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Nucleolar localization/retention signal is responsible for transient accumulation of histone H2B in the nucleolus through electrostatic interactions

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ABSTRACT

The majority of known nuclear proteins are highly mobile. The molecular mechanisms by which they accumulate inside stable compartments that are not separated from the nucleoplasm by membranes are obscure. The compartmental retention of some proteins is associated with their biological function; however, some protein interactions within distinct nuclear structures may be non-specific. The non-specific retention may lead to the accumulation of proteins in distinct structural domains, even if the protein does not function inside this domain. In this study, we have shown that histone H2B-EGFP initially accumulated in the nucleolus after ectopic expression, and then gradually incorporated into the chromatin to leave only a small amount of nucleolus-bound histone that was revealed by removing chromatin-bound proteins with DNase I treatment. Nucleolar histone H2B had several characteristics: (i) it preferentially bound to granular component of the nucleolus and interacted with RNA or RNA-containing nucleolar components; (ii) it freely exchanged between the nucleolus and nucleoplasm; (iii) it associated with the nuclear matrix; and (iv) it bound to interphase prenuclear bodies that formed after hypotonic treatment. The region in histone H2B that acts as a nucleolar localization/retention signal (NoRS) was identified. This signal overlapped with a nuclear localization signal (NLS), which appears to be the primary function of this region. The NoRS activity of this region was non-specific, but the molecular mechanism was probably similar to the NoRSs of other nucleolar proteins. All known NoRSs are enriched with basic amino acids, and we demonstrated that positively charged motifs (nona-arginine (R9) and nona-lysine (K9)) were sufficient for the nucleolar accumulation of EGFP. Also, the correlation between measured NoRS activity and the predicted charge was observed. Thus, NoRSs appear to achieve their function through electrostatic interactions with the negatively charged components of the nucleolus. Though these interactions are non-specific, the functionally unrelated retention of a protein can increase the probability of its interaction with specific and functionally related binding sites.

1. Introduction

The cell nucleus is a highly organized biological structure that is likely formed by stochastic self-organization [1–4]. At present, a key question is how macromolecules find their target sites inside the cell nucleus and how they accumulate in these target sites, which are not separated from the nucleoplasm by membranes.

The majority of nuclear proteins examined thus far are highly mobile; they diffuse rapidly in the nucleoplasm and typically exchange quickly with their binding sites (for review see: [5]). Diffusion coefficients for molecules, such as GFP or dextrans, that do not bind to chromatin or nuclear structures range between ~1 and 100 $\mu m^2/s$, depending on the size and shape of the molecule [6–9]. In contrast to inert, non-binding marker proteins, the overall mobility of biologically active molecules is reduced by a factor of 10–100 [10–13]. In the process of interacting with high-affinity binding sites, these molecules become immobilized for a short period of time during which they exert their biological functions. For example, the mobility of GFP-fibrillarin within the nucleolus is approximately 10 times slower than that of fibrillarin in the nucleoplasm (the diffusion coefficients are 0.046 $\mu m^2/s$ in the nucleolus and 0.53 $\mu m^2/s$ in the nucleoplasm) [10]. Similar results were obtained for GFP-UBF, GFP-nucleolin, GFP-Rpp29, GFP-B23 and GFP-S5 [11].

Fluorescence photobleaching and activation experiments show that the residence time of proteins on chromatin is seconds or less [13,14]. Certain proteins, such as core histones, are exceptions and are largely immobile [15]. These measurements, and the known ability of the proteins to diffuse rapidly through the nucleus, suggest that molecules find their target sites by three-dimensional random scanning of the nuclear space [13].

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It is thought that the retention of a protein at its binding site is associated with its biological function, and the duration of retention approximately equals the length of a single act of biological activity. However, one can assume that a portion of protein interactions with the structures may be non-specific; for example, protein accumulation might be not associated with a particular protein function. Indeed, even a completely inert molecule like ovalbumin showed at times short-lived binding events with a decay time of ~6.3 ms within the nucleoli [16]. If the protein interacts non-specifically with the components inside the macroscopic structure where its functional binding sites are distributed, this non-specific accumulation can increase the probability of an interaction with functional binding sites. It is clear that the identification and investigation of such non-specific interactions can be particularly difficult because they may play a secondary role in protein accumulation. A study that investigates the proteins that do not have an active functional role, but still accumulate in a given structure, would be useful.

Some proteins that are found in the nucleolus are not necessary, at first glance, for known nucleolar functions. For example, it was demonstrated that certain variants of histone H1 might accumulate in the nucleolus [17–19]. This nucleolar accumulation may occur due to non-specific interactions and may have no direct functional significance. If so, these proteins may be a suitable model for the study of weak, non-specific interactions. To eliminate the possibility of some function for the nucleolar accumulation of histone, we have chosen histone H2B for our experiments, which, to our knowledge, never accumulates in the nucleolus. Here, we have shown that histone H2B-EGFP initially accumulated in the nucleolus after ectopic expression, and later incorporated into the chromatin. Nucleolar histone H2B freely exchanged between the nucleolus and nucleoplasm, and therefore, behaved similarly to nucleolar proteins. We identified a region in histone H2B that acted as a nucleolar localization/retention signal (NoRS). This signal was a part of the nuclear localization signal, and its presence in histone was not dictated by functional necessity. NoRSs similar to the ones described in the current work might be responsible for non-specific retention of proteins in the nucleolus. The possible mechanisms of their action are described.

