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Detection and characterization of ubiquitylated H2B in mammalian cells

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ABSTRACT

Histone H2B ubiquitylation was shown to be associated with actively transcribed genes in mammalian cells and has been suggested to be involved in transcriptional regulation. Despite the limited applicability of genetic tools to analyze H2B ubiquitylation in mammals, several biochemical and immunological approaches have been successfully implemented to study this modification. Here we describe several techniques to detect ubiquitylated H2B in mammalian cells and to dissect its genomic localization.

1. Introduction

While the identification of histone H2B ubiquitylation in mammals [1] preceded the identification of histone ubiquitylation in yeast [2] by almost two decades, our understanding of mammalian H2B ubiquitylation has evolved much less rapidly. This is partly due to the fact that mammals have numerous copies of histone genes [3] versus a single copy in yeast. Thus, in yeast, mutating the ubiquitylation site of the single H2B gene can yield important insights about ubiquitylated H2B, such as its distribution throughout the genome and its effects on gene expression (for an elaborate description of analysis of H2B ubiquitylation in yeast see Trujillo et al. and Chandrasekharan et al. in this issue of "Methods"). In mammals, the site-specific mutation of numerous H2B genes (e.g. at least 17 in humans, Ref. [3]) cannot be implemented. However, other methods can be successfully utilized to study H2Bub in mammalian cells as described below.

In spite of lacking genetic tools comparable to those available for yeast studies, valuable information has accumulated about H2B ubiquitylation in mammalian cells over the years. The ubiquitylation site was determined to be lysine 120 of human H2B [4] and the enzymes responsible for this ubiquitylation were identified as Rad6 (the H2B E2, Ref. [5]) and hBre1/RNF20 (the H2B E3, Ref. [6]). H2B deubiquitylating enzymes were described as well (e.g. Ref. [7]). However, an association of H2Bub with specific genomic loci could not be demonstrated for many years. More recently, the generation of specific anti-H2Bub antibodies paved the way to demonstrating H2Bub association with the transcribed regions of transcriptionally active genes in mammalian cells [8]. Notably, while H2Bub seems to be associated with a broad spectrum of genes, its depletion (by siRNAs to hBre1) affects the expression of only a subset of genes [9], similarly to what has been found in yeast [10]. Importantly, studies of mammalian chromatin templates *in vitro*, as well as *in vivo* studies, demonstrated transcription-dependent H2B ubiquitylation and the dependency of H3K4 and H3K79 methylation on H2B ubiquitylation [5].

In this chapter we describe methods for the detection of H2Bub in mammalian cells and for the characterization of its genomic localization.

2. Production of H2Bub-specific monoclonal antibodies

To date, H2Bub is the only reported ubiquitin–protein conjugate to which monoclonal antibodies were intentionally generated. It would thus be of interest to detail the procedure of generating these antibodies. While the immunization and hybridoma generation procedures follow similar lines to reported protocols, there may be additional considerations involved in the choice of immunogen and the hybridoma screening steps as detailed below. Therefore, in the sections concerning immunization and hybridoma generation, we focused on particular issues relevant to the production of H2Bub-specific antibodies. The detailed protocol used for generation of mouse monoclonal antibodies is as previously described [11].

2.1. Immunization

2.1.1. Choice of immunogen

Antibodies prepared by immunization with denatured peptides or proteins may not react with native proteins. However, in the

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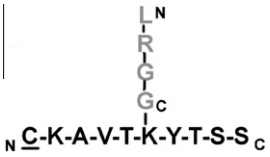

Fig. 1. Branched peptide used for immunization. The amino acid sequences in black and grey are derived from human H2B and ubiquitin, respectively. The underlined Cysteine was added to the sequence to facilitate conjugation to KLH.

case of H2Bub, using the full length native ubiquitylated H2B for immunization will likely shift the immune response towards private epitopes of either ubiquitin or H2B, located far from the ubiquitylation site. We therefore chose to use a short branched peptide comprising sequences of both mammalian H2B and mammalian ubiquitin together, derived from the vicinity of the ubiquitin conjugation site on human H2B [8] (Fig. 1). We chose the peptide arm length around the conjugated lysine to be 4 amino acids, theoretically allowing the complementarity determining regions of an antibody to recognize up to 9 amino acid residues (5 residues of H2B, including the conjugated lysine itself, and 4 last residues of ubiquitin). We did not check additional peptides with different arm lengths, though they may constitute successful immunogens as well (e.g. [12]). A cysteine was added to the N-terminus of the H2B sequence to facilitate conjugation to KLH (keyhole limpet hemocyanin, see Section 2.1.4).

2.1.2. Synthesis of immunogen

Solid-phase peptide synthesis was used throughout chain assembly of the peptide [13] using an automated solid-phase multiple peptide synthesizer (AMS422; Abimed Analyzer-Technik Gmbh) and following the commercial protocols of the company.

