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Research article

THE LECTIN-BINDING PATTERN OF NUCLEOLIN AND ITS INTERACTION WITH ENDOGENOUS GALECTIN-3

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Abstract: Unlike nuclear nucleolin, surface-expressed and cytoplasmic nucleolin exhibit Tn antigen. Here, we show localization-dependent differences in the glycosylation and proteolysis patterns of nucleolin. Our results provide evidence for different paths of nucleolin proteolysis in the nucleus, in the cytoplasm, and on the cell surface. We found that full-length nucleolin and some proteolytic fragments coexist within live cells and are not solely the result of the preparation procedure. Extranuclear nucleolin undergoes N- and O-glycosylation, and unlike cytoplasmic nucleolin, membrane-associated nucleolin is not fucosylated. Here, we show for the first time that nucleolin and endogenous galectin-3 exist in the same complexes in the nucleolus, the cytoplasm, and on

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Abbreviations used: AAA - *Aleuria aurantia* agglutinin; DSA - *Datura stramonium* gglutinin; GNA - *Galanthus nivalis* agglutinin; H antigen - Fucα1-2Galβ1-4GlcNAcβ1-3Gal-; LEA - *Lycopersicon esculentum* agglutinin; Lex - Galβ1-4(Fucα1-3)GlcNAcβ-; LTA - *Tetragonolobus purpureus* agglutinin; MAA-II - *Maackia amurensis* agglutinin; SNA-I - *Sambucus nigra* agglutinin; PHA-E - *Phaseolus vulgaris* erythroagglutinin; PHA-L - *Phaseolus vulgaris* leucoagglutinin; PNA - peanut agglutinin; T antigen - Galβ1–3GalNAcα1 -Ser/Thr; Tn antigen - GalNAcα1 -Ser/Thr; UEA-I - *Ulex europaeus* agglutinin; VVA - *Vicia villosa* agglutinin

the cell surface of melanoma cells. Assessments of the interaction of nucleolin with galectin-3 revealed nucleolar co-localization in interphase, suggesting that galectin-3 may be involved in DNA organization and ribosome biogenesis.

Keywords: Glycosylation of nucleolin, Galectin-3, Melanoma, Mass spectrometry, Confocal microscopy, Lectin assay, Co-immunoprecipitation

INTRODUCTION

Nucleolin is a ubiquitous non-histone nucleolar phosphoprotein shown to be overexpressed in rapidly dividing cells and cancer cell lines [1–3]. It is a multifunctional protein involved in numerous cell processes, such as proliferation and growth, transcription, cytokinesis, nucleogenesis, signal transduction, mRNA regulation, apoptosis, induction of chromatin condensation, and replication [4].

Nucleolin is composed of three main domains. There is the N-terminal domain, which is rich in acidic residues and is the site of numerous phosphorylations by casein kinase 2 (CK2) and cyclin dependent kinase 1 (CDK1). It is also the site where the bipartite nuclear localization signal (NLS) is localized. The central domain contains four RNA recognition motifs (RBDs) that are determinants of RNA-binding specificity. Finally, there is the C-terminal domain, which is rich in arginine and glycine residues (RGG or GAR domain) that participate in interactions with ribosomal proteins [5, 6]. This domain exhibits helicase activity, and contributes to non-specific RNA binding [7]. As a nucleolar protein, it is usually found in dense fibrillar components (DFC) and granular components (GC) of nucleoli, where it participates in multiple stages of early pre-rRNA processing, such as rDNA transcription, pre-rRNA processing, and assembly of ribosomal proteins and rRNAs [6, 8]. Phosphorylation of nucleolin by CK2 is important in the regulation of rDNA transcription [9, 10].

In the nucleoplasm, nucleolin has been found associated with several genes transcribed by Pol II. It is involved in many aspects of gene expression, including chromatin remodeling, DNA recombination and replication, gene transcription, and regulation of the telomerase [4, 11].

Despite the lack of an export signal, nucleolin shuttles to the cytoplasm, where it takes part in regulation of translation and mRNA stability [12–17]. In some cancer cells where nucleolin is overexpressed in the cytoplasm, aberrant stabilization by nucleolin leads to upregulation of the anti-apoptotic Bcl-2 protein, elevating resistance to chemotherapy and helping cancer cells avoid apoptosis [18, 19]. Cytosolic nucleolin also has been shown to suppress the translation and induction of p53 after DNA damage [20], thereby facilitating the generation of viable genetically altered cells and possibly leading to malignancy. Shuttling of nucleolin between the nucleus and cytoplasm supports the import and/or export of several nucleolar components or proteins, such as ribosomal proteins.

Although nucleolin lacks a transmembrane domain or signal sequence, a large number of reports have identified nucleolin on the cell membrane [21–28] in close association with the intracellular actin cytoskeleton. Upon stimulation of cell proliferation, cytoplasmic nucleolin is translocated to the surface [29]. Cell surface nucleolin serves as a receptor, with potential roles in cell migration and adhesion and in virus infection [30]. Cell surface nucleolin is a receptor for gp120 of HIV-1 [21], intimin-gamma of enterohemorrhagic *Escherichia coli* O157-H7, various growth factors, such as midkine, pleiotrophin and lactoferrin [27, 31, 32], DNA aptamers [18], and DNA nanoparticles. It may be a key transporter for all of these compounds from the membrane through the cytoplasm to the nucleus [23]. Nucleolin has been reported as a ligand for L- and P-selectins [25, 33]. It acts as the polylactosaminoglycan-binding receptor on macrophages [34] and as a endostatin receptor that mediates the anti-angiogenic and antitumor activities of endostatin [35]. Plasma membrane nucleolin has been reported to act as a binding molecule for agents involved in proliferation, such as the tumor-homing peptide F3 in the endothelial cells of angiogenic blood vessels [24]. Therefore, the protein has been indicated as a shuttle molecule between the cell surface and nucleus, and indeed as a mediator for extracellular regulation of nuclear activity [11]. Prediction of MHC class-I binding sites in nucleolin may make it a potential target for T-cells and thus a candidate for immunotherapy [28].

It was recently demonstrated that nucleolin undergoes complex *N*- and *O*-glycosylation [28, 36–38]. Two *N*-glycosylation sites localized in the RBD 1 and 3 domains were defined, and five potential *O*-glycosylation sites in the N-terminal domain were predicted [36]. The presence of *O*-glycans was confirmed by the presence of T and Tn antigen and fucosyl-containing *O*-glycan chains. Since extranuclear nucleolin differs from the nuclear form by its glycosylation, it was hypothesized that glycosylation may help to regulate nucleolin traffic, functions and degradation [36, 39].

