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NMR and mass spectrometry studies of putative interactions of cell cycle proteins pRb and CDK6 with cell differentiation proteins MyoD and ID-2

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Abstract

Cell growth and differentiation require precise coordination of cell cycle and differentiation proteins. This can be achieved by direct interactions between proteins, by indirect interaction in multiprotein complexes, or by modulation of gene expression levels of partner proteins. Contradictory data abound in the literature regarding the binding between some central cell cycle proteins, pRb, and CDK6, with myogenic differentiation promoting, MyoD, and inhibiting, Id-2, factors. We have tested these interactions using pure proteins and in vitro biophysical and biochemical methods, which included mass spectrometry, nuclear magnetic resonance (NMR), the affinity chromatography pull-down assays, and gel filtration chromatography. Using this multimethod approach, we were able to document interactions between pRb and HPV-E7, pRb and SV40 large T antigen, CDK6 and p19, and MyoD and DNA. Using the same methods, we could unambiguously show that there is no direct protein–protein interaction in vitro between the small pocket domain of pRb and the bHLH domain of MyoD, the small pocket domain of pRb and Id-2, and CDK6 and a 15-amino-acid peptide from the C-terminal domain of MyoD. Indirect interactions, through additional binding partners in multiprotein complexes or modulation of gene expression levels of these proteins, are therefore their probable mode of action.

Keywords: MyoD; Id-2; Retinoblastoma protein; CDK6; Interaction; Complex

1. Introduction

The proper execution of cell differentiation requires interplay between differentiation signals and the cell cycle [1]. A large body of evidence indicates that protein–protein interactions are important in pathways that initiate and terminate cell cycle [2]. Proteins from two subclasses of the HLH (helix–loop–helix) family–the bHLH (basic HLH) proteins and the Id proteins (Id, inhibitors of differentiation)–are crucial for the coordination of differentiation processes in the cell cycle [3]. These proteins form both homo- and heterodimers. Dimerization and interaction with the basic region are essential for DNA binding and transcriptional activity [4,5].

The bHLH subclass contains MyoD, myogenin, E12, E47, E2-5/ITF1, NeuroD/BETA2, and TAL [5]. MyoD plays a key role in the differentiation of all skeletal muscle lineages [6]. A forced expression of MyoD in a variety of different cell backgrounds, including normal, transformed, and tumor cell lines, was shown to induce growth arrest. This feature was correlated with the presence of the MyoD bHLH domain [7]. In vitro protein binding and immunoprecipitation studies indicated that both MyoD and myogenin bind to the so-called small pocket domain of underphosphorylated pRb directly through the bHLH domain [8]. Several other in vivo studies however could find no evidence for such binary interactions suggesting that if there is any direct interaction, it appears to be weak, making the significance of the in vitro MyoD–pRb interaction questionable

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[9–12]. Using the two-hybrid system, Zhang et al. [11] could not detect direct MyoD or myogenin–pRb interactions. These authors however found that MyoD interacted with human CDK4 and CDK6 through a conserved 15-amino-acid region situated at the C-terminus of MyoD. The C-terminus of MyoD (15 amino acids fragment) was shown to be sufficient to inhibit CDK4-dependent phosphorylation of pRb [11,12]. We decided therefore to investigate the direct MyoD–pRb and MyoD–CDK6 interactions using multiple biophysical methods described below.

Id-1 to Id-4 are another category of mammalian HLH proteins [13]. These proteins promote cell proliferation by binding to transcription factors from the bHLH subclass of proteins and inhibit their ability to bind DNA [14]. Id-2 and MyoD have a high degree of sequence homology. Only Id-2, and not other Id proteins, was reported to disrupt the antiproliferative effects of pRb proteins via direct interactions [15]. The HLH domain of Id-2 was shown to interact with pRb (small pocket domain), p107, and p130 [15,16].

CDK4 and CDK6 are physiological pRb kinases, which are inhibited by the INK4 (p14, p16, p18, and p19) and CIP/KIP (p21, p27) family of inhibitors [17,18]. This inhibition prevents the phosphorylation of pRb and keeps pRb in its active growth-suppressing state. CDK6 shows a high amino acid homology with CDK4, and these two proteins are also expected to have similar 3D structures [19,20]. The pRb inhibits cell cycle progression by interacting with transcription factors such as E2F [2] and is essential for the myogenesis processes [21]. It is also a target of viral oncoproteins like HPV16 E7, SV40 large T antigen and, adenovirus E1A [2,22].

