

Rho GTPase Rac1: Molecular Switch within the Galectin Network and for N-Glycan α 2,6-Sialylation/O-Glycan Core 1 Sialylation in Colon Cancer *in Vitro*

(apoptosis / cancer / glycosylation / invasion / lectin / migration)

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Abstract. The Rho GTPase Rac1 is a multifunctional protein working through different effector pathways. The emerging physiological significance of glycan-lectin recognition gives reason to testing the possibility for an influence of modulation of Rac1 expression on these molecular aspects. Using human colon adenocarcinoma (SW620) cells genetically engineered for its up- and down-regulation (Rac1⁺ and Rac1⁻ cells) along with wild-type and mock-transfected control cells, the questions are addressed whether the presence of adhesion/growth-regulatory galectins and distinct aspects of cell surface glycosylation are affected. Proceeding from RT-PCR data to Western blotting after two-dimensional gel electrophoresis and flow cytometry with non-crossreactive antibodies against six members of this lectin family (i.e. galectins-1, -3, -4, -7, -8 and -9), a reduced extent of the presence of galectins-1, -7 and -9 was

revealed in the case of Rac1⁻ cells. Application of these six galectins as probes to determination of cell reactivity for human lectins yielded relative increases in surface labelling of Rac1⁻ cells with galectins-1, -3 and -7. Examining distinct aspects of cell surface glycosylation with a panel of 14 plant/fungal lectins disclosed a decrease in α 2,6-sialylation of N-glycans and an increase in PNA-reactive sites (i.e. non-sialylated core 1 O-glycans), two alterations known to favour reactivity for galectins-1 and -3. Thus, manipulation of Rac1 expression selectively affects the expression pattern within the galectin network at the level of proteins and distinct aspects of cell surface glycosylation.

Introduction

The members of the Rho family of GTPases (Rac, Rho and Cdc42) are molecular switches regulating key cellular activities, among them apoptosis/cell cycle progression and directional movements by cytoskeleton remodelling (Wherlock and Mellor, 2002; Jaffe and Hall, 2005; Heasman and Ridley, 2008). Involving the downstream effectors p21-activated kinases and c-Jun amino-terminal kinases as well as transcription factors (e.g. nuclear factor- κ B), a Rho protein such as Rac1 can also affect gene expression (Coso et al., 1995; Perona et al., 1997; Montaner et al., 1998; Burrige and Wennerberg, 2004; Heasman and Ridley, 2008; Machesky and Sansom, 2012). Owing to the emerging significance of Rac1 in clinically relevant processes, e.g. wound healing (its engineered deficiency impairing myofibroblast formation and delaying cutaneous wound closure (Liu et al., 2009)) or melanoma occurrence (Hodis et al., 2012; Krauthammer et al., 2012; for general review on malignancy, please see Gómez del Pulgar et al., 2005), delineating the routes to reach the specific end point of interest poses an attractive challenge with promising therapeutic perspective.

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Abbreviations: ConA – *Canavalia ensiformis* agglutinin, DBA – *Dolichos biflorus* agglutinin, Gal-1–9 – galectin-1–9, LCA – *Lens culinaris* agglutinin, LEA – *Lycopersicon esculentum* lectin, MAA-I – *Maackia amurensis* agglutinin I, PHA-E – *Phaseolus vulgaris* agglutinin (erythroagglutinin), PHA-L – *Phaseolus vulgaris* agglutinin (leukoagglutinin), PNA – peanut agglutinin, PSA – *Pisum sativum* agglutinin, PSL – *Polyporus squamosus* lectin, RT-PCR – reverse transcriptase-polymerase chain reaction, SNA – *Sambucus nigra* agglutinin, UEA-I – *Ulex europaeus* agglutinin I, VAA – *Viscum album* agglutinin.