Interestingly, there are conspicuous functional similarities between nucleolin and galectin-3. Both proteins contain a nuclear localization signal but lack a signal sequence. They are localized on the cell surface and in intracellular spaces (cytoplasm and nuclear matrix), and they shuttle between the nucleus and cytoplasm. Both nucleolin and galectin-3 are secreted via a non-classical pathway. Both proteins are multifunctional molecules. Numerous proteins are capable of interacting with nucleolin or galectin-3. Like nucleolin, galectin-3 is able to bind DNA and RNA [40], and in the nucleus galectin-3 acts as a pre-mRNA splicing factor [41]. In this study, we sought to assess the glycosylation profiles of nucleolin from membrane, cytoplasmic and nuclear fractions. In view of the similar distributions of nucleolin and galectin-3 in the cell, we also addressed the potential interactions of these two proteins.

MATERIALS AND METHODS

Materials

Biotinylated, FITC-conjugated and agarose-bound lectins, and Vectashield Hardset mounting medium with DAPI were purchased from Vector. Anti-mouse IgG/AP, anti-rabbit IgG/AP and rabbit anti-histone H2B polyclonal antibody were from Chemicon. Immunoprecipitation Kit (Protein G) was purchased from Roche. Mouse anti-human L1CAM mAbs, clone UJ127.11, RPMI 1640 with GlutaMax-I medium (Gibco), foetal bovine serum (Gibco), Protein Assay Kit, ExtrAvidin/AP, normal goat serum and protease inhibitor cocktail were obtained from Sigma. Mouse anti-human nucleolin mAbs MS-3, mouse anti-human nucleolin mAbs D-6, mouse anti-Galectin-3 mAbs (clone A3A12) and mouse anti-Galectin-3 mAbs (clone 9C4) were from Santa Cruz Biotechnology. Alexa Fluor Cy3-conjugated goat anti-mouse mAbs, goat anti-mouse IgG-AlexaFluor488 and StreptAvidin-AlexaFluor488 were from Invitrogen. Anti-β-tubulin was purchased from Pharmingen. Trypsin was from Biocentrum. ProteoExtract Subcellular Proteome Extraction Kit was from Calbiochem (Merck Millipore). All other chemicals were of the highest purity and were purchased from Sigma-Aldrich.

Cell lines and cell culture conditions

The metastatic melanoma cell line Ma-Mel-27 was donated by Professor D. Schadendorf of the Klinik für Dermatologie, Venerologie und Allergologie of the Universitätsklinikum Essen in Germany. Mycoplasma free cells were maintained in RPMI 1640 medium with GlutaMax-I, supplemented with 10% foetal calf serum, 100 units/ml penicillin and 100 μg/ml streptomycin. Cells were grown in monolayers in a 95% air/5% CO_2 atmosphere at 37ºC in a humidified incubator. Mycoplasma infection of the cell culture was verified using commercial PCR testing and confocal microscopy.

Cell extract preparation

The whole cell protein extract was prepared on ice by sonication (Bandelin Electronic) of cells in a buffer consisting of 50 mM Tris/HCl (pH 7.5), 1 mM EDTA, protease inhibitor cocktail (20 μl/ml) and 1 mM phenylmethylsulfonyl fluoride, and then Triton X-100 (final concentration 1%) and protamine sulfate (final concentration 0.3%) were added. The homogenates were incubated for 30 min with shaking on ice. The cell extracts were clarified by centrifugation at 18,000 x g for 20 min at 4ºC. The protein concentrations were determined using a Protein Assay Kit.

For fresh cell extract preparation, Ma-Mel-27 cells were grown until they reach 80% confluence in 30-mm culture dish. The cells were then washed twice with cold PBS, scraped with a rubber policeman and treated with 75 μl of boiling 2 x Laemmli buffer contained 2 mM EDTA and 10% β-mercaptoethanol. The sample was heated for 10 min at 100ºC and then 15 μl of sample was loaded into a polyacrylamide gel electrophoresis well.

Subcellular fractionation

Subcellular fractionation was done using ProteoExtract Subcellular Proteome Extraction Kit according to the manufacturer's instruction. Each fraction was desalted via MeOH/$CHCl_3$ precipitation and subjected to 10% sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE). After electrotransfer on a PVDF membrane, immunodetection of nucleolin was performed using mAbs MS-3 (1:6000 dilution). Proteins specific to each fraction were detected using antibodies against L1CAM (mAbs UJ127.11; 1:12500 dilution) for the membrane fraction; tubulin (mAbs anti-tubulin; 1,5:1000 dilution) for the cytosolic fraction; and H2B (pAbs anti-Histone H2B; 1:1000 dilution) for the nuclear fraction. Suitable anti-mouse IgG/AP (1:4000 dilution) or anti-rabbit IgG/AP (1:4000) were used as secondary antibodies. The conjugated alkaline phosphatase was detected via NBT/X-phosphate staining.

Lectin precipitation and western blotting

Whole cell extract (300 μg of total protein) was diluted with an incubation buffer consisting of 10 mM HEPES, 150 mM NaCl, 0.1 mM $CaCl_2$, 0.01 mM $MgCl_2$ (pH 7.5) at a 1:1 (v/v) cell extract to buffer ratio, and incubated overnight at 4ºC with 5 μl of biotinylated lectins or with 40 μl of agarose-bound lectins. Afterwards, the samples with biotinylated lectins were mixed with 30 μl of Streptavidin-agarose for additional 4 h at 4ºC. All of the precipitates were washed three times with incubation buffer, and once with PBS with subsequent centrifugations (1500 rpm, 3 min). Precipitated glycoproteins were liberated by boiling in 2 x Laemmli buffer containing 10% β-mercaptoethanol and 2 mM EDTA at 100ºC for 8 min.

In parallel, negative controls were created using lectins preincubated with 0.4 M acetic acid (2 h, RT), and then incubated with whole cell extract (final concentration of acetic acid: 0.2 M). Electrophoresis in 10% SDS-PAGE, electrotransfer onto PVDF membrane and immunodetection of nucleolin using anti-nucleolin mAbs (D-6; 1:3000 dilution) were performed. Rabbit anti-mouse IgG/AP (1:4000 dilution) was used as a secondary antibody and the conjugated alkaline phosphatase was detected via NBT/X-phosphate staining.

