COMMUNICATION

A fucose-containing *O*-glycoepitope on bovine and human nucleolin

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In this paper, we demonstrate the existence and localiza-
tion of fucosyl-containing *O*-glycoforms of nucleolin in cul-
tured bovine endothelial cells (CVEC) and malignant cul-
tured human A431 cells. The tool for this discovery was an
antibody raised against gp273, a glycoprotein ligand for the
sperm–egg interaction in the mollusc bivalve *Unio elongat-
ulus*. The function and immunological properties of gp273
mainly depend on clustered Lewis-like, fucose-containing
O-glycans. Here an anti-gp273 antibody was used to evalu-
ate whether glycoepitopes similar to those of gp273 are part
of potential ligands of selectins in endothelial cells. We found
that anti-gp273 strongly and exclusively interacted with a
110 kDa protein in CVEC and A431 tumor cells. After par-
tial purification, mass spectrometry identified the protein
as nucleolin. This was confirmed by comparing anti-gp273
and anti-nucleolin antibody immunoblotting after nucleolin
depletion. We confirmed that anti-gp273 binding to nuclear
and extranuclear nucleolin was against a fucose-containing
O-glycoepitope by immunoblot analysis of the protein after
chemically removing *O*-glycans and by lectin-blot analysis
of control and nucleolin-depleted samples. Using anti-gp273
IgG, we detected nucleolin on the plasma membrane and
cytoplasm. *O*-Glycosylation may regulate the plethora of
functions in which nucleolin is involved.

Keywords: A431 human cancer cells/CVEC/glycoepitopes/
nucleolin/RNA-interference

Introduction

Glycoepitopes may be shared by different proteins with overlap-
ping functions (Tessier-Lavigne and Goodman 1996). We have
been studying for many years a glycoprotein of the vitelline
coat of oocytes of the mollusc bivalve *Unio elongatulus*. The
glycoprotein, gp273 (mass 273 kDa) (Focarelli et al. 1997),
was found to be the ligand molecule for sperm-egg recognition
(Focarelli and Rosati 1995). The carbohydrate content of gp273

was calculated to be ∼3.5% by mass and to be consist of fu-
cose, mannose, galactose, glucose, *N*-acetyl-galactosamine, and
N-acetylglucosamine (Di Patrizi et al. 2002). The *N*-linked gly-
cans liberated by means of PNGase F and analyzed by a combi-
nation of HPLC, ^{1}H NMR spectroscopy, and mass spectrometry
revealed oligomannose glycans that constitute the greatest part
of the carbohydrate content. Fucose, galactose, and *N*-acetyl-
galactosamine were presumed to be part of *O*-glycans and car-
bohydrate analysis of gp273 after the removal of *N*-glycans
confirmed their presence in the remaining *O*-glycoprotein
(unpublished results). The presence of *O*-glycans bearing
these sugars was also confirmed by lectin-screening studies
(Focarelli et al. 2003). Using purified anti-gp273 immunoglobu-
lins, we found that the anti-gp273 epitope consists of *O*-glycans
containing Lewis-like structures with fucose (Focarelli et al.
2003) and sulfation (data not shown) as determinant. Since
sulfo-Le(x)/Le(a) fuco-oligosaccharides (Yuen et al. 1992; Varki
1994; Galustian et al. 2002) are indicated as ligand(s) for selectin
recognition, we investigated whether anti-gp273 also labeled
glycoepitopes with function of ligands in selectin-mediated in-
teractions in vascular endothelial cells.

We analyzed bovine cultured coronary venular endothelial
cell (CVEC) extracts for reactivity to anti-gp273. Intriguingly,
we found that the sole protein specifically recognized by anti-
gp273 in these cells was nucleolin. The antibody was then shown
to identify the same protein in cultured human epidermoid car-
cinoma A431 cells. It was also found to be a valid tool for
identifying and characterizing nucleolin glycoforms located in
the nucleus, on the plasma membrane and in the cytoplasm.

Material and methods

Cell lines

Bovine CVEC and human epidermoid carcinoma A431 were
plated in 100 mm diameter dishes in DMEM supplemented, re-
spectively, with 4500 mg/L glucose and 10% fetal calf serum
(FCS) (Hyclone, Logan, UT) or with 1000 mg/L glucose and
10% bovine calf serum (BCS) (Hyclone). CVEC were main-
tained in culture in gelatin-coated dishes. Media were used after
the addition of 2 mM L-glutamine and antibiotics (100 U/mL
penicillin and 100 μg/mL streptomycin). Cell lines were
obtained from American Type Culture Collection (ATCC,
Rockville, MD). All reagents were purchased from Sigma
(St. Louis, MO). Cells were split 1:3 three times (CVEC) and
1:8 twice a week (A431).