Focusing on colorectal carcinoma, genetically engineering Rac1 levels in human colon adenocarcinoma (SW620) cells combined with mRNA array analysis of 1423 genes identified five types of transcript differentially regulated at $P < 0.0002$ when comparing the effects of Rac1 silencing/over-expression, especially two target genes of Wnt signalling (i.e. *NKD1* and *SI0044*) (Gómez del Pulgar et al., 2007). Since measuring tumorigenicity and mortality after orthotopic injection of stably modified lines into nude mice revealed increased Rac1 expression to enhance progression (Espina et al., 2008), detailed inspection of each of the markedly Rac1-regulated genes is warranted, prompting us to take the detected transcriptional regulation of the glycogene among this set to the level of protein presence and cell surface presentation. Here we present the follow-up analysis for galectin-1 (Gal-1; $P = 0.00016$), a member of a family of adhesion/growth-regulatory lectins (Kaltner and Gabius, 2012). These proteins, by virtue of their capacity to bind distinct carbohydrate determinants, are able to translate information encoded in the glycan chains of cellular glycoconjugates into effects (for recent reviews on different aspects of the sugar code, please see Gabius, 2009; for an introduction to glycopathology, please see Gabius and Kayser, 2014). In view of the growing awareness of the physiological potential of orchestrating lectin-glycan expression we examined this lectin family and the possibility of Rac1-dependent alteration of the cellular glycophenotype.

Gal-1 is a multifunctional protein with context-dependent activities inside (nucleus, cytoplasm) and outside (surface, matrix) cells (Smetana et al., 2013). Nuclear localization and interaction with small nuclear ribonucleoprotein particles implicates the lectin in splicing (Haudek et al., 2010; Kodet et al., 2011). Its cytoplasmic rendez-vous with oncogenic H-Ras ensures its routing as well as enhanced and prolonged Raf1 and mitogen-activated protein kinase stimulation (Elad-Sfadia et al., 2002; Rotblat et al., 2004; Villalobo et al., 2006). As a glycan-binding cell surface effector, it can trigger signalling to induce p21/p27-dependent cell cycle arrest or caspase-8-mediated anoikis in carcinoma cells (Fischer et al., 2005; André et al., 2007; Sanchez-Ruderisch et al., 2011), affect cell motility and invasion depending on the tumour cell type (Camby et al., 2002; Hittlet et al., 2003; Roda et al., 2009) and convert fibroblasts to myofibroblasts nourishing a tumour-promoting microenvironment (Dvořánková et al., 2011; Valach et al., 2012). Having mentioned the role of Rac1 in wound healing above, the Gal-1 presence and tissue reactivity to lectin were found to undergo changes during progression of this process analysed for skin and trachea (Gál et al., 2011; Grendel et al., 2012).

The SW620 cells studied here are known to express Gal-1 and other members of the galectin family, which are also present in colon carcinomas *in vivo* (André et al., 1999; Lahm et al., 2001; Nagy et al., 2003; Saal et al., 2005; Gebert et al., 2012; Dawson et al., 2013). In addition to Gal-1 monitoring by RT-PCR, two-dimen-

sional gel electrophoresis and flow cytofluorimetry, the evidence for a network gave reason to similarly study galectins-2, -3, -4, -7, -8 and -9. Considering their extracellular functionality by targeting certain glycoconjugates (glycolipids such as ganglioside GM1 or sulphatide, glycoproteins such as fibronectin or its receptor, $\alpha_5\beta_1$ -integrin) as counterreceptors, we then obtained information on the extent of cell reactivity to galectins by assessing the cells' capacity to bind Gal-1 and the other listed family members using flow cytofluorimetry. Hereby, galectin-relevant aspects of the cell surface glycome were quantitatively characterized. To broaden the comparative glycophenotyping, 14 plant/fungal lectins were similarly used as sensors to trace the Rac1-dependent impact on cell surface glycosylation (for an introduction to N- and O-glycosylation, please see Patsos and Corfield, 2009; Zuber and Roth, 2009). Of note, such changes are not only known to be able to modulate reactivity to endogenous lectins, but also glycoprotein functions and even their trafficking/stability (Gabius et al., 2011, 2012). Our experiments therefore took the previously noted Rac1 effects on gene transcription for Gal-1 to the level of the protein presence and beyond Gal-1 in this family of lectins. Moreover, they led to revealing changes in N- and mucin-type O-glycan sialylation, adding at least three galectins and two features of cell surface glycosylation to the list of cellular targets of Rac1.