Reciprocal co-immunoprecipitation and western blotting

Subcellular extracts (300–700 μl) that contained proteins in their native state were immunoprecipitated with anti-nucleolin mAbs (D-6; 1.2–2 μg) or with anti-galectin-3 mAbs (9C4; 2–2.5 μg) for 18 h at 4ºC. Subsequently, 30 μl of Protein G-agarose (Immunoprecipitation Kit) was added to each of the immunoprecipitates and incubated for additional 7 h at 4ºC. The beads were washed three times in 1 ml PBS containing 1 mM $CaCl_2$ and 1 mM $MgCl_2$, and then the bound proteins were eluted by boiling in 2 x Laemmli buffer containing 10% β-mercaptoethanol, 2 mM EDTA at 100ºC for 8 min. Electrophoresis and western blotting were performed as described above. Immunodetection of galectin-3 on blot was performed using the mouse monoclonal anti-galectin-3 antibody (9C4; 1:1000 dilution).

Protein identification by mass spectrometry (MS) based on peptide sequencing

Sample preparation and MS measurements

We used lectin precipitation for nucleolin isolation, because the IgG heavy chains of anti-nucleolin co-migrate in SDS-PAGE with some proteolytic fragments of nucleolin. Protein bands of interest, corresponding to VVA-positive protein bands, were excised from the SDS-PAGE gel and chopped into cubes (ca. 1 mm x 1 mm). The obtained pieces were destained by washing several times in 25% and 50% acetonitrile, reduced at 56ºC for 45 min in 50 mM DTT and alkylated for 2 h at room temperature in darkness using 55 mM iodoacetamide. Excess reagents were washed out with 50% acetonitrile in ammonium bicarbonate buffer and gel pieces were dried in 100% acetonitrile followed by drying for 15 min in SpeedVac. After that, gel pieces were subjected to in-gel digestion with 15 µl 10 ng/µl trypsin solution. Digestion was carried out at 37ºC overnight. The peptides were extracted from the gel by sonication. The extracts were evaporated until dry and resuspended in 2% ACN with 0.05% TFA. The resulting peptide mixtures were analyzed using UltiMate 3000RS LCnanoSystem (Dionex) coupled with MicrOTOF-Q II mass spectrometer (Bruker) using Appollo Source ESI nano-sprayer equipped with low-flow nebulizer. The peptides were injected on a RP-C18 precolumn (Acclaim PepMap Nano trap Column) using 2% ACN with 0.05% TFA as a mobile phase, and further separated on a 15 cm × 75 µm RP column (Acclaim PepMap 100 µm 100 Å Nano Series TM Column) using a gradient 2–40% ACN in 0.05% FA for 60 min. Each peptide separation was preceded by a blank run to ensure the absence of cross-contamination. Mass spectrometry was operated in standard DDA (data dependent acquisition) MS/MS mode with fragmentation of the most intensive precursor ions.

Analysis of mass spectrometry data

Mascot Generic format (.mgf) file was generated by preprocessing the raw data with Data Analysis 4.0 software (Bruker). The obtained peak lists were used to search the non-redundant protein database of the SwissProt and NCBI (with the taxonomy restriction: *Homo sapiens* – 20,257 sequences) using in-house Mascot server (v.2.3.0, Matrix Science). The following search parameters were applied:

Enzyme specificity – trypsin
Permitted number of missed cleavages – 1 or 2
Fixed modification – carbamidomethylation (C)
Variable modifications – oxidation (M)
Protein mass – unrestricted
Peptide mass tolerance – ± 20 ppm
Fragment mass tolerance – ± 0.05 Da
Searches were performed among the peptides with a charge of 2+, 3+ or 4+.

Immunofluorescence and confocal microscopy

Cells were prepared for confocal microscopy according to Hoja-Łukowicz 2009 et al. [28] and examined using a LSM 510 META, Axiovert 200 M, ConfoCor 3 confocal microscope (Carl Zeiss MicroImaging). FITC-conjugated lectins (dilution 1:200) or biotin-labelled lectins (1:300 dilution): GNA, DSA, LEA, PHA-E, PHA-L, MAA-II, SNA-I, AAA, UEA-I, LTA and PNA were used for co-localization studies. In case of biotinylated lectins additional incubation with StreptAvidin-AlexaFluor488 (1:300 dilution) was performed. In the controls, lectins pre-blocked with the corresponding hapten or with 0.2 M acetic acid (2 h, RT) were used (Table 1). In additional control, lectin incubation was omitted to reveal non-specific staining due to StreptAvidin binding.

Galectin-3 was stained with mouse monoclonal anti-Galectin-3 for 2 h (clone A3A12; 1:100 dilution in 2% BSA/PBS), and then with anti-mouse IgG-AlexaFluor488 (dilution 1:100 in 2% BSA/PBS) for additional 2 hrs. The quantitative analysis of co-localization was performed using profile display mode (LSM image browser, Zeiss, Oberkochen, Germany) and the intensity profile graphs were drawn.

Table 1. Lectins used in fluorescence staining and their inhibitory compounds.

Lectin	Composition of blocking solution
GNA	0.5 M methyl α-D- mannopyranoside in 1% BSA/PBS
DSA	0.5 M D(+)glucosamine hydrochloride in 1% BSA/PBS
LEA	0.5 M D(+)glucosamine hydrochloride in 1% BSA/PBS
PHA-E	1 M N-Acetyl-D-galactosamine in 1% BSA/PBS
PHA-L	1 M N-Acetyl-D-galactosamine in 1% BSA/PBS
MAA	glycophorin A (1mg/ml) in PBS
SNA-I	0.2 M CH_3COOH in 1% BSA/PBS
AAA	0.5 M L(-)fucose in 1% BSA/PBS
LTA	0.5 M L(-)fucose in 1% BSA/PBS
UEA-I	0.5 M L(-)fucose in 1% BSA/PBS
PNA	0.5 M D(+)galactose in 1% BSA/PBS

BSA, bovine serum albumin

RESULTS

Subcellular localization of nucleolin in melanoma cells

Human melanoma Ma-Mel-27 cells were fractionated into cytosolic, membrane and nuclear fractions, each of which was subjected to SDS-PAGE and further immunoblotted with anti-nucleolin antibody (Fig. 1A). The molecular mass of nucleolin is predicted to be 77 kDa, but nucleolin is known to migrate in SDS-PAGE as a ~105-kDa protein band (instead of 77 kDa), because of the presence of very large acidic amino acid clusters in its N-terminal domain. These impede correct

binding of SDS on the protein [5]. We observed a 107-kDa band corresponding to the full-length form of nucleolin. We found many degradation fragments with molecular weights below 107 kDa. To check whether the observed degradation fragments resulted from autoproteolysis of nucleolin or from the isolation and extraction procedures, we prepared fresh whole cell extract by directly treating a cell culture monolayer with boiling Laemmli sample buffer. The immunodetected peptide bands of 107, 98, 89, 85, 59 (weak band), 55 and 49 (weak band) kDa (Fig. 1B), supported the coexistence of untruncated and truncated forms of nucleolin within live cells. This is in accord with the well-known instability and self-cleaving activity of nucleolin, even in the presence of added protease inhibitors [42, 43].