Processing cell proteins

Cells were grown to 80% confluence or the exponential phase
of growth. Adherent cells were washed three times with

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cold phosphate-buffered saline (PBS, 150 mM NaCl, 50 mM KH_2PO_4, pH 7.4) on cell culture plates and scraped with 120 μL of lysis buffer (10 mM Tris–HCl, pH 8.0, 1% Triton-X 100, 1 mM ethylenediaminetetraacetic acid (EDTA), 1 mM phenylmethylsulfonylfluoride (PMSF), protease inhibitor cocktail (Sigma-Aldrich)). Extranuclear and nuclear fractions were obtained as follows: 3×10^6 adherent trypsinized cells were centrifuged at $900 \times g$ for 15 min. Pellets were resuspended in 7 mL buffer E (20 mM Tris–HCl, pH 7.6, 150 mM NaCl, 7.5 mM $MgCl_2$, 5 mM β-mercaptoethanol, 0.5% Triton X-100, and protease inhibitor cocktail) and incubated on ice for 15 min. Nuclei were pelleted by centrifuging at $1000 \times g$ for 15 min. The supernatant collected contained the extranuclear fraction. Nuclei were washed once with buffer E and resuspended on ice in buffer I (20 mM Tris–HCl, pH 7.6, 50 mM KCl, 40 mM NaCl, 1 mM EDTA, 5 mM β-mercaptoethanol, 1% Triton X-100, 20% glycerol, and protease inhibitor cocktail) for 15 min. The mixture was centrifuged at $1000 \times g$ for 15 min. The nuclear fraction was collected. Finally, the nuclear and extranuclear fractions were centrifuged at $12,000 \times g$ for 20 min to remove the remaining insoluble material. Protein concentration was determined by a bicinchoninic acid (BCA) protein assay reagent kit (Sigma-Aldrich).

Fast performance liquid chromatography

CVEC and A431 cellular lysates were pooled separately and fractioned (3 mg for each run) by anion-exchange chromatography on a UNO Q-6 column (BioRad Microscience, Cambridge, MA) in a BioLogic HR Chromatography System (BioRad Microscience), eluted at a flow rate of 3 mL/min under a linear gradient produced by mixing 10 mM Tris–HCl, pH 8.0 (eluent A), and 1 M NaCl and 10 mM Tris–HCl, pH 8.0 (eluent B), as follows: isocratically with A for 13 mL, followed by a linear gradient 0–100% B for 64 mL, isocratically with 100% B for 6 mL. Proteins were detected at 280 nm with a UV monitor. CVEC and A431 fractions were collected, dialyzed, and lyophilized for further analysis.

Antibodies

The antibodies were as follows: anti-nucleolin (MS-3 mouse monoclonal, 1:200 dilution for immunoblotting and 1:50 for immunofluorescence, Santa Cruz Biotechnology, Inc., CA); anti-actin (polyclonal rabbit IgG, 1:5000, Sigma-Aldrich); anti-gp273 (polyclonal rabbit IgG, 1:1000 dilution for immunoblot and 1:50 for immunofluorescence) obtained as previously described (Focarelli et al. 2003); secondary antibodies, horseradish peroxidase-conjugated goat anti-mouse IgG (Fab specific) (1:3000, Sigma-Aldrich) and goat anti-rabbit IgG (1:3000, BioRad Microscience); and secondary antibodies coupled to Alexa Fluor dyes A488 (goat anti-mouse IgG, 1:100) and A555 (goat anti-rabbit IgG, 1: 100) (Invitrogen, Eugene, OR).

Sodium dodecyl sulfate–polyacrylamide gel electrophoresis and immunoblot

Proteins were separated on 8% sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE), stained with silver nitrate or colloidal Coomassie, or transferred to nitrocellulose. The nitrocellulose membranes were blocked with 3% nonfat powdered milk in TBS (20 mM Tris–HCl, pH 7.5, 500 mM NaCl) and incubated for 1 h at room temperature (RT)

with anti-nucleolin antibody MS-3 or anti-gp273. After several washes with TBS–Tween 0.2% (TTBS), blots were incubated for 1 h at RT with the respective secondary antibodies conjugated with horseradish peroxidase. After extensive rinsing in TTBS, labeled proteins were developed using an Immun-Star HRP Chemiluminescent Kit (BioRad Microscience) according to the manufacturer's instructions. Images were digitalized with CHEMI DOC Quantity One program.

Lectin-screening studies

The same amount of the total CVEC protein extract or of the corresponding nuclear and extranuclear fractions were separated on 8% SDS–PAGE, transferred to nitrocellulose, and analyzed with the following biotin-conjugated lectins: peanut agglutinin (PNA), *Artocarpus integrifolia* agglutinin (Jacalin), *Ulex europaeus* agglutinin (UEA-1), concanavalin A from *Canavalia ensiformis* (ConA) (Sigma-Aldrich), *Lotus tetragonolobus* lectin (LTL), and *Aleuria Aurantia* lectin (AAL) (VECTOR UK).

The nitrocellulose membrane was first incubated in the blocking solution, washed twice in Tris-buffered saline (TBS), and then incubated overnight at 4°C with lectins. The blot was then extensively washed with TBS and the recognized glycoproteins revealed by incubation with horseradish peroxidase-conjugated streptavidin (GE Healthcare, UK) for 1 h at RT.