Material and Methods

Cell culture

Human colon adenocarcinoma (SW620) cells were cultured in RPMI 1640 medium (Sigma, Munich, Germany) supplemented with 10% foetal calf serum (Biobrom, Berlin, Germany), 2 mM L-glutamine, 100 U/ml penicillin and 100 μ g/ml streptomycin. Mock transfection was performed using the pcDNA3.1 vector without the galectin-encoding cDNA insert (Invitrogen, Karlsruhe, Germany), setting a concentration of 1 mg/ml of neomycin (G418; Roth, Karlsruhe, Germany) during selection, lowered to 200 μ g/ml for routine culture of cell clones, as described for the Rac1-over-expressing clone (Rac1⁺) and the clone carrying the short hairpin RNA against human *rac1* (Rac1⁻) (Gómez del Pulgar et al., 2007).

RNA extraction and RT-PCR profiling

Total RNA from cell extracts was obtained by RNAeasy mini columns (Qiagen, Hilden, Germany), then checked for integrity by agarose gel electrophoresis, freed of any residual DNA contamination by treatment with RNase-free DNase I (Roche Diagnostics, Mannheim, Germany) and used as template for SuperscriptTM II RNase H⁻ Reverse Transcriptase (Invitrogen) as described (Lohr et al., 2007). PCR amplification was directed by primer sets specific for human galectins-1, -2, -3, -4, -7, -8 and -9, respectively, using *Taq* DNA

polymerase in 30 cycles as described (Lahm et al., 2001), followed by gel electrophoresis in 2% agarose at 40 V for 80 min and visualization of bands.

Two-dimensional gel electrophoresis and Western blot-based galectin detection

Samples of extracted protein were prepared and processed by isoelectric focusing (pI range: 3–10) in an IPGphor™ unit (GE Healthcare, Freiburg, Germany) at about 42 kVh, followed by further separation of the mixture of extract (glyco)proteins according to mobility in 12.5% running gel using a Hoefer SE-600 system (Hoefer, Inc., Holliston, MO), fixation of gels and electrotransfer of the (glyco)proteins to nitrocellulose as described (Purkrábková et al., 2003). Galectin identification was accomplished with non-crossreactive polyclonal anti-galectin antibodies, obtained by in-house production combined with chromatographic removal of fractions from each immunoglobulin G preparation that were cross-reactive with a second (or more) member(s) of the family, at a concentration of 1 µg/ml when using recombinant galectins (3–6 ng) or 400 µg protein per extract sample and a goat anti-rabbit immunoglobulin G-horseradish peroxidase conjugate (0.5 µg/ml; Sigma) by enhanced chemiluminescence detection, driven by a substrate mixture with 1.25 mM sodium salt of luminol in 2 ml 0.1 M Tris-HCl (pH 8.6), 0.2 ml 6.7 mM p-coumaric acid in dimethyl sulphoxide and 0.6 µl H₂O₂ (30% v/v) for 2 min at room temperature. Exposure periods of the processed blot to CL-XPosure™ X-ray film (Pierce, Bonn, Germany) were routinely set to 8 minutes.

