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Localization of nuclear actin in nuclear lipid microdomains of liver and hepatoma cells: Possible involvement of sphingomyelin metabolism

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Abstract

Nuclear actin has been implicated in different nuclear functions. In this work, its localization in nuclear membrane, chromatin and nuclear lipid microdomains was investigated. The implication of sphingomyelin metabolism was studied.

Nuclear membrane, chromatin and nuclear lipid microdomains were purified from hepatocyte nuclei and H35 human hepatoma cell nuclei. The presence of β-actin was analyzed with immunoblotting by using specific antibodies. Sphingomyelinase, sphingomyelin-synthase, and phosphatidylcholine-specific phospholipase C activities were assayed by using radioactivity sphingomyelin and phosphatidylcholine as substrate.

The results showed that β-actin is localized in nuclear lipid microdomains and it increases in cancer cells. Evidence is provided to the difference of phosphatidylcholine and sphingomyelin metabolism in various subnuclear fractions of cancer cell nuclei compared with normal cells. Our findings show increase of sphingomyelin-synthase and inhibition of sphingomyelinase activity only in nuclear lipid microdomains.

Nuclear lipid microdomains, constituted by phosphatidylcholine, sphingomyelin and cholesterol, play a role as platform for β-actin anchoring. Possible role of sphingomyelin metabolism in cancer cells is discussed.

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Introduction

The actin is a structural protein underlying the proper development, cell size and shape and has a prominent influence on governing the molecular mechanism and regulating different cell functions (1). Six different isoforms of actin encoded by specific genes are present in mammals (2). In particular, α-skeletal actin is particularly expressed in skeletal muscle, α-cardiac actin in cardiac muscle, α-smooth actin and γ-smooth actin smooth muscle. β-cyto actin and γ-cyto actin are expressed in all tissue (3).

The presence of β-actin in the nucleus has been a controversial issue for many years due to the hypothesis of possible contaminations during purification procedure (4). Today the presence and the role of actin in the nucleus is clear; it is present as a component of all three RNA polymerases, as a constituent of nuclear ribonucleoprotein particles and as a regulatory component of chromatin remodeling (5). Nuclear actin is present in a monomeric state and influences its own polymerization state by binding transcriptional activators, which move between cytoplasm and the nucleus (4). It is present in nuclear complexes for DNA synthesis and chromatin remodeling and for nuclear import/export proteins, such as importin (4). The role of actin in the activity of RNA polymerase II was described (6). The size of actin (approximately 42 kDa) does not permit the passive diffusion through the nuclear pore; exportin 6 permits the nuclear export of actin and importin 9 permits the maintenance of nuclear actin levels (7). In this way, actin may play a role as a sensor of extracellular signals in order to transduce signals to the chromatin state and moderate genome organization of living cells (5).

Nuclear lipid microdomains (NLMs) are specific areas of inner nuclear membrane that act as platforms for chromatin anchoring (8), DNA duplication (9) and RNA transcription (10). In addition, NLMs regulate nuclear vitamin D3 uptake by influencing differentiation of embryonic hippocampal cells (11). In hepatoma cells, NLMs play a role as resting place of dexamethasone to delay cell proliferation (12). NLMs are vesicle structures with 300-600 nm average diameter; they contain cholesterol, phosphatidylcholine (PC) and sphingomyelin (SM) with 1:1:1 ratio similar to lipid rafts present in cell membrane (8). The SM metabolism in NLMs of normal liver was demonstrated (8) but until now we have no information on it in hepatoma cells. Nuclear SM is localized in chromatin (Chr), nuclear membrane (NM), nuclear matrix (13) and in NLMs (8); it is synthesized by SM-synthase (SM-synthase) and catabolized by sphingomyelinase (SMase, 13).

Until now we do not have information about the localization on nuclear actin. In this study, with the immunoblotting analysis that simultaneously show the β-actin in NM, Chr, and NLM, we demonstrate for the first time the specific localization of nuclear actin in NLM. We provide evidence that β-actin is higher in H35 hepatoma NLM than hepatocyte (H) NLM. Additionally, we used enzyme activity assay to study the SM and PC metabolism in different subnuclear fractions. Notably we demonstrate the higher activity of SM-synthase and lower activity of SMase in NLM from H35 cells than in NLM from H. The correlation between β-actin and SM metabolism in NLM from normal and cancer cells is discussed.