Identification of nucleolin by in-gel cleavage and mass spectrometry

The silver-stained electrophoretically resolved protein bands of CVEC and A431 anion-exchange chromatography fraction, corresponding to anti-gp273 positivity in blot, were excised from the gel, destained, and dehydrated. Proteolytic digestion of the gel-immobilized proteins was performed overnight at 37°C with 0.2 μg/μL trypsin. Peptide mass fragments were determined by mass spectra obtained using an ETTAN MALDI-TOF mass spectrometer from Amersham Biosciences (Uppsala, Sweden). The identity of the polypeptides was assigned by comparison of the mass fingerprint with the predicted mass fingerprint of nucleolin in the database.

siRNA transfection

Three *Silencer* pre-designed siRNAs (directed against exons 3, 8, and 13 of human nucleolin) and a *Silencer* negative control siRNA were purchased from Ambion (Austin, TX). Cells were plated 2.2×10^5 in 6-well plates, 6×10^5 in 100 mm diameter dishes, or seeded 30×10^3 on cover slips and transfected transiently using lipofectamine (Invitrogen, Carlsbad, CA, USA) and 500 nM (final concentration) of either nontargeting siRNA or siRNAs directed toward the mRNA of nucleolin. Cells were assayed after 24, 48, and 72 h.

Fluorescence microscopy

30×10^3 cells were seeded on cover slips in a 24-multiwell plate and cultured for 24 h in the appropriate medium. Cells were washed three times with cold PBS and fixed with 4% paraformaldehyde (PFA) in PBS for 2 h at 4°C, and then washed in PBS. After fixing, to monitor intracellular nucleolin, cells were permeabilized in 0.5% Triton X-100 in PBS for 5 min at 4°C and washed three times in PBS. After blocking no specific binding in 3% goat serum for 30 min, cells were incubated

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with anti-nucleolin and anti-gp273 diluted in 1% goat serum for 1 h at RT. Cells were then washed in PBS and bound antibodies were detected with secondary antibodies coupled to Alexa Fluor dyes for 30 min at RT. Other experiments were carried out incubating cells with anti-nucleolin, followed by anti-gp273 and the respective secondary antibodies. Nuclei were identified with Hoechst 3342 1 μg/mL (Sigma-Aldrich). Finally, the cover slides were mounted in Mowiol 4-88 (Calbiochem, La Jolla, CA) and visualized by fluorescence microscopy Leitz Diaplan (Leica, Heidelberg, Germany) and confocal microscope LSM-510 META (ZEISS, Jena, Germany). The images were processed in Photoshop 5.5 (Adobe Systems, Mountain View, CA).

### Enzyme and chemical oligosaccharide cleavage

Complete deglycosylation of CVEC glycoproteins was obtained by a chemical treatment with anhydrous trifluoromethanesulfonic acid (TFMS) (Sigma-Aldrich). Aliquots of 2 mg of lyophilized and completely dried proteins form CVEC lysates were dissolved in 300 μL of anhydrous and precooled TFMS containing 10% (v/v) of anisole (Sigma-Aldrich). Reaction proceeded 3 h at 2–8°C on ice. Before stopping the reaction, 4 μL of a 0.2% (w/v) bromophenol blue (Biorad) solution was added as an indicator. Neutralization was performed at −15°C, in a methanol-dry ice bath, dropping a precooled solution of distilled water/methanol 1:1 containing the 60% (v/v) of pyridine until the color of solution turned to blue. Before further analysis, the samples were dialyzed extensively against distilled water.

N-Linked oligosaccharides chains were released from the protein backbone by PNGase F from *Flavobacterium meningosepticum* (Sigma-Aldrich). Aliquots of 120 μg of CVEC lysates were dissolved in 20 mM NaH_2PO_4, pH 7.2, containing 50 mM β-mercaptoethanol and 0.1% (w/v) SDS. Samples were denatured by heating to 100°C for 5 min. After cooling on ice, the solution was incubated with 0.5% (w/v) Nonidet P-40 and PN-Gase F (5 U/mL) for 16 h at 37°C. As control, an equivalent amount of sample was incubated under the same conditions in the absence of the enzyme.

Release of *O*-linked chains was carried out by nonreductive mild alkaline hydrolysis (β-elimination). Cellular lysates were incubated in 50 mM NaOH at 37°C for different time intervals and analyzed by western blot. As control, an equivalent amount of sample was incubated under the same conditions without NaOH.

