk8 sequence is 99% identical to zebrafish cdk8 (*Danio rerio*) with only 4 amino acid variations. Although we have not yet established the sequence of walleye cyclin C, comparisons of known cyclin Cs show that they are the most highly conserved of all cyclins among metazoan species (Hoeppner et al., 2005). The sequence of zebrafish cyclin C is 94% identical to that of human cyclin C. The conservation of cyclin C and cdk8 sequences explains the ability of the rv-cyclin to function across vertebrate species, including HeLa cells, and indicates the importance of this function in gene regulation.

Fig. 1A serves to illustrate two important aspects of rv-cyclin structure: one is the remote sequence identity of the rv-cyclin to *all* known cyclins, none of which has greater than 20% identity with rv-cyclin. Yet structure prediction tools consistently identify its cyclin box fold with high confidence levels. A second important aspect, apparent from the alignment, is the exclusion of the rv-cyclin carboxy-terminal AD from alignment with any cyclin sequence. The AD is a separate functional domain distinct from the cyclin box fold.

Mutations at conserved residues, marked by asterisks in Fig. 1A, are known to disrupt the binding of cyclins A and C to their cdk partners (Hoeppner et al., 2005). To determine whether the aligned residues, K80 and E111 of rv-cyclin, effect its binding to cdk8, they were each substituted with alanine and the mutated constructs were tested for co-IP with cdk8 (Fig. 1B). In addition, a double mutation, K80A/E111A (DM), a loss-of-function mutation within the rv-cyclin AD, V260S, and a carboxy-truncated form of the rv-cyclin, amino acids 1–255, were also tested. V260S blocks rv-cyclin pulldown of TAF9 and CBP/p300 but not of Mediator component, Med23 (Sur2) (Rovnak and Quackenbush, 2006), and the 1–255 construct contains the entire predicted cyclin box fold and excludes the AD. HA-tagged human cyclin C and wild-type and mutated forms of rv-cyclin were co-expressed with FLAG-tagged human cdk8 in HeLa cells, and whole cell lysates were subjected to immune precipitation with anti-cdk8 antibody. The resultant precipitates were analyzed by Western blot for co-IP of individual HA-tagged constructs and for IP of FLAG-tagged cdk8. Anti-cdk8 co-IPs of mutants K80A, E111A, and DM were greatly reduced. The V260S mutation and deletion of the carboxy end had little or no effect on anti-cdk8 co-precipitation (Fig. 1B).

The rv-cyclin binds cdk8 directly

GST fusions with wild-type rv-cyclin and with human cyclin C were prepared and tested for their ability to pulldown expressed, FLAG-tagged cdk8 from HeLa cell lysates. After incubation and washing, proteins bound to glutathione Sepharose were tested for kinase activity *in vitro* with γ^{32}P-ATP and GST-RNAPII CTD fusion protein (GST-CTD) as substrate. After incubation and additional washes, the proteins that were pulled down and the bound GST substrate were eluted in sample buffer, separated on polyacrylamide gels, and blotted on nylon membranes. After autoradiography to determine the phosphorylation status of the CTD, blots were blocked and probed for cdk8 and for input CTD substrate (Fig. 2A). Both GST-rv-cyclin and GST-cyclin C were able to pulldown active cdk8 as determined by radiolabeling of the CTD and detection of cdk8 with specific antibody. GST protein alone did not pulldown cdk8 or kinase activity. Pulldowns of alternative CTD kinases, cdk7 and cdk9, were not detected by Western blot (data not shown). To further confirm direct rv-cyclin–cdk8 interaction, pulldown assays were performed with purified, His-tagged cdk8 protein. In this case, the 6-His tagged cdk8, bound to nickel-charged (Ni) Sepharose, was used to pulldown soluble GST fusion proteins. Both GST-rv-cyclin and GST-cyclin C, but not GST alone, bound to the cdk8 demonstrating direct interaction of rv-cyclin with cdk8 (Fig. 2B). Soluble GST fusion proteins were pre-cleared with Ni Sepharose to exclude nonspecific binding.