Materials and Methods

Animals and Cells

Thirty-day-old Sprague Dawley rats of either sex (Harlan Nossan, Milan, Italy) kept at normal light-dark periods were used. They had free access to pelleted food and water prior to killing between 9 and 10 a.m. All treatments were made according to the international regulation of National Institutes of Health. H35 hepatoma cells were obtained from the European Collection of Animal Cell Cultures (Salisbury, Wilts, United Kingdom).

Materials

Dulbecco's modified Eagle's medium (DMEM), bovine serum albumin (BSA), dithiothreitol, fetal bovine serum (FBS), phenylmethylsulfonylfluoride (PMSF), methanol, 3-(4,5-dimethyl-thiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), 2-propanol, metyl-tert-butyl ether, formic acid, and chloroform were obtained from Sigma Chemical Co. (St. Louis, Missouri, U.S.A.); PC (L-3-phosphatidyl N-methyl-^{3}H choline 1,2 dipalmitoyl, 81.0 Ci/mmol) was from Amersham Pharmacia Biotech (Rainham, Essex, UK); Ecoscint A was from National Diagnostic (Atlanta, Georgia, U.S.A); anti-LaminB and anti-β-actin were obtained from Santa Cruz Biotechnology, Inc. (California, USA).

Nuclei, nuclear membrane, chromatin and nuclear lipid microdomain purification

For H nuclei preparation, rat liver was homogenized, filtered and nuclei were isolated as previously reported (14). For hepatoma cell nuclei preparation, H35 hepatoma cells were seeded, cultured and N were isolated according to Albi *et al.*, 2005 (15). NM and Chr were purified as previously reported (16). NLM were purified as reported by Cascianelli et al., 2008 (8).

Protein content

Total protein concentration was analyzed by using bovine BSA as a standard as previously reported (16).

Electrophoresis and Western Blot analysis

40μg of protein from N, Chr and NLMs were submitted to SDS-PAGE electrophoresis in 10% polyacrylamide slab gel. Electrophoresis and immunoblotting analyses were performed to detect lamin B and β-actin according to Bartoccini et al., 2011 (11). The area density was evaluated by densitometry scanning and analysis with Scion Image (11).

Enzyme activity assay

SMase, SM-synthase, and PC-PLC activities were assayed as previously described by using radioactive SM or PC as substrate (8).

Statistical analysis

Data were expressed as mean ± S.D. of three independent experiments performed in duplicate and *t* test was used for statistical analysis. $P < 0.001$ was considered as statistically significant.

Results

Nuclear actin is localized in nuclear lipid microdomains

NM, Chr and NLMs were purified in order to ascertain the subnuclear localization of β-actin. Data of protein content were reported as μg/g liver for H preparations and μg/10^{6} cells for H35 preparations (**Fig.1**).

For this reason to make a real quantitative difference between H and H35 preparations was really hard. However, the distribution of protein content in NM, Chr, and NLM was similar in H and H35 cells, about 38-40% in NM, 55-60% in Chr and 1.5% in NLM. Then, we set out to investigate the possible presence of β-actin in subnuclear preparations. Thus, immunoblotting analysis was used to highlight simultaneously the presence of β-actin NM, Chr, and NLM from H and H35. Since NLMs are specific domains of inner nuclear membranes, laminB was analyzed as marker of NM and NLM purification; Chr was used as control (17). By comparing H and H35 cells, LaminB band resulted similar in H and H35 cells (**Fig. 2a,b**). Next, we focused on the results showing the localization of β-actin. Surprisingly, it was found to localize notably in NLM (**Fig. 2a,b**). We observed more intense staining for β-actin protein in H35 than H (**Fig.2a,b**).

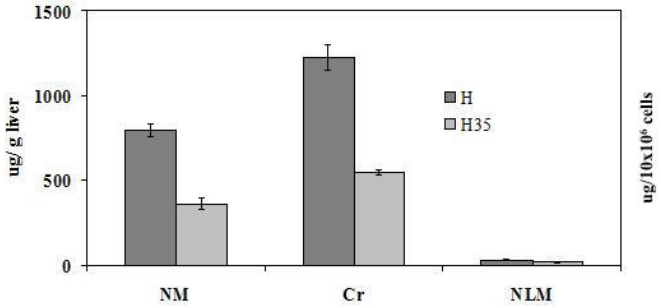

Figure 1. Protein content in nuclear membrane (NM), chromatin (Chr), and nuclear lipid microdomains (NLM) from hepatocytes and H35 hepatoma cells. Left ordinate for NM and Chr, in the right ordinate for NLM. The data are expressed as mean+SD of three experiments performed in duplicate. Significance *P< 0.001 versus NM and Chr.

