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The mammalian heterochromatin protein 1 binds diverse nuclear proteins through a common motif that targets the chromoshadow domain

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Abstract

The HP1 proteins regulate epigenetic gene silencing by promoting and maintaining chromatin condensation. The HP1 chromodomain binds to methylated histone H3. More enigmatic is the chromoshadow domain (CSD), which mediates dimerization, transcription repression, and interaction with multiple nuclear proteins. Here we show that KAP-1, CAF-1 p150, and NIPBL carry a canonical amino acid motif, PxVxL, which binds directly to the CSD with high affinity. We also define a new class of variant PxVxL CSD-binding motifs in Sp100A, LBR, and ATRX. Both canonical and variant motifs recognize a similar surface of the CSD dimer as demonstrated by a panel of CSD mutants. These in vitro binding results were confirmed by the analysis of polypeptides found associated with nuclear HP1 complexes and we provide the first evidence of the NIPBL/delangin protein in human cells, a protein recently implicated in the developmental disorder, Cornelia de Lange syndrome. NIPBL is related to Nipped-B, a factor participating in gene activation by remote enhancers in *Drosophila melanogaster*. Thus, this spectrum of direct binding partners suggests an expanded role for HP1 as factor participating in promoter–enhancer communication, chromatin remodeling/assembly, and subnuclear compartmentalization.

Keywords: Chromatin; Heterochromatin; HP1; Epigenetic; Gene regulation; Gene silencing; Chromodomain; Chromoshadow domain; NIPBL; Cornelia de Lange syndrome

The packaging of DNA into chromatin in eukaryotes plays a critical role in regulating genomic processes such as replication, recombination, and gene transcription. Much is known about the fundamental unit of chromatin structure, the nucleosome, including a prototypical crystal structure [1]. The amino-terminal tails of the core histones, which extend beyond the globular core and DNA, have emerged as the key regulators of nucleosome assembly and DNA packaging. It has been hypothesized that post-translational modifications (acetylation, methylation, phosphorylation, etc.) of the nucleosomal histones specify a code that can be correlated with the transcriptional activity of genes [2,3].

The modification of histone tails and the incorporation of alternate histone isoforms into the nucleosome likely provide signals for higher order chromatin assembly/disassembly processes. However, it is unclear how these marked nucleosomes and other nuclear factors are differentially assembled into higher order

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chromatin structures that vary in transcriptional potential. It is a reasonable hypothesis that the mechanisms for the assembly of nucleoprotein complexes will be less dependent upon direct protein–DNA interactions and more dependent upon protein–protein interactions. With this in mind, we have focused our studies of chromatin function on the heterochromatin protein 1 (HP1) family of proteins.

The HP1 proteins are non-histone, chromosomal proteins that have conserved roles in epigenetic regulation of gene expression. In humans, the family is composed of three members; HP1α, β, and γ. The prototypical HP1 was discovered as an abundant antigen localized to the chromocenters of polytene chromosomes in *Drosophila melanogaster* and was later shown to be a gene dosage-dependent regulator of position effect variegation (PEV) [4,5]. The phenomenon of PEV is observed when an expressed euchromatic gene is juxtaposed next to a block of silent heterochromatin. This silent state is thought to then spread into the adjacent euchromatin in an HP1-regulated manner in some cells leading to stable gene silencing and variegated patterns of expression.

HP1 proteins possess an N-terminal chromodomain (CD), a central linker region, and a C-terminal chromoshadow domain (CSD). Both domains act as protein–protein interaction domains. The methylation of lysine 9 in histone H3 has been shown to create a direct and specific binding site for the HP1 CD [6–8]. A histone methyl transferase for lysine 9 in histone H3 N-terminal tail is encoded by *Su(var)3–9* and *Clr4* in *D. melanogaster* and *Schizosaccharomyces pombe*, respectively. Each has been shown to be essential for epigenetic silencing and localization of HP1 proteins [9–11].

A parallel series of studies have characterized the biochemical and biological activities of the HP1 C-terminal CSD, which is found solely in the HP1 family of CD-containing proteins. We and others have recently shown that the HP1 CSD exists as a homodimer [12–14]. This dimer binds with high affinity to a consensus monomeric pentapeptide motif of the form, PxVxL, which has also been designated the HP1BD or MIR [14,15]. This motif occurs in the KAP-1 corepressor, the p150 subunit of chromatin assembly factor-1 (CAF-1 p150), and was recently identified in the human $TAF_{II}130$ component of TFIID [16], DNA polymerase-α [17], and Su(var)3–7 [18], and AF10 [19].