Flow cytometric cell surface analyses

Quantitation of cell surface presentation of galectins was performed in a FACScan instrument (Becton-Dickinson, Heidelberg, Germany) with suspension samples of 4×10^5 cells. Labelling for immunodetection was performed with solutions containing non-crossreactive anti-galectin antibodies (10 µg/ml) as first-step reagents and fluorescein thiocarbamyl-labelled conjugate of goat anti-rabbit immunoglobulin G (Sigma) as described (André et al., 1999, 2007). Biotinylated plant lectins (PHA-E, PHA-L, LEA, SNA, DBA, MAA-I, PNA, jacalin and UEA-I) were from Alexis (Grünberg, Germany), biotinylated lectins from *Canavalia ensiformis* (ConA), *Lens culinaris* (LCA), *Pisum sativum* (PSA), *Polyporus squamosus* (PSL) and *Viscum album* (VAA) and biotinylated/fluorescent galectins were prepared and tested for maintained sugar specificity by a solid-phase assay using (neo)glycoconjugates for matrix establishment and cytochemical staining of specimens with known reactivity as described (André et al., 2003, 2011; Habermann et al., 2011; Schlötzer-Schrehardt et al., 2012). Fluorescent streptavidin/R-phycoerythrin conjugate (1 : 40; Sigma) was used for detection of cell-bound biotin, controls for carbohydrate-dependent binding and titrations to reach saturation were routinely performed as described (Kopitz et al., 2013).

Results

From RT-PCR profiling of galectin gene expression to cell surface presentation of galectins

The pattern of mRNA presence in the galectin network was determined with a set of seven galectin-type-specific primers pairs. Confirming previous monitoring of the SW620 (wild-type) cell line (Lahm et al., 2001), transcription of galectin genes goes beyond the most often studied genes for Gal-1 and -3 (Fig. 1). Lack of a signal in the case of Gal-2 in all four cell preparations gives an example for qualitatively different regulation within this lectin family. The mock-transfected cells presented the same pattern as the wild-type cells, as did the Rac1⁺ cells (Fig. 1). In comparison, drastic differences in expression of the genes for Gal-1, -7 and -9 were detectable after 30 cycles when testing material

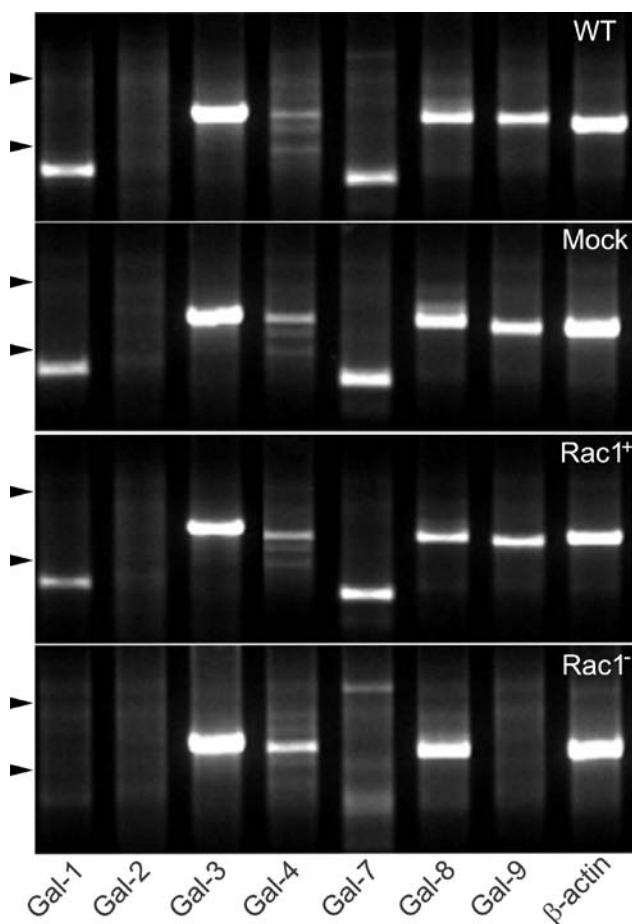


Fig. 1. Profiling of the extent of mRNA presence from transcription of galectin genes in the colon adenocarcinoma (SW620) wild-type cells and their variants. The main band in each lane represents the amplification product from RT-PCR using galectin-type-specific primer sets, consistently occurring at the position predicted for the respective cDNA. Amplification of β -actin-specific cDNA served as a loading and quality control. Calibration for length is given for 500 bp and 1000 bp by arrowheads.

from $Rac1^{-}$ cells (Fig. 1). Evidently, the microarray data on *gal1* gene transcription (Gómez del Pulgar et al., 2007) were reflected in the RT-PCR analysis. What's more, these experiments revealed an effect of silencing $Rac1$ expression posttranscriptionally on the relative abundance of mRNA presence for Gal-7 and -9. To what extent proteins were detectable in cell extracts was examined by two-dimensional gel electrophoresis combined with Western blot-based detection, with recombinant proteins as controls.