Results and discussion

Different amounts of SDS-separated Triton X-100 protein extracts of CVEC were tested with anti-gp273 IgG and only one protein running at an apparent MW of 110 kDa was immunoreactive (Figure 1A). 20 μg whole protein was found sufficient for anti-gp273 IgG to reveal positivity when used at a dilution of 1:1000. Immunoblot experiments with the same amount of protein of Triton X-100 extract of bovine liver and muscle cells did not show any reactive species to anti-gp273 (data not shown). Anion-exchange chromatography analysis of Triton X-100 cell extract of CVEC indicated that the anti-gp273 positive peptide mainly eluted at a NaCl concentration of 0.6 M (Figure 1A). Figure 1A shows enrichment of nucleolin in the 0.6 M NaCl fraction of CVEC and also reveals that when the protein was abundant,
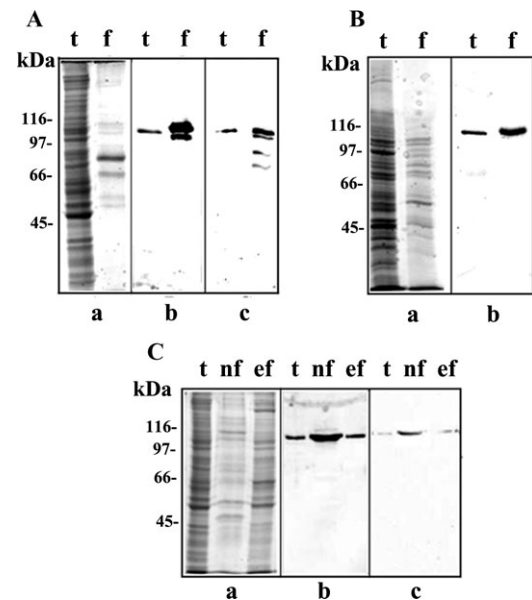
Fig. 1. Recognition of nucleolin by anti-gp273 in CVEC and A431. (**A**) Immunoblot analysis of a total lysate (*t*) of CVEC and of the fraction eluting at 0.6 M NaCl (*f*) after FPLC fractionation of the lysate. Twenty micrograms of protein was separated on 8% SDS–PAGE, stained with colloidal Coomassie (a), transferred to nitrocellulose, and incubated with anti-gp273 (b), followed by the respective secondary antibody. All gel regions positive to anti-gp273 in the FPLC fraction were used for MALDI analysis. In c, 40 μg of the same samples used for a and b, analyzed with the anti-nucleolin antibody MS-3. (**B**) Immunoblot analysis of a total lysate (*t*) of A431 cells and of the corresponding 0.6 M NaCl fraction (*f*) after FPLC fractionation of the lysate. Twenty micrograms of protein was separated on 8% SDS–PAGE, stained with colloidal Coomassie (a), transferred to nitrocellulose, and incubated with anti-gp273 (b), followed by the secondary antibody. (**C**) Twenty micrograms of a total CVEC lysate and nuclear (*nf*) and extranuclear fractions (*ef*), obtained as indicated in *Material and methods*, were separated on 8% SDS–PAGE, stained with colloidal Coomassie (a), transferred to nitrocellulose, and incubated with anti-gp273 (b), followed by the corresponding secondary antibody. In c, detection of nucleolin in 40 μg of the same nuclear (*nf*) and extranuclear fractions (*ef*) by the anti-nucleolin antibody MS-3.

it immunoblotted as a broadband with an apparent MW ranging from 105 to 120 kDa. All immunoreactive portions of the gel were analyzed by mass spectrometry and were found to match a nucleolin-related protein (accession number: gi76682880). We, therefore, tested whether anti-gp273 also labeled nucleolin in human cultured cells. To do so, we analyzed the protein extract of human epidermoid carcinoma A431 cells with the same antibody dilution used for CVEC. We only found a positive peptide running at about 110 kDa (Figure 1B). We also analyzed the anti-gp273 positive protein of the human A431 cells by mass spectrometry after partial purification by fast performance liquid chromatography (FPLC) (Figure 1B). The results confirmed those of CVEC since the anti-gp273 positive protein matched nucleolin (accession number: P19338).
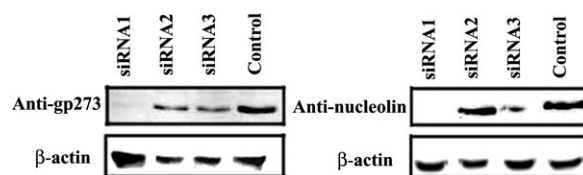
To confirm that anti-gp273 IgG recognized nucleolin, we analyzed whole Triton X-100 extract of CVEC, the corresponding nuclear and extranuclear fractions, and the 0.6 M NaCl FPLC fraction with anti-gp273 and the commercial anti-nucleolin monoclonal antibody MS-3 (Figure 1A and C). We used MS-3 because it is directed against the entire nucleolin peptide; however, since the antibody MS-3 is directed against human nucleolin, cross-reactivity of the antibody with bovine nucleolin was
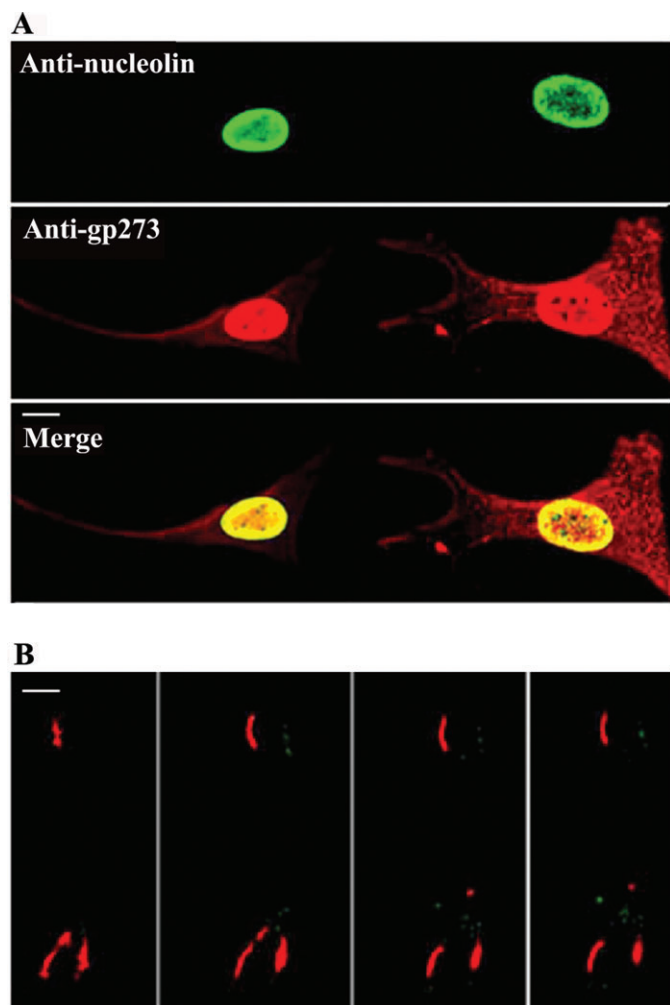
Fig. 2. Detection of intracellular and membrane-associated nucleolin recognized by anti-gp273 IgG by confocal immunofluorescence laser microscopy. In **A**, permeabilized cells incubated with anti-gp273 IgG and with the anti-nucleolin antibody MS-3 followed by the corresponding secondary antibodies conjugated, respectively, with rhodamine and with fluorescein. In **B**, merge of four sequential sections of nonpermeabilized cells incubated in both the antibodies as in **A**. Scale bar is 15 μm.