On the other hand, the presence of additional bands in the membrane (72, 68, 53, 46 kDa) and nuclear (72, 68, 53 kDa) fractions along with the disappearance of the full-length form in the membrane fraction (Fig. 1A) result from degradation during the sustained isolation of subcellular fractions and the extraction steps.

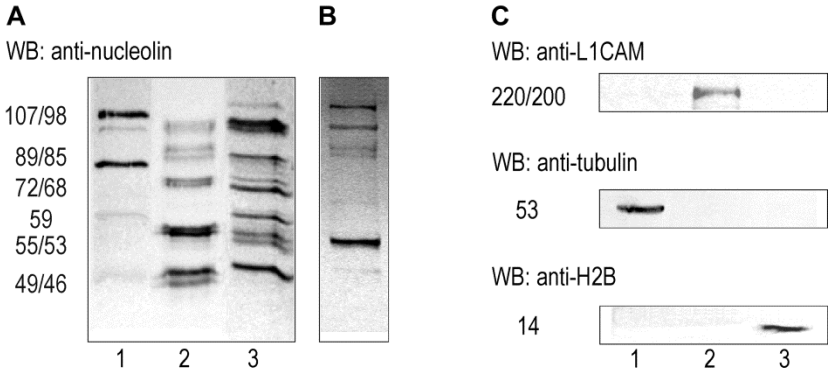

Fig. 1. Nucleolin expression in Ma-Mel-27 cells. A – Subcellular localization of nucleolin analyzed in cell fractions. Cells were fractionated into cytosolic (1), membrane (2) and nuclear (3) fractions with the ProteoExtract Subcellular Proteome Extraction Kit, and each fraction was immunoblotted with anti-nucleolin antibody. B – Nucleolin detection in freshly prepared cell extract. Fresh whole cell extract was prepared by directly treating a cell culture monolayer with boiling Laemmli sample buffer and then electrophoresed. Nucleolin was analyzed by immunoblotting. C – Determination of subcellular fraction purity. The purity of isolated subcellular fractions was confirmed by western blotting with antibodies for fraction-marker proteins: L1CAM for cell membrane (2), β-tubulin for cytosol (1), and H2B for nuclei (3). Peptide bands were visualized using AP-conjugated secondary antibody with subsequent colorimetric detection. Molecular masses (kDa) are indicated on the left-hand side of the blots.

In this case, the proteolytic degradation pattern of nucleolin was specific to the particular cell compartment. The nucleolin in the cytosolic fraction was the least degraded. We found a much stronger signal for higher molecular weight polypeptides for cytoplasmic (107 kDa and 85 kDa) or nuclear nucleolin (98 kDa and 85 kDa) than for membrane-associated nucleolin (Fig. 1A). However, there were two additional bands at positions of 89 kDa and 46 kDa in the

membrane fraction (Fig. 1A, line 2). Moreover, in both cytosolic and nuclear fractions there was one additional band at 59 kDa. The identity and purity of each fraction were monitored by immunodetection of tubulin (cytosol-positive control), L1CAM (membrane-positive control) and histone H2B (nuclear-positive control) (Fig. 1C). Histone H2B was detected exclusively in the nuclear fraction, demonstrating that the nucleolin detected in the cytosolic and membrane fractions was not due to nuclear contamination. L1CAM was expressed at high levels in the membrane fraction. The lack of immunodetected tubulin in the membrane and nuclear fractions supports the purity of all the subcellular fractions.

Lectin reactivity of nucleolin in melanoma cells

To examine the glycosylation profile of nucleolin, we used a set of lectins for lectin precipitation and confocal microscopy analysis. Lectin precipitation followed by on-blot immunodetection of nucleolin showed a lectin-dependent pattern of bands corresponding to the particular proteolytic degradation fragments of nucleolin (Fig. 2). The 46-kDa band showed no glycosylation for the lectin probes we used. Moreover, no 89- or 85-kDa bands were observed in the total cell lysate, perhaps due to their low abundance or degradation.

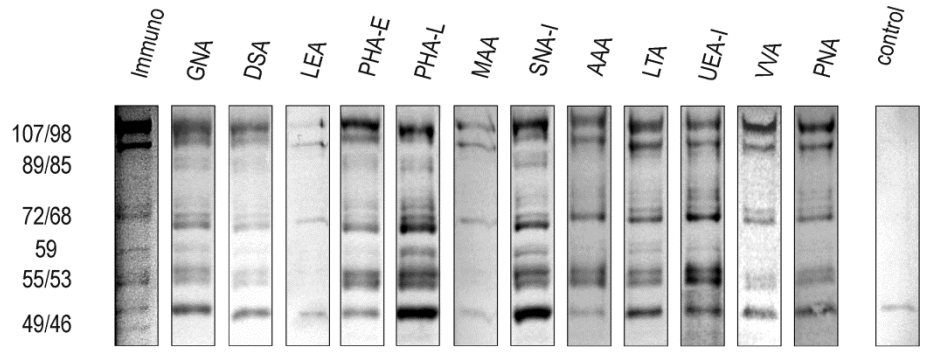

Fig. 2. Western blot analysis of nucleolin glycosylation. Immunodetection of nucleolin in whole-cell extract from cell line Ma-Mel-27 (Immuno), in lectin precipitates, and in a control sample obtained by precipitation with preinhibited lectin (Control). Lectin abbreviations and binding specificities: GNA, *Galanthus nivalis* agglutinin (recognizes high mannose and hybrid type oligosaccharides); DSA, *Datura stramonium* agglutinin (recognizes two or three *N*-acetyllactosaminyl repeats); LEA, *Lycopersicon esculentum* agglutinin (recognizes three or more *N*-acetyllactosaminyl repeats); PHA-L, *Phaseolus vulgaris* leucoagglutinin (recognizes GlcNAcβ1-6-branched triantennary and tetraantennary complex type glycans); PHA-E, *Phaseolus vulgaris* erythroagglutinin (recognizes bisecting GlcNAc residue); MAA-II, *Maakia amurensis* agglutinin (recognizes sialic acid α2-3-bound to terminal galactose residue); SNA-I, *Sambucus nigra* agglutinin (tetramer; recognizes sialic acid α2-6-bound to terminal galactose or to N-acetylgalactosamine); AAA, *Aleuria aurantia* agglutinin (recognizes fucose residues); UEA-I, *Ulex europaeus* I agglutinin (recognizes α1-2-bound to terminal galactose residue). The molecular masses (kDa) of full-length nucleolin and its proteolytic fragments are indicated on the left-hand side of the figure.