2. Materials and methods

2.1. Plasmids

Histone H2B-EGFP plasmid (Addgene plasmid 11680) [20] was used for transfection and for plasmid construction. To obtain nucleolar-targeting fusion proteins, PCR amplificated fragments (Supplementary methods) of histone H2B were treated with HindIII and BamHI and ligated into either pEGFP-C1 vector (Clontech) or pEGFP-N1 vector (Clontech). To obtain nucleolar-targeting fusion proteins, the oligonucleolides (Supplementary methods) were annealed and ligated into HindIII and BamHI-digested pEGFP-C1 vector (Clontech). pEGFP-C1 was used as a control plasmid. For histone H2BΔ22-41 construction, the histone H2B cDNA coding sequence was excised from the histone H2B-GFP as a 389-bp Acc65I-BamHI fragment and inserted between the BamHI and Acc65I sites of pBluescript(KSII+) plasmid (Stratagene) to produce pBluescript(KSII+)/H2B. The 467-bp fragment, excised from of the pBluescript(KSII+)/H2B plasmid with DdeI, filled in with Klenow, followed by treating with NspI was inserted in to the pBluescript (KSII+)/H2B, treated with SspBI, filled in with Klenow fragment, followed by treating with NspI, to produce pBluescript(KSII+)/H2BΔ22-41. The H2BΔ22-41 cDNA coding sequence excised from the plasmid pBluescript(KSII+)/H2BΔ22-41 as a 326-bp Acc65I-BamHI fragment and inserted between the BamHI and Acc65I sites of pEGFP-N1 plasmid (Clontech) to produce pEGFP-histone H2B(Δ22-41).

2.2. Cell culture and transfection

HeLa cells were grown in Dulbecco's Modified Eagles Medium supplemented with L-glutamine, 10% fetal calf serum (HyClone) and antibiotic/antimycotic solution (Sigma). Cellular transfection was performed with Lipofectamine 2000 reagent (Invitrogen), according to the manufacturer's instruction. NaCl extraction was carried out as described elsewhere [21]. To inhibit rRNA transcription, the cells were grown in medium with 0.1 μg/ml actinomycin D. To induce interphase prenucleolar body formation, the cells were incubated in 20% Hanks solution for 15 min and were then transferred into complete culture medium for 30 min.

2.3. Correlative light and electron microscopy

For correlative light and electron microscopy, the cells were grown on round photo-etched coverslips (Electron Microscopy Sciences) and fixed in 4% glutaraldehyde (Pelco International) in 0.1 M Sörensen phosphate buffer. Immediately after fixation, the coverslips were imaged with an Attofluor chamber (Invitrogen). Cells expressing the protein of interest were revealed using an Axiovert 200M fluorescent microscope (Carl Zeiss) and photographed using PlanNeofluar 5×/0.13 and PlanNeofluar 40×/0.75 objectives. The former objective allowed us to find the cells in phase contrast microscopy, using lines and numbers as reference points, and the latter objective was needed to select overexpressing cells in ultrathin section. The surrounding cells in which pEGFP fluorescence was not detected were used as a negative control. The coverslips were retrieved from the chamber, and the cells were further fixed in glutaraldehyde for 1.5 h, postfixed in 1% OsO_4 (Sigma) and embedded in Epon (Fluka). After Epon polymerization and removal of the coverslips by consecutive immersion into liquid nitrogen and boiling water, the cells of interest were selected under phase-contrast and cut into ultrathin sections using an LKB Ultratome III. The sections were stained with lead citrate and examined using an HU-12 electron microscope (Hitachi).

2.4. Western blotting

For immunoblotting, the cells were lysed in Laemmli's sample buffer, boiled for 3 min, resolved on a 12.5% SDS-polyacrylamide gel and transferred to a nitrocellulose membrane (Sigma) using a semi-dry transfer system (Helicon). The membranes were blocked for 60 min with 1% bovine serum albumin (BSA, MP Biomedicals). The blots were incubated for 1.5 h with primary anti-GFP antibodies (Abcam), washed with buffer containing 50 mM Tris–HCl (pH 7.6) and 200 mM NaCl and incubated for 1 h with secondary antibodies conjugated to alkaline phosphatase (Sigma). After washing, the blots were incubated in developing NBT/BCIP solution (Sigma).

2.5. Immunocytochemistry and microscopy

For immunofluorescent labeling, cells were fixed in 3.7% formaldehyde at room temperature for 15 min and then permeabilized with 0.5% Triton X-100 in PBS for 5 min. After washing in PBS, the cells were incubated in 1% BSA for 45 min and then incubated with primary antibodies for 60 min at 25 °C. Anti-B23 and anti-Sc-35 antibodies were purchased from Sigma, and anti-A3 antibodies were characterized elsewhere [22]. For negative controls, PBS was used instead of the primary antibodies. After several washes in PBS containing 0.1% BSA and 0.05% Tween 20, the cells were incubated with Alexa 555-conjugated antibodies (Invitrogen) for 45 min. The cells were washed in PBS, stained with a 0.1 μg/ml solution of DAPI (Sigma) and embedded in Mowiol (Calbiochem) containing an anti-bleaching agent, DABCO (Sigma). The preparations were observed using an Axiovert 200M microscope (Carl Zeiss) equipped with an Apochromat 100×/1.4 oil immersion objective. Stacks of images were recorded with an ORCAII-ERG2 cooled CCD-camera (Hamamatsu). Deconvolution was carried out using Axiovision 3.1 software (Carl Zeiss). For final presentation, all images were transferred into Adobe Photoshop 5.0 LE. The intensity of cell fluorescence was measured using ImageJ