Fig. 3. Depletion of the nucleolin isoforms recognized by anti-gp273 and by anti-nucleolin by the same nucleolin RNA 1 silencer. Immunoblot showing that the nucleolin isoforms identified by anti-gp273 are depleted by the same siRNA depleting the nucleolin identified by the antinucleolin antibody. 6×10^5 cells, plated in 100 mm diameter dishes, were transfected transiently using lipofectamine and three *Silencer* predesigned siRNAs (siRNA1, siRNA2, and siRNA3, *Materials and methods*) and a negative control siRNA. After 72 h treatment, 20 μg protein of the total CVEC lysate was used for analysis with anti-gp273 and 40 μg for that with anti-nucleolin. β-Actin is shown as a control.
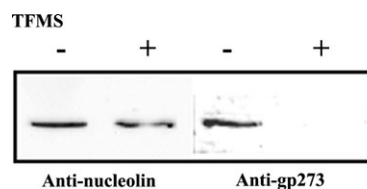

Fig. 4. Importance of glycans in forming the epitope(s) of the nucleolin isoforms recognized by anti-gp273 IgG. Immunoblot with anti-gp273 and with anti-nucleolin showing that the removal of glycans completely abolishes reactivity of nucleolin to anti-gp273. The total CVEC protein extract was incubated with (+) TFMS containing 10% (v/v) of anisole for 3 h and, after treatment, 20 μg and 40 μg protein, respectively, were used for immunoblot analysis with anti-gp273 and anti-nucleolin. Control samples (−) were incubated under the same conditions in the absence of TFMS.

first ascertained to be positive in an equal amount of protein extract of bovine cells and human A431 (data not shown). The results confirmed that anti-nucleolin only recognized a peptide running at about 110 kDa, as anti-gp273, in all samples (Figure 1A and C) and that the reactive species was highly enriched in the 0.6 M NaCl FPLC fraction (Figure 1A). Here, nucleolin forms running below 100 kDa only recognized by anti-nucleolin were found (Figure 1A). Forms running below 100 kD recognized by the anti-nucleolin antibody but not by anti-gp273 were also frequently observed in the nuclear fraction (Figure 5A).

Double immunofluorescence analysis of Triton X-100 permeabilized CVEC confirmed that anti-gp273 interacts with nuclear nucleolin since the signal of both antibodies was present in this cell region (Figure 2A). On the other hand, anti-gp273 IgG but not anti-nucleolin revealed nucleolin in the cytoplasm and at cell periphery (Figure 2A). Analysis of nonpermeabilized cells showed that anti-gp273 but not anti-nucleolin identified nucleolin located in the plasma membrane (Figure 2B). Inter-

estingly, the anti-gp273 signal was observed in one restricted area in small round cells, in limited areas of small ellipsoidal cells (Figure 2B), and diffused in large flat cells with many protrusions.

To confirm that anti-gp273 was directed against nucleolin, three different siRNA sequences directed against distinct exons of human nucleolin and a siRNA control were used, followed by immunoblot analysis of the cell extracts and of corresponding nuclear and extranuclear fractions with anti-nucleolin and anti-gp273. Of these three siRNA, only one (the same for both the antibodies: siRNA 1, directed against 8 exon) almost completely abolished nucleolin expression after 72 h of depletion (Figure 3), and no differences in the timing of expression decay were observed between nuclear and extranuclear nucleolin. The same siRNA was checked and proved to deplete nucleolin in A431 cells used in the same way as a human control. No depletion at all was observed with the control siRNA (Figure 3).