The GNA-positive bands confirmed the presence of high mannose or high mannose and hybrid-type oligosaccharides. The DSA-positive bands confirmed the presence of *N*-acetyllactosamine species with two or three *N*-acetyllactosaminyl repeats attached to C-2 and 6 of α-mannose on the C-6 side. The LEA-positive bands suggest the presence of poly-*N*-acetyllactosamine units [44]. The PHA-E- and PHA-L-positive bands confirmed the presence of bisected complex *N*-glycans and the presence of GlcNAcβ1-6-branched triantennary and/or tetraantennary complex-type glycans respectively. The reactivity of nucleolin with MAA-II and SNA-I respectively showed the presence of α2-3- and α2-6-linked sialic acids in nucleolin. We used AAA, LTA and UEA-I lectins to disclose the presence of fucose and monosaccharide residues to which fucose had been bound. All these lectins recognize L-fucosyl residues but AAA binds Fucα1-6 linked to the proximal GlcNAc residue (core fucose) as well as the Fucα1-2Galβ1-4GlcNAc sequence (blood group H(0) determinant) and the Galβ1-4(Fucα1-3)GlcNAc sequence (Le^x determinant). LTA and UEA-I are reported to prefer Le^x/H and H determinant respectively, but terminal α2-3-sialylation of Le^x structure limits LTA binding [45]. The presence of *O*-linked glycans was detected using VVA lectin (recognizes GalNAc α-O-Ser/Thr; Tn antigen) and PNA lectin (recognizes Galβ1-3GalNAcα-O-Ser/Thr; T antigen).

Since preincubation of lectins with appropriate haptenic sugars before lectin precipitation did not block the binding sites of lectins, even when the sugar concentration was elevated up to 20 times in comparison with the concentration recommended by the supplier (data not shown), we used 0.4 M acetic acid to inhibit the binding sites of lectins. Almost total inhibition was observed in the case of all of the lectins. Note that the intensity of the 49-kDa band, despite its presence in the control, was significantly weaker in comparison with the same band in each lectin-positive probe (a typical control sample is shown in Fig. 2 Control). It could mean that this part of the bound lectin, namely, the part that does not bind in the presence of acetic acid, is bound to specific sugar residues, and another part, the part that binds in the presence of acetic acid, is bound nonspecifically to nucleolin. To confirm that the observed degradation fragments were of nucleolin origin, VVA-agarose precipitated and CBB-stained protein bands, which corresponded to nucleolin bands in western blot, were excised from the gel and subjected to nanoLC-MS/MS analysis (Fig. 3A). Protein identification based on peptide sequence analysis in the SwissProt database within the Mascot search engine showed the presence of nucleolin in all of the studied bands (Fig. 3B). This result also supports the high specificity of the antibody. The matched peptides covered between 5.2 and 35.1 % (2–31 peptides) of the protein sequence (Suppl. Fig. 1 in Supplementary material at http://dx.doi.org/10.2478/s11658-014-0206-4)). In all of the analyzed bands, the other proteins were also identified (data not shown).

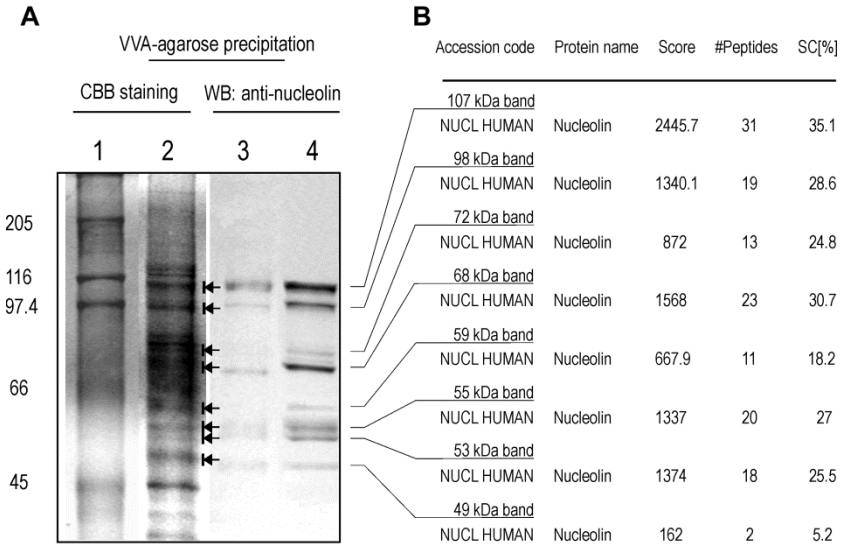

Fig. 3. Mass spectrometry analysis of in-gel tryptic digest of full-length nucleolin and proteolytic fragments, isolated from total lysate of Ma-Mel-27 cells using VVA-agarose. A – The CBB-stained protein bands of interest, isolated by VVA-agarose precipitation (shown by arrows; lane 2) and corresponding to western blot-positive protein bands in VVA-agarose precipitation (line 3) and in whole cell extract (line 4) were excised from the gel and subjected to nanoLC-MS/MS analysis. Molecular masses (kDa) of high molecular weight markers (line 1) are indicated on the left-hand side of the gel. B – The acquired spectra were analyzed using Bruker Data Analysis software and interpreted using Mascot search engine against Swiss-Prot and NCBI sequence databases (with the taxonomy restriction: *Homo sapiens*). Peptides determined by sequencing were found to correspond to a high degree of certainty to human nucleolin. # Peptide, the number of the identified peptides (based on MS/MS spectra); SC[%], the percentage of the protein's sequence that is represented by the identified peptides.

The subcellular co-localization of nucleolin with lectin-positive carbohydrate structures in Ma-Mel-27 melanoma cells was assayed by double fluorescence (Fig. 4A–C). Nucleolin was localized mainly in the nucleus, although some fluorescence was detected in the cytoplasm and on the cell surface. Extranuclear nucleolin was more abundant on the cell surface than in the cytoplasm. Lectin staining was observed almost exclusively on the cell surface and in the cytoplasm (Fig. 4C). Interestingly, the cell surface of Ma-Mel-27 cells was UEA-I-negative (data not shown). This result is in agreement with our earlier findings on glycosylation of cell surface L1CAM molecules in other human melanoma cells [46]. Cells not exposed to lectins showed no obvious fluorescence (Suppl. Fig. 2B and D), indicating that the staining observed in cells was not due to a nonspecific StreptAvidin binding with cell protein(s). All of the used lectins were shown to bind specifically to Ma-Mel-27 cells, as revealed in competitive blocking studies with hapten sugars or with the use of 0.2 M acetic acid. Only in the case of some blocked lectins was a very weak staining observed (Suppl. Fig. 2).