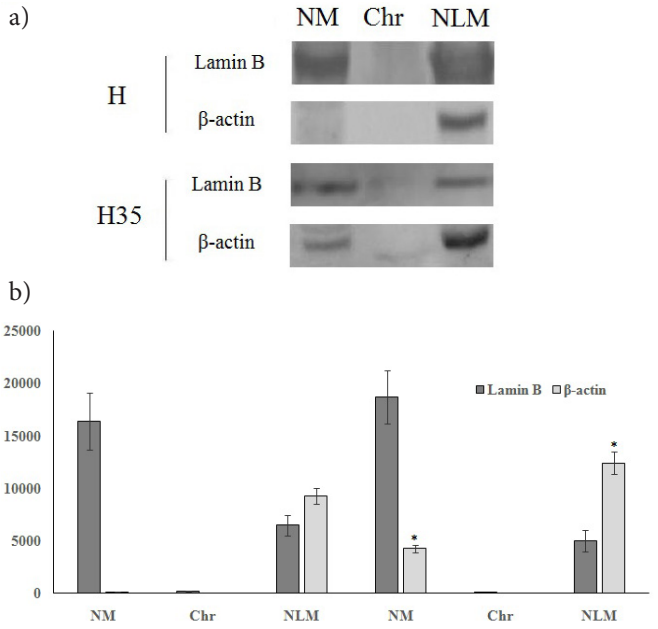

Figure 2. LaminB and β-actin detection in nuclear membrane (NM), chromatin (Chr), and nuclear lipid microdomains (NLM) from hepatocytes and H35 hepatoma cells. a) immunoblots of proteins probed with specific antibodies and visualized by ECL. Apparent molecular weight for LaminB and β-actin was 68KDa, and 43KDa, respectively; b) the area density was evaluated by densitometry scanning and analysis with Scion Image, the data represent the mean + S.D. of three experiments performed in duplicate. (Significance, *P< 0.001 versus H preparations).