We next concentrated our efforts in characterizing the anti-gp273 epitope(s) of nucleolin. First of all, we checked whether the peptide backbone participated to the epitope formation, by analyzing whether gp273 and nucleolin had similar peptide sequences. This was done by testing gp273 with anti-nucleolin and by checking homologies in a database of trypsin-digested fragments of gp273. The results indicated that gp273 and nucleolin do not share peptide sequences, since anti-nucleolin did not react at all with purified gp273, even at high concentrations (data not shown), and no relationship with other known proteins
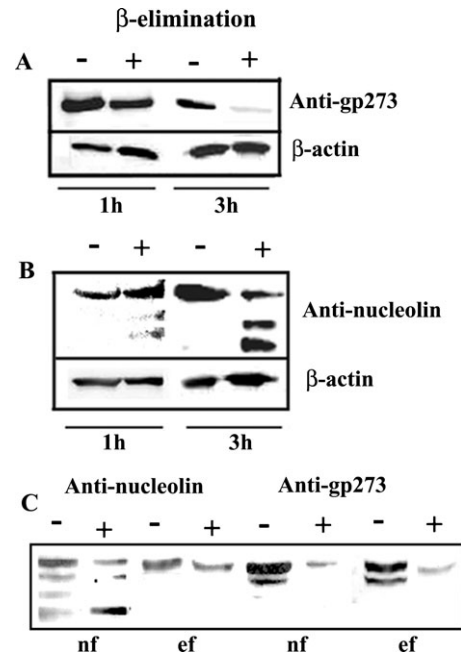
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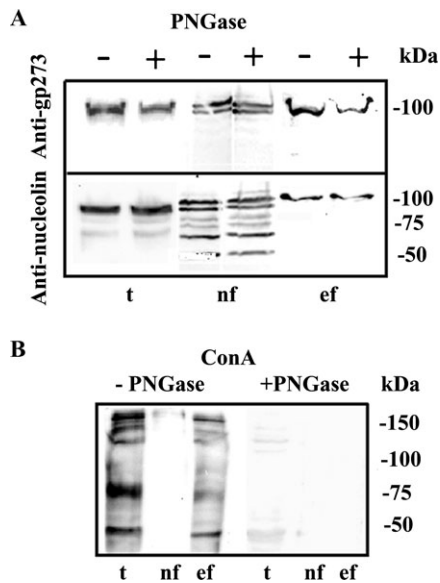
Fig. 5. Partial contribution of *N*-glycans to the formation of the anti-gp273 epitope(s) of the extranuclear nucleolin. Immunoblot analysis with anti-gp273 and anti-nucleolin of CVEC lysate (*t*) and of the corresponding nuclear (*nf*) and extranuclear fractions (*ef*) showing that the removal of *N*-glycans only slightly lowers the anti-gp273 reactivity of the extranuclear nucleolin. (**A**) Immunoblot with anti-gp273 and the anti-nucleolin antibody of a total CVEC protein extract and of the corresponding nuclear and extranuclear fractions incubated with 0.5% (w/v) Nonidet P-40 and PNGase F (5 U/mL) for 16 h at 37°C. Control samples were incubated under the same conditions in the absence of the enzyme. Twenty grams of protein was always used for detection of nucleolin with anti-gp273 and 40 µg for detection of nucleolin with the anti-nucleolin antibody. The nuclear and extranuclear fractions, obtained as indicated in *Materials and methods*, were separated on 8% SDS–PAGE, transferred to nitrocellulose, and incubated with anti-gp273 and anti-nucleolin followed by the corresponding secondary antibodies. (**B**) Lectin-blot analysis with ConA of 40 µg protein of a total CVEC protein extract (*t*) and of the corresponding nuclear (*nf*) and extranuclear fractions (*ef*) incubated with or without PNGase F (5 U/mL).

Fig. 6. Identification of *O*-glycans as part of the epitope(s) of the nuclear and extranuclear isoforms of nucleolin recognized by anti-gp273 IgG. Immunoblot analysis with anti-gp273 and anti-nucleolin of CVEC lysate (*t*) and of the corresponding nuclear (*nf*) and extranuclear fractions (*ef*) showing that the removal of *O*-glycans greatly lowers the anti-gp273 reactivity of the nuclear and extranuclear nucleolin. (**A**). Immunoblot analysis with anti-gp273 of 20 µg protein of a total CVEC extract incubated in the presence (+) or absence (−) of 50 mM NaOH at 37°C for 1 and 3 h. β-Actin is shown as a control. (**B**) Immunoblot analysis with anti-nucleolin MS-3 of 40 µg protein of a CVEC extract treated as a sample in **A**. β-Actin is shown as a control (**C**). Immunoblot analysis with anti-gp273 and anti-nucleolin of 20 and 40 µg protein, respectively, of nuclear and extranuclear CVEC fractions incubated in the presence or absence of 50 mM NaOH at 37°C for 3 h.

was identified for gp273 by MALDI spectrometry and database analysis.