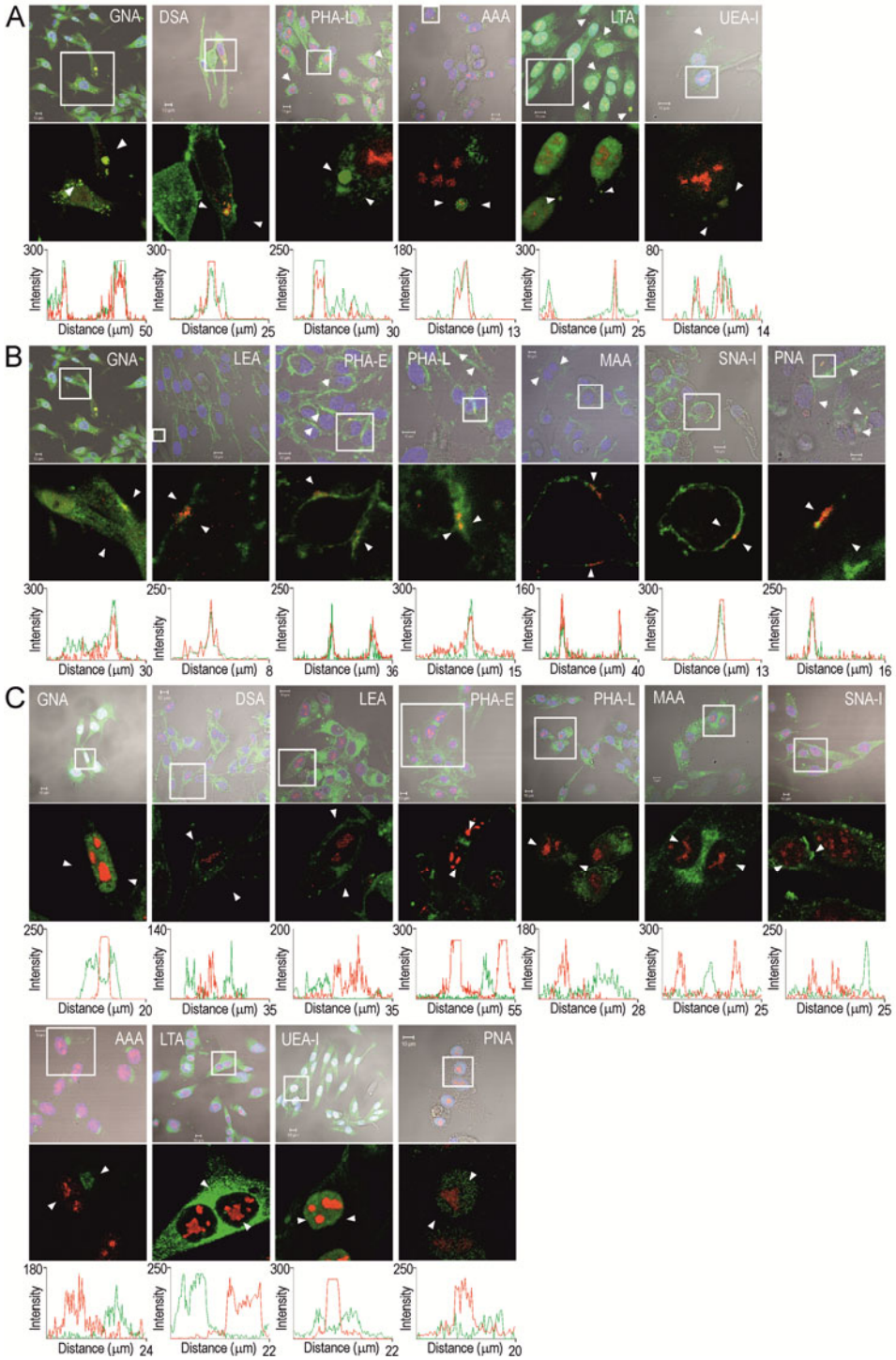

Fig. 4. Confocal microscopy images of doubly labeled Ma-Mel-27 cells in different subcellular locations. Cells were cultured in fresh medium for 5 h before labeling. A – Nucleolin detection in cytoplasm. Cells were fixed with 3% PFA before simultaneous incubation with mAb MS-3 (dilution 1:100) and with FITC-labeled lectin (dilution 1:100) or with biotinylated lectin (1:300 dilution) in 1% BSA/PBS (overnight, RT). B – Detection of cell-surface clustered nucleolin. Living unpermeabilized cells were simultaneously incubated with anti-nucleolin mAb MS-3 (1:25 dilution) and with FITC-labeled lectin (1:100 dilution) or with biotinylated lectin (1:300 dilution) in 10% NGS, 2% BSA/PBS containing 25 mM NaN3 for 1 h at 37ºC before fixation with 2% PFA. C – Nucleolin detection in the nucleus. Cells were fixed with 2% PFA/Triton before simultaneous incubation with mAb MS-3 (1:100 dilution) and with FITC-labeled lectin (1:100 dilution) or with biotinylated lectin (1:300 dilution) in 1% BSA/PBS (overnight, RT). For biotinylated lectins an additional incubation with StreptAvidin-AlexaFluor488 was performed. The bound lectin was revealed as green stain. The bound anti-nucleolin antibody was revealed using Cy3-labeled goat anti-mouse antibodies (red). Overlapping of the two stains, observed as yellow regions (arrows), confirmed the presence of specific glycan residues in nucleolin molecules. Nuclei were counterstained with DAPI (blue). The co-localization ratio was quantified by measuring the fluorescence intensity overlap with confocal software. Graphs depict the intensities (arbitrary units) of signals from nucleolin (red line) and lectin (green line) staining between arrowheads in magnified views. White open squares indicate areas magnified and shown below. Lectin abbreviations and binding specificities as in Fig. 2.

When the images of cells stained for nucleolin and a given lectin were merged, specks of co-localized nucleolin and lectin were seen as scattered dots within the cytoplasm and on the cell surface (Fig. 4A and B). No co-localization was observed in the nucleoli (Fig. 4C). Overlapping of the two stains, observed as yellow regions, confirmed the presence of specific glycan residues in the nucleolin and supported the biochemistry results. Interestingly, unlike cytoplasmic nucleolin, membrane-bound nucleolin was not fucosylated. To quantify the significance of co-localization, we analyzed the intensity profile graphs of nucleolin co-localization with a given lectin using the profile display mode (LSM image browser, Zeiss).