The contradictory data on signal transduction between differentiation HLH factors and cell cycle regulatory proteins prompted us to re-investigate these interactions. Since the assays reported in the literature were based not on isolated and homogenously pure proteins, we decided to use pure proteins and check for binary associations using direct in vitro experiments. These experiments included NMR spectroscopy, gel filtration chromatography, mass spectrometry, and affinity chromatography pull-down assays. These interactions were checked on isolated constructs that either corresponded exactly to the constructs used in the literature or were truncated constructs that covered the parts of the proteins that were reported to be interaction sites. We show here that there was no direct protein–protein interaction in vitro among the various domains of pRb and MyoD, pRb and Id-2, and CDK6 and MyoD, which were claimed to bind to each other.

2. Materials and methods

2.1. Cloning and plasmids

All Id-2, MyoD, and pRb constructs were cloned into the pET30 Xa/LIC vector (Novagen) according to the manufacturer's manual. All protein sequences were identical with those of *Homo sapiens*. Short His-tag (6× H) and DAPase (dipeptidyl aminopeptidase) (Qiagen) digestion stop sites (KK, KR or RK) were introduced by Quick-Change Mutagenesis Kit (Stratagene). The pRb ABC-C had 6 His, a DAPase digestion stop site, and pRb sequence 379–791. The pRb ABC-CL construct spanned residues 379–578 and 642–791 of pRb. Construct MCNC consisted of 6 His, a DAPase digestion stop site, and MyoD sequence 99–173. The Id-2 construct covered residues 2–132. The CDK6 construct was cloned between the *Bam*HI and *Eco*RI sites into the pAcG2T vector. The CDK6 construct consisted of full-length CDK6 cloned on the C-terminal part of GST. The HPV16 E7 full-length construct was cloned into pET8 vector and had C-terminal 6 His. The HPV16 E7-short was cloned into pET30 Xa/LIC (Novagen) vector and spanned residues 21–40 (the same sequence like E71 peptide). The p19 construct spanned the full length of the p19 protein. All constructs used in this work are summarized in Fig. 1.

2.2. Protein expression

Id-2, MyoD, pRb, and E7 constructs were expressed in *Escherichia coli* strains BL21 STAR™ (DE3) (Invitrogen). Cells were cultured according to standard procedures [23] in LB or M9 minimal medium [23] supplemented with 1% glucose, 10 mM K_2PO_4, pH 7.2, 1 mM $MgSO_4$, kanamycin (MyoD, Id-2, pRb, and E7), ^{15}N-labeled NH_4Cl_2 for uniform ^{15}N labeling, and for specific ^{15}N labeling, ^{15}N-labeled amino acids were used. The temperature of pRB cultures were decreased to 18 °C at $OD_{600nm}=0.4$. Cultures were induced at $OD_{600nm}=0.7$ with 1 mM IPTG and grown for 4 h at 37 °C or for 12 h at 18 °C (pRb). After this time, cells were spun and pellets were frozen.

CDK6 was expressed in *Spodoptera frugiperda* strain Sf9. Infective viruses carrying GST-CDK6 were produced, and the right titer was checked. Protein expression was performed in 4 l (cell density 2 mln/1000 ml) at 27 °C. After 58 h, cells were harvested and frozen.

2.3. Purification, DAPase, thrombin cleavage

E. coli cells, which overexpressed any of pRb constructs or the full-length E7, were lysed in a lysis buffer (50 mM NaH_2PO_4, pH 8, 300 mM NaCl, 10 mM βME, 10 mM imidazole; additionally, the lysis buffer was supplemented with protease inhibitor cocktail (one tablet of "Complete" without EDTA, Roche, per 50 ml lysate)), sonicated, and centrifuged. Supernatants were passed through NiNTA (Qiagen) in the first step of purification. The sample was loaded onto the column and washed with lysis buffer supplemented with 20 mM imidazole. Elution was performed with lysis buffer supplemented with 250 mM imidazole. The pRb:ABC-C and ABC-CL constructs were subjected to the MonoQ (Amersham-Pharmacia) anionic exchange chromatography