We then evaluated the contribution of glycan chains in forming the nucleolin epitope labeled by anti-gp273 IgG. This was first done by analyzing the protein after the complete removal of carbohydrates with trifluoromethanesulfonic acid (TFMS). The results confirmed that the anti-gp273 IgG recognize a glycoepitope of nucleolin as it occurs for gp273 since deglycosylated nucleolin was no longer reactive to anti-gp273 IgG whereas it was still recognized by anti-nucleolin (Figure 4).

The relative role of *N*- and *O*-glycans in forming the anti-gp273 nucleolin epitope was then investigated by immunoblot analysis of CVEC extract and respective nuclear and extranuclear fractions after the enzyme removal of *N*-glycans (Figure 5) and the chemical removal of *O*-glycans (Figure 6). *N*-Glycans resulted to only partially participate in the anti-gp273 epitope formation of the extranuclear nucleolin forms since 16 h PNGase F treatment slightly lowered immunoreactivity to anti-gp273 of nucleolin included in the total CVEC extract and in the extranuclear fraction but not that of nucleolin included in the nuclear fraction (Figure 5A). Immunoblot analysis of the same samples with the anti-nucleolin antibody indicated that neither the 110 kDa nucleolin form nor all lower forms changed reactivity

to this antibody after *N*-glycans removal (Figure 5A). The removal of *N*-glycans by PNGase was tested and proved by lectin-blot analysis with ConA of the total lysate and corresponding fractions treated with or without PNGase (Figure 5B). ConA analysis also explained why the removal of *N*-glycans from the nuclear CVEC fraction did not interfere with anti-gp273 and anti-nucleolin reactivities; these glycans are, in fact, absent from proteins present in this fraction (5B). Differently from *N*-glycans, *O*-glycans were found to be determinant in the formation of anti-gp273 epitope of nucleolin. One hour of mild alkaline treatment of the CVEC total extract resulted in a partial lowering of the anti-gp273 110 kDa immunoreactivity and 3 h resulted in a strong decrease in it (Figure 6A). By using anti-nucleolin, we observed a gradual decrease in the 110 kDa reactive band and, in parallel, an increasing immunoreactivity of the bands at lower MW. These lower bands were not identified at all by anti-gp273 (Figure 6B). Analysis of nuclear and extranuclear fractions after 3 h of mild-alkaline treatment revealed that *O*-glycans participate in the anti-gp273 glycoepitope of both nuclear and extranuclear nucleolin. In both fractions, anti-gp273 reactivity was, in fact, found to strongly decrease as a consequence of this treatment (Figure 6C). By using anti-nucleolin, we observed that the decrease in the 110 kDa band and the increase in bands at lower MW only occurred in the nuclear

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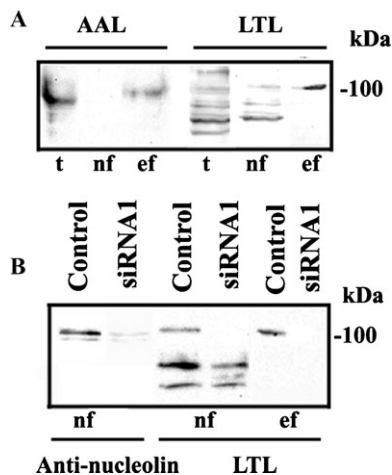
Fig. 7. Identification of fucose as a key component of the anti-gp273 *O*-glycoepitope of the nuclear and extranuclear nucleolin. (**A**) Lectin-blot with AAL and LTA of a total CVEC protein extract (*t*) and of the corresponding nuclear (*nf*) and extranuclear fractions (*ef*). Twenty micrograms of protein was separated on 8% SDS–PAGE, transferred to nitrocellulose, and then incubated overnight at 4°C with the lectins. The blot was then extensively washed with TBS and the recognized glycoproteins revealed by incubation with horseradish peroxidase-conjugated streptavidin for 1 h at RT. (**B**) Immunoblot with the anti-nucleolin antibody of the nuclear fraction and lectin-blot with LTL of the nuclear (*nf*) and extranuclear fractions (*ef*) of CVEC transfected transiently using siRNA1. 6×10^5 cells, plated in 100 mm diameter dishes, were transfected transiently using lipofectamine and the *Silencer* siRNA1 and 40 μg of the nuclear and extranuclear fractions, obtained as indicated in *Materials and methods*, were separated on 8% SDS–PAGE, transferred to nitrocellulose, and incubated with anti-nucleolin or biotin-conjugated LTL followed by the corresponding secondary antibody or by streptavidin. Control:negative control siRNA.

fraction whereas reactivity in the extranuclear fraction only present at 110 kDa remained unchanged (Figure 6C).