Galectin-3 co-immunoprecipitates with nucleolin in human melanoma cells

Since galectin-3 has been shown in different subcellular compartments in malignant human cells, and since alterations of the galectin-3 assembly and localization play additional roles in cell transformation, we examined whether galectin-3 is a binding partner for nucleolin. To determine whether endogenous galectin-3 interacts with nucleolin, subcellular extracts from Ma-Mel-27 cells were subjected to immunoprecipitation with anti-nucleolin D-6 monoclonal antibody (D-6 is a mouse monoclonal antibody raised against amino acids 271-520 of nucleolin of human origin) or with anti-galectin-3 monoclonal antibody (clone 9C4; a mouse monoclonal antibody raised against full-length human galectin-3). Then the immunoprecipitates were Western blotted with anti-galectin-3 and anti-nucleolin. We found reciprocal co-immunoprecipitation of both proteins from all studied subfractions (Fig. 5A). This result demonstrates that galectin-3 coexists with nucleolin in the same complexes in cytoplasm, on the cell surface and in the nucleus of human melanoma cells.

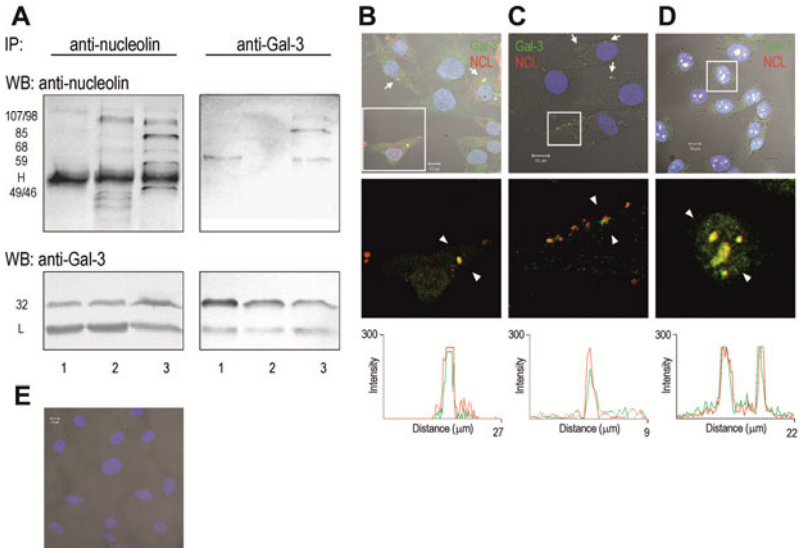

Fig. 5. Nucleolin interaction with endogenous galectin-3 in Ma-Mel-27 cells. A – Reciprocal co-immunoprecipitation of galectin-3 and nucleolin in subcellular fractions: cytosolic (1), membrane (2) and nuclear (3). Immunoprecipitates were electrophoresed and immunoblotted with anti-nucleolin or anti-galectin-3. The molecular masses (kDa) of full-length nucleolin, its proteolytic fragments and galectin-3 are indicated on the left-hand side of the gel. H, heavy chain of IgG; L, light chain of IgG. B–D – Confocal micrographs of Ma-Mel-27 cells double-labeled with anti-galectin-3 antibody (green) and anti-nucleolin antibody (red) in different subcellular locations. Arrows indicate co-localization (yellow) of galectin-3 with nucleolin present in (B) cytoplasm, on the cell surface (C), and in nucleoli (D). The co-localization ratio was quantified by measuring the fluorescence intensity overlap with confocal software. The graphs below show the overlap of fluorescence intensity peaks between arrowheads in magnified views of nucleolin (red line) and lectin (green line). E – Negative non-specific reaction of control cells with fluorochrome-conjugated secondary antibodies. White open squares indicate areas magnified and shown below.

Galectin-3 co-localizes with nucleolin in human melanoma cells

To see whether endogenous galectin-3 co-localizes with nucleolin in Ma-Mel-27 melanoma cells, we employed immunofluorescence in confocal microscopy (Fig. 5B-D). In agreement with our immunoaffinity purification results, we observed similar subcellular distributions and co-localization of galectin-3 and nucleolin in human melanoma cells. Nucleolin was strongly and specifically stained as large dots in the nucleus, indicating its nucleolar location. The results showed that the total pool of galectin-3 in interphase nuclei co-localized extensively with nucleolin at nucleolar sites (Fig. 5D). This is consistent with previous studies that found galectin-3 in the nucleoli of different cells [4]. Both extranuclear nucleolin and galectin-3 were weakly to moderately positive, and more abundant in cytoplasm then on the cell surface, where they appeared only as occasional punctate staining (Fig. 5B and C). Cells not exposed to antisera recognizing nucleolin or galectin-3 showed no obvious fluorescence (Fig. 5E), indicating that the staining seen in cells was not due to

a nonspecific cross-reaction of the secondary antibody with a cell protein. The overlapping of intensity profiles confirmed that endogenous galectin-3 and nucleolin co-localize in the cytoplasm, on the cell surface and within nucleoli in melanoma cells. These findings were confirmed in WM1205Lu metastatic melanoma cells (data not shown).

DISSCUSION

In this study, we demonstrated interrelationships between the subcellular localization, degradation and glycosylation of nucleolin. Nucleolin cleavage products have not yet been characterized fully, but several proteolytic sites have been mapped within the N-terminal domain of nucleolin [5]. We have shown for the first time differences in the proteolytic degradation pattern of nucleolin in the context of its subcellular localization. Several serine and threonine residues of nucleolin are highly phosphorylated by different kinases. Nucleolin has been shown to be phosphorylated by casein kinase II (CK2) during interphase [47] and by cell-cycle-control CDC2/Cdk1 kinase during mitosis [10]. Nucleolin is also a specific substrate of protein kinase C-ζ (PKC- ζ) [48], PI3K [49], and Rho-associated kinase (ROCK) [49]. Cell surface nucleolin is phosphorylated by casein kinase-like ectoprotein kinase [11]. The regulatory roles of these phosphorylations are not well understood. The presence of p34cdc2 phosphorylation sites improves the efficiency of nuclear translocation when they are dephosphorylated and enhances cytoplasmic localization when phosphorylated. These results indicate that the changes in the phosphorylation state of nucleolin during the cell cycle possibly are a regulatory element of nucleolin localization. Phosphorylation also makes nucleolin highly susceptible to proteolytic degradation [50]. Nucleolin fragments were produced through thiol protease cleavage of this molecule [51]. The differences in nucleolin degradation patterns are probably attributable to utilization of different phosphorylation sites by each of the mentioned kinases. Here, we showed that unlike membrane-bound and nuclear nucleolin, cytoplasmic nucleolin undergoes limited proteolysis and consists mainly of full-length nucleolin. This observation supports the finding that PRL-3 phosphatase can dephosphorylate nucleolin in the cytoplasm, suppressing phosphorylated nucleolin in the cytoplasm and causing accumulation of nucleolin in the nucleolus [52]. On the other hand, in the case of extranuclear nucleolin, the phosphorylation sites T105, T106 and T113 can also serve as glycosylation sites [36] and when they are glycosylated, they no longer can be phosphorylated. We cannot rule out that N- and O-glycans being in proximity of other phosphorylation sites may cause potential steric hindrance for kinases action. These may be reasons for which we found that the extranuclear pool of glycosylated nucleolin was a less heterogenous mixture of the full-length molecule and its proteolytic fragments than the nuclear pool of non-glycosylated protein. Therefore, we may hypothesize that the changes in the phosphorylation/glycosylation state of nucleolin are possibly a regulatory

element of the molecular mechanism responsible for its subcellular localization and degradation.