In this study, we have mapped the CSD interaction motifs in LBR, Sp100, ATRX, HP1-BP74, and NIPBL using mutagenesis and peptide competition. HP1 CSD binding by either canonical or variant PxVxL motifs in these proteins displays varying affinities and we propose that each of these nuclear partners interacts with the HP1 CSD exclusively. Several of these interactions are readily observed in vivo by co-immunoprecipitation of FLAG-HP1α, supporting the idea that multiple proteins bind HP1α in the nucleus. Together, these results expand the consensus sequence for targeting the HP1 CSD and implicate HP1 in diverse nuclear pathways. This study also reveals an interaction between HP1 and NIPBL in human cells, which may hold insight into the mechanism behind the defects arising from mutations in NIPBL that are associated with Cornelia de Lange syndrome [20,21].

Materials and methods

Plasmids. Construction of plasmids used in this study and oligonucleotide primers used for cDNA amplification or mutagenesis are contained in supplementary material available online.

GST protein-binding assays. The GST and polyhistidine fusion proteins were purified from bacteria under native conditions and the recombinant polyhistidine CSD proteins were subjected to size exclusion chromatography on a Superdex 200 column (GE-Healthcare-Amersham) as described previously [14]. The polyhistidine fusions were also produced in vitro using pcDNA3.1-based templates and ^{35}S-Translabel (ICN) in a TnT Quick Coupled Transcription/Translation System (Promega). A KAP-1 peptide, amino acids 478–497, was synthesized and purified on a C18 column by reverse-phase HPLC. A mutant version of the KAP-1 peptide corresponding to the double substitution RV487,488EE was also synthesized and both were dissolved in 50% DMSO at a stock concentration of 4 mM.

For binding reactions, equal amounts of GST fusion proteins (2 μg) were incubated with either a recombinant partner protein (5 μg) or in vitro translated partner protein (10 μl). In the peptide competition experiments, the peptide was added to an aliquot of the in vitro translated partner protein, which was then added to the GST fusion protein. The final concentrations of the KAP-1 peptides in the binding reactions were 40, 20, 10, and 5 μM. All reactions were carried out and analyzed as previously described [14].

Transient expression and immunopurification of FLAG-HP1α complexes. Human embryonic kidney 293 (HEK293) cells were transfected at 70% confluency using Lipofectamine as described by the manufacturers (Invitrogen). Thirty-six hours after transfection, cells were harvested and nuclear extracts were prepared as described [22], with the addition of DNAaseI (50 U/ml) and RNAaseA (50 μg/ml). The clarified extract was adjusted to 200 mM NaCl by addition of 5 vols. of binding buffer and then immunoprecipitated with FLAG-M2 monoclonal antibody resin (Sigma) for 2 h. The immune-complexes were washed twice in binding buffer with 500 mM NaCl for 30 min, thrice in 100 mM NaCl binding buffer, and thrice in 100 mM NaCl, 50 mM Tris–HCl, pH 8.0. Elution was performed twice with 200 μg/ml FLAG peptide (Sigma) in 100 mM NaCl, 50 mM Tris–HCl, pH 8.0, for 30 min. Aliquots of the eluate were separated on NuPAGE 4–12% Bis-Tris gradient gels (Invitrogen) and transferred to PVDF membranes. Membranes were probed with antisera to KAP-1, CAF1 p150 (Oncogene Research Products), BRG1, HP1α (Upstate Biotechnology), Sp100 or NIPBL. The antiserum to NIPBL was raised against a bacterially expressed GST-NIPBL (a.a. 906–1091) antigen in the Wistar Institute Hybridoma Core Facility.

Mass spectrometry analysis. Polypeptides specifically co-immunoprecipitated with the wild-type FLAG-HP1α protein were excised from gels stained with colloidal blue and digested in situ with trypsin. The peptides were analyzed using microcapillary reverse-phase HPLC nano-spray tandem mass spectrometry on a Finnigan LCQ quadrupole ion trap mass spectrometer at the Wistar Institute Protein Microchemistry/Mass Spectrometry Facility. The MS/MS spectra were compared against protein sequence data bases using SEQUEST [23].