In order to achieve information about the carbohydrates of nucleolin glycoforms recognized by anti-gp273, we analyzed 40 μg of total CVEC lysate and corresponding nuclear and extranuclear fractions by means of lectins. At this protein concentration, besides the already described ConA, only lectins from *Lotus*, *Ulex*, and *Aleuria* resulted in the binding of peptides of CVEC nuclear and/or extranuclear fractions. All these lectins recognize L-fucosyl residues but lectins from *Lotus* and *Ulex* are described to prefer (α-1,2)-linked fucose residues, whereas the lectin from *Aleuria* binds preferentially to fucose linked (α-1,6) to *N*-acetylglucosamine. AAL-positive glycopeptides were mainly found in the extranuclear fraction in a MW ranging between 120 and 150 kDa (Figure 7A); UEA-positive peptides were detected both in nuclear and extranuclear fractions at high and low MW (data not shown) and four LTL-positive peptides were observed in the nuclear fraction in a MW ranging between 50 and 110 kDa and only one with an apparent MW of 110 kDa in the extranuclear fraction (Figure 7A). Lectin analysis after PN-Gase and mild-alkaline treatment revealed that fucosyl residues positive to LTL and UEA took part of *O*-glycans whereas those positive to AAL were prevalently part of *N*-glycans since reactivity to LTL and UEA was abolished by mild-alkaline treatment whereas AAL positivity was lowered by PNGase treatment (data not shown). Since positivity to LTL and AAL was prevalently in the MW range of nucleolin, we then analyzed by means of these lectins CVEC nuclear and extranuclear fractions after depletion of nucleolin by means of siRNA1. Positivity to AAL resulted to be only partly lowered in the depleted sample with respect to the control one, thus suggesting the presence in the 120–150 kDa range of AAL-positive glycoproteins other than nucleolin. Instead analysis with LTL clearly indicated that a positive peptide at MW of 110 kD completely disappeared from nuclear and extranuclear fractions in the nucleolin depleted samples (Figure 7B).

In this paper, we demonstrate that anti-gp273 strongly and specifically reacts with nuclear and extranuclear nucleolin glycoforms and that cross-reactivity mainly depends on fucosyl-containing *O*-glycan chains. It is possible that fucosyl-containing *N*-glycans also but at a very low degree participate to this glycoepitope in the extranuclear nucleolin. Nucleolin has been reported as ligand for L-selectin in myeloid and hematopoietic progenitor cells (Harms et al. 2001) and as a P-selectin receptor on human colon carcinoma cells (Reyes-Reyes and Akiyama 2008). This should support our hypothesis that anti-gp273 labels ligand glycoepitopes for selectins. However, nucleolin is a complex, multifunctional protein and its *O*-glycans probably have additional functions. Nucleolin is highly expressed by exponentially growing eukaryotic cells and was primarily found on the nucleus (Lapeyre et al. 1987). Only recent studies have indicated that cell surface forms also exist (Said et al. 2002; Sinclair and O'Brien 2002), though there is little convincing morphological data of this location so far. Anti-gp273 was proved to strongly label the protein on the plasma membrane, besides detecting it in the nucleus and in the cytoplasm. 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 endothelial cells of angiogenic blood vessels (Christian et al. 2003). The protein has therefore been indicated as a shuttle molecule between the cell surface and nucleus and indeed as a mediator for the extracellular regulation of nuclear activity (Srivastava and Pollard 1999). The structural features that govern this shuttle activity are not well known. It was recently proposed that *N*- and *O*-glycosylation of part of the extranuclear nucleolin (Carpentier et al. 2005) helps regulate this nucleolin traffic. Our results indicate that *O*-glycoforms are not limited to the extranuclear environment but are also present in the nucleus and that in both forms fucosyl residues are key components. Our results also suggest that, besides the most representative 110 kDa form, other minor, more or less *O*-glycosylated nucleolin forms exist in the cells. This can be deduced from the fact that in the highly nucleolin-enriched fraction obtained after FPLC anion-exchange fractionation, the reactivity to anti-gp273 included peptides running above and slightly below 110 kDa. Instead no one of the nucleolin forms at lower MW, prevalently present in the nuclear fraction, and reported in the literature as products of degradation processes are recognized by anti-gp273. Nucleolin traffic, functions, and degradation may, therefore, be regulated by the extent of *O*-glycosylation and/or fucosylation. This process could occur in concomitance with, or in alternative to the phosphorylation mechanism already known to modulate the nuclear functions of nucleolin (Morimoto et al. 2007). Indeed, potential sites of *O*-glycosylation have been indicated through bioinformatics tools in the TPXKK N-terminal region of the protein where also the phosphorylation sites have been detected (Carpentier et al. 2005).

Summarizing, the anti-gp273 antibody turns out to be a tool to highlight the evolutionary significance of the *O*-glycoepitope responsible of carbohydrated-based cross-reactivity and to study whether and how *O*-glycosylation regulate the multifaceted functions played by nucleolin in cell proliferation and malignancy.

Funding

Conflict of interest statement

None declared.

Abbreviations

CVEC, coronary venular endothelial cell; EDTA, ethylenediaminetetraacetic acid; FPLC, fast performance liquid chromatography; PFA, paraformaldehyde; PMSF, phenylmethylsulfonylfluoride; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis.

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