We reported that cytoplasmic and membrane-bound nucleolin, unlike nuclear-localized nucleolin, bears Tn antigen in cultured human melanoma cell line Ma-Mel-27 [28]. Now, in a confocal microscopy approach, we used a wide spectrum of lectins, molecules that recognize and bind specific mono- and oligosaccharides, to show the localization-dependent differences in the glycosylation pattern of nucleolin. Unlike nucleolar nucleolin, the extranuclear pool of nucleolin was *N*- and *O*-glycosylated. Our observations are in agreement with earlier studies [36, 39, 53].

We found that cell surface nucleolin possesses high mannose and/or hybrid type *N*-glycans, bisected and β1-6-branched *N*-glycans with polylactosamine extension, and T antigen and α2-3/6-linked sialic acid. The cytoplasmic nucleolin possesses high mannose and/or hybrid type *N*-glycans, β1-6-branched *N*-glycans and, unlike cell surface nucleolin, core fucosylated *N*-glycans as well as Le^x and H antigens. A comparison of the co-localization study with the lectin precipitation results reveals apparent discrepancies in relation to the AAA, LTA and UEA-I lectin probes. With double fluorescence staining we showed that only cytoplasmic nucleolin was fucosylated (Fig. 4A, AAA, LTA and UEA-I). Surprisingly, proteolytic fragments with molecular weights of 72, 68, 55 and 53 kDa present in the membrane fraction but not in the cytoplasmic fraction were AAA-, LTA- and UEA-I-positive. These discrepancies might be explained by the fact that proteins from intracellular membrane vesicles (exosome, endosome, microsome and lysosome) are present in membrane protein fraction 2, as intracellular organelles remain intact after extraction of cytosol fraction 1 (Figs 1A and 2). Cytoplasmic nucleolin is translocated to the surface through an unconventional secretory pathway in small secretory vesicles [29]. It has been suggested that fucosylation modulates the turnover of nucleolin by protecting it against proteolysis or autoproteolysis [37]. Our results showing differences in fucosylation and differences in the degree of degradation of cytoplasmic and membrane-bound nucleolin provide support for that suggestion. Although nucleolin glycosylation is absolutely required for its cell surface localization [53] and self-interaction [54], the consequences of this post-translational modification for nucleolin functioning are still unknown. As surface protein nucleolin serves as a receptor for various extracellular ligands involved in cell proliferation, differentiation, adhesion, mitogenesis and angiogenesis, it is also possible that glycosylation of nucleolin may be required for these interactions.

To the best of our knowledge this report gives the first evidence that nucleolin interacts with galectin-3 on the cell surface, in the cytoplasm and in the nucleolus. Galectin-3 binds type 1 or type 2 Galβ1-3(4)GlcNAc units with higher affinity for polylactosamine structures. Fucosylation, sialylation or further modification of terminal galactose by α1-3-linked Gal or GalNAc do not affect binding [55]. Galectin-3 shuttles between the cytoplasm and nucleus due to the presence of targeting signals recognized by importin(s) and exportin-1.

Within the nucleus, galectin-3 can be found diffused in the nucleoplasm and associated within structures corresponding to subnuclear domains. The cytoplasmic versus nuclear distribution of galectin-3 depends on the cell type (normal or cancerous), the proliferative state [56], and on the presence or absence of an interacting partner [57]. A great number of ligands have been reported for galectin-3. Some of them are compartment-specific but others are present together with galectin-3 in both the cytoplasm and nuclear subcellular compartments. Here we demonstrated that this interaction also takes place in the cell membrane. Although galectin-3 contains a carbohydrate recognition domain (CRD), most proteins bind galectin-3 via protein–protein interactions. In the case of nuclear nucleolin these interactions are most likely to be protein–protein, but cytoplasmic and membrane nucleolin may also be recognized by their glycans. We did not perform assays in the presence of lactose, but in fact most of the protein–protein interactions of galectin-3 with its ligands were perturbed by lactose, suggesting that the binding sites overlap. The ability of galectin-3 to form higher order oligomers and to function as a scaffold protein promotes heterotypic protein interactions and provides the formation of a cluster with its partners. Proteins that are recruited to the cluster increase local molecular concentrations and thus can express their functions with higher activity, in space and time [58–60]. Therefore, we can hypothesize that multifaceted functions of nucleolin in melanoma cell proliferation and malignancy is promoted by recruitment of nucleolin to the galectin-3 cluster, in which, in case of extranuclear nucleolin, nucleolin glycan residues can fine tune galectin-3 binding.

We disclosed the interactions between nucleolin and galectin-3 by two methods: co-immunoprecipitation and co-localization. In co-immunoprecipitation, mediation via an intermediary molecule cannot be ruled out but the confocal microscopy images confirm the co-localization of these two proteins. We suggest that the galectin-3−nucleolin complex may participate in nucleologenesis. Additional studies are required to answer the question of whether or not this interaction is direct or indirect. The other components of the complexes should be investigated in future. Further work should also address the functional significance of this interaction.

CONCLUSIONS

These results provide evidence for different paths of nucleolin degradation in the nucleus, in the cytoplasm, and on the cell surface. We showed that, besides the most representative 107-kDa form, other minor and more or less *N*- and *O*-glycosylated nucleolin forms coexist in the cell. Only extranuclear nucleolin undergoes *N*- and *O*-glycosylation. Unlike cytoplasmic nucleolin, membrane-associated nucleolin is not fucosylated. We uncovered a potential association between galectin-3 and nucleolin in Ma-Mel-27 melanoma cells. Galectin-3 was found as a novel binding partner of nucleolin in the nucleolus, in the cytoplasm,

and on the cell surface of human melanoma cells. The nucleolar co-localization of galectin-3 with nucleolin in interphase suggests that galectin-3 may be involved in DNA organization and ribosome biogenesis.

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