The amino side-chain of the lysine building block was masked by a protecting group which is capable of selective removal under mild conditions, while leaving other protecting groups intact. Following removal of the protective group of the amino side chain of the conjugated lysine, the sequence derived from the ubiquitin sequence (LRGG) was assembled using the same synthesis protocol. The fully synthesized branched peptide was removed from the polymeric carrier, along with the protecting groups, by acidolysis with trifluoroacetic acid. The crude peptide was purified by preparative HPLC and analyzed by mass spectrometry, amino acid analysis and analytical HPLC.

2.1.3. Choice of animal species

While we discuss here the generation of mouse monoclonal antibodies against mammalian H2Bub, it is important to note that polyclonal antibodies against H2Bub or other ubiquitin conjugates may also be generated in a variety of mammalian hosts. Indeed, rabbit polyclonal antibodies targeting yeast H2Bub were recently described [14].

2.1.4. Immunization procedure

(1) Peptide should first be conjugated to keyhole limpet hemocyanin (KLH) and should then be prepared in complete Freund's adjuvant (CFA) according to standard procedures [11], to increase the immunogenicity of the peptide.
(2) Four 3-month old BALB/c female mice were immunized. Mice should be injected subcutaneously with 50–100 μg of the conjugated preparations in CFA (approximately 50–100μL).
(3) Repeat injections as indicated in (2) several times at intervals of two weeks. Immune reaction should be monitored by bleeding and analysis of serum reactivity (see Section 2.4.1 below).
(4) Three weeks following the last injection as in (3), mice should receive two injections on two consecutive days.

2.2. Fusion of mouse cells with Polyethylene Glycol

(1) Spleens are removed three days after the last injection and spleen lymphocytes are collected. NS0/1 myeloma cells [15] in logarithmic growth phase are used as fusion partner.
(2) 100×10^6 cells from each individual spleen are mixed with 20×10^6 NS0/1 myeloma cells, centrifuged, and resuspended for 1 min in 2 ml of 37 °C 41% Polyethylene Glycol (PEG 1500, Serva), followed by 1 min incubation at 37 °C.
(3) Slowly and carefully wash fused cells with serum free Dulbecco's Modified Eagle's Medium (DMEM, Gibco, catalog number 11995) and resuspend in DMEM-HAT (DMEM supplemented with 1 mM pyruvate, 2 mM glutamine, 10 U/ml penicillin, 20 μg/ml streptomycin, 15% heat inactivated horse serum, 10^{-4} M hypoxanthine, 4×10^{-7} M aminopterin, and 1.6×10^{-5} M thymidine).
(4) Following fusion, distribute cells into 96-well microtiter plates at a concentration of 2×10^4 viable myeloma cells per well and maintain in a humidified incubator with 8% CO_2 in air.

2.3. Hybridoma maintenance and cloning

(1) Medium should be replaced at least once a week after onset of fusion.
(2) Ten to 14 days after fusion DMEM-HAT medium is replaced by DMEM-HT medium (same as DMEM-HAT but without the aminopterin), and 10 days later by regular serum-containing DMEM.
(3) When confluent, hybridomas can be transferred to 24 well plates.
(4) We used cloning under conditions of limiting dilutions rather than soft agar cloning, by diluting the hybrid cells to less than one cell/well. Cloning was repeated for a few cycles to ensure secretion of the monoclonal antibody.

2.4. Screening of immune reactivity

2.4.1. Screening of mouse serum reactivity

Following repeated immunization, mice were bled and serum was used to probe individual strips of SDS–PAGE-separated U2OS whole cell lysates (prepared by the method described in Section 3.1.2) blotted onto a nitrocellulose membrane. As H2Bub is ubiquitous in mammalian cells, other mammalian cell lines can be used as well (e.g. HEK293, H1299). While other methods were considered for screening (e.g. ELISA), it is noteworthy that a part of the immune response was directed against H2B (and to a lesser degree against ubiquitin), and thus it was necessary to resolve the lysate proteins by SDS–PAGE according to their size. Use of polyclonal serum from mice showing strong H2Bub-specific immune response showed a preference for H2Bub (~24 kDa) compared to H2B (~17 kDa) in Western blots. Specifically, the 1:100–2:100 ratio between H2Bub and H2B signals commonly seen in Western blots of mammalian cell extracts was shifted in favor of H2Bub to a ratio of 10:100–20:100 or more. A standard can be generated by probing a strip with anti-H2B antibodies and comparing the resulting H2Bub/H2B signal ratio to that observed when using the mouse serum for probing.

2.4.2. Screening of hybridoma clones

Hybridoma supernatants can be collected for analysis as early as 10–14 days after fusion, as soon as vigorous growth and change of medium color are observed. Hybridoma supernatants should be