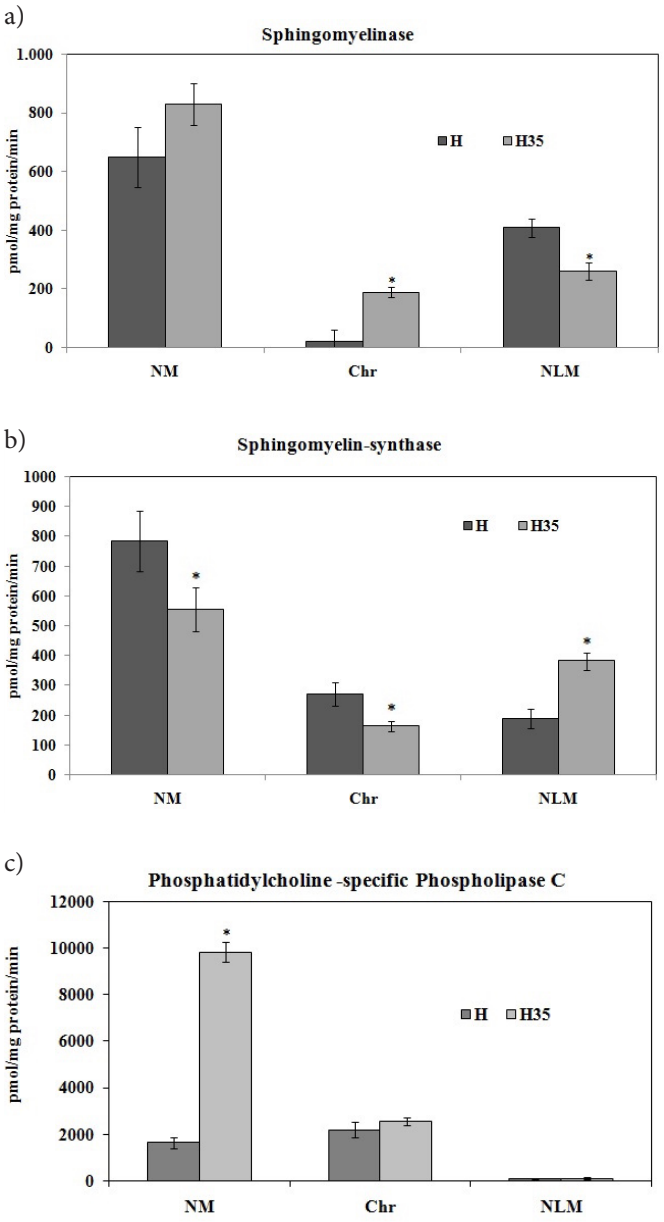

Figure 3. Sphingomyelinase (a), sphingomyelin-synthase (b), and phosphatidylcholine-specific phospholipase C (c) in nuclear membrane (NM), chromatin (Chr), and nuclear lipid microdomains (NLM) from hepatocytes and H35 hepatoma cells. The activity was measured as pmol/mg protein/min, the data represent the mean + S.D. of three experiments performed in duplicate. (Significance, *P< 0.001 versus H preparations).

Intranuclear Sphingomyelin Metabolism

Once the nuclear region which was necessary for β-actin localization was identified, we studied the SM metabolism in the different subnuclear fractions. Since PC-PLC activity in NLM was present only in trace amount (8), it was used for comparison. By using the results in H preparations as controls, we showed that in NM of cancer cells the SMase activity unchanged, SM-synthase was inhibited, and PC-PLC was strongly stimulated (**Fig. 3a,b,c**). In Chr, SMase activity increased and SM-synthase decreased without changes of PC-PLC (**Fig. 3a,b,c**). Unexpectedly, in NLM the behavior of SMase and SM-synthase was opposite to that of Chr (**Fig.3a,b**); PC-PLC remained very low (**Fig. 3c**).

Discussion

The nucleus contains more than 30 actin-binding proteins which are not able to form filaments but had evolved nuclear-specific functions (18). There is an evolving body of literature supporting a role for β-actin in cell nucleus, as a regulator of chromatin remodeling (5), of transcription process (4, 6), and of signal transduction from cytoplasm to chromatin (5).

Therefore several groups are currently working on the function of nuclear β-actin. In this study, we attempted to delineate the domains responsible for nuclear β-actin localization. Therefore our work has identified for the first time the association of nuclear β-actin with specific lipid microdomains present in inner NM that acts as platform for Chr anchoring, DNA duplication and transcription (8, 9, 10). Therefore it is possible that the β-actin, binding to NLM, plays a role of key molecule involved in the active chromatin function. Additionally, in this study, we uncovered an enrichment of β-actin in the NM of cancer cells and consequently a strong enrichment in the NLM. You can assume that cancer cells need more nuclear actin attached to the NLM for more intranuclear activities. Since SM is the most important lipid in NLM in cell nucleus and its metabolism is correlated with cell proliferation and cancer (13), we attempted to delineate the change of SM metabolism in NLM in association with that of β-actin, by supposing that in cancer cells NLM might enrich in SM content to facilitate its function as platform for nuclear activities. In line with our hypothesis, SM-synthase that uses ceramide and PC to form SM freeing diacylglycerol (DAG, (14)) increases while SMase that degrades SM freeing ceramide (14) decreases. These findings support our recent results showing an increase of SM and decrease of ceramide content in NLM from H35 cells compared with NLM from H (17). Specifically, we showed a shift from 20:0 SM to 16:0 SM in NLM from cancer cells and hypothesized that it might influence the content of functional proteins in the microdomains (17). Furthermore we demonstrated in H35 NLM an increase of STAT3, Raf1, and PKCζ proteins involved in hepatocarcinogenesis (17). Therefore, in this study, we showed for the first time the change of SM metabolism in NLM of cancer cells that can justify our previous results. We highlighted that the changes of SM in NLM are completely different from those of other sub-nuclear districts. In fact, PC-PLC activity notably changes in NM. Of note and quite surprisingly, the activity of PC-PLC in Chr and NLM from cancer cells are unchanged respect to that from normal cells, indicating that only NM is a source of DAG from PC in H35 cells. In Chr, SMase activity increases and SM-synthase activity decreases; the opposite happens in NLM. This apparent contradiction could be explained with the different roles of SM in Chr (14, 19) and in NLM (8,9,10).

Taken together, these results strongly suggest that a specific regulation of the SM metabolism in NLM is responsible for the β-actin anchoring in NLM of cancer cells

This could suggest a couple of possibilities: a) the SM content in NLM is critical to the attack of β-actin; or b) the overexpression of β-actin in cancer cells requires the high level of SM in NLM. These are interesting possibilities that will be investigated in future studies.

Conflict of interest statement and Ackowledgements

The authors declare no conflict of interest. The authors would like to thank University of Udine for the financial support.

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