At the level of protein, data from Western blotting were fully in line with a marked reduction of the Gal-1 presence. The main spot of human Gal-1 and additional variants separated by isoelectric focusing were seen after extract processing of cells (wild-type, mock-transfected and $Rac1^{+}$) at rather similar intensity under identical conditions, whereas prolonged exposure times were required for recording the chemiluminescence signal when testing the same quantity of extracted protein from $Rac1^{-}$ cells (Fig. 2, left panel). In contrast, extents of Gal-3 presence (the main spot is protein without phosphorylation, the minor spot at lower pI value protein with Ser phosphorylation; please see Hamann et al., 1991 for its detection in human SL66 fibroblasts) appeared rather similar for all cell types, similarly as the respective RT-PCR signals (Fig. 2, right panel). Maintaining identical conditions, no respective signal for phosphorylation of Gal-3 in $Rac1^{-}$ cells was recorded. Performing the immunodetection protocol after Western blotting for Gal-4, -7, -8 and -9 consistently disclosed medium to weak signals for Gal-8 and Gal-4, respectively, a marked difference in the case of Gal-7, as seen for Gal-1, and a slight difference for Gal-9, with $Rac1^{-}$ cells harbouring less galectin than the other cell preparations (not shown).

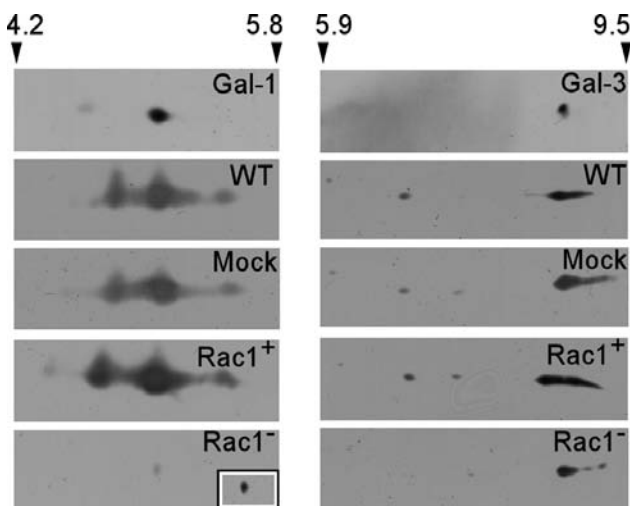


Fig. 2. Protein pattern after two-dimensional gel electrophoretic analysis combined with Western blot-based detection of recombinant human galectins (Gal-1 at 3 ng, Gal-3 at 6 ng) and their presence in extracts (400 μ g protein) of wild-type (WT), mock-transfected (Mock), $Rac1^{+}$ and $Rac1^{-}$ cells. Inset: exposure time extended from routinely used 8 to 16 min to trace positivity.

When looking at the cell surface presentation of galectin by cytofluorimetry, special care was exercised to monitoring all four types of SW620 cells with aliquots of the same antibody-containing solutions under identical culture parameters, analysing the suspensions always in parallel, each cell batch repeatedly and several preparations from different cell stocks, this to ensure reproducibility within the limits of accuracy of the method. The availability of non-crossreactive antibodies excluded ambiguities in interpretation of signals. Titrations of antibody concentration were done for each galectin and cell type to find out the linear range for the relationship of antibody concentration to the extent of positivity, where comparative measurements will be most sensitive for spotting differences.

The assessment of quantitative data on the Gal-1 cell surface presence broadened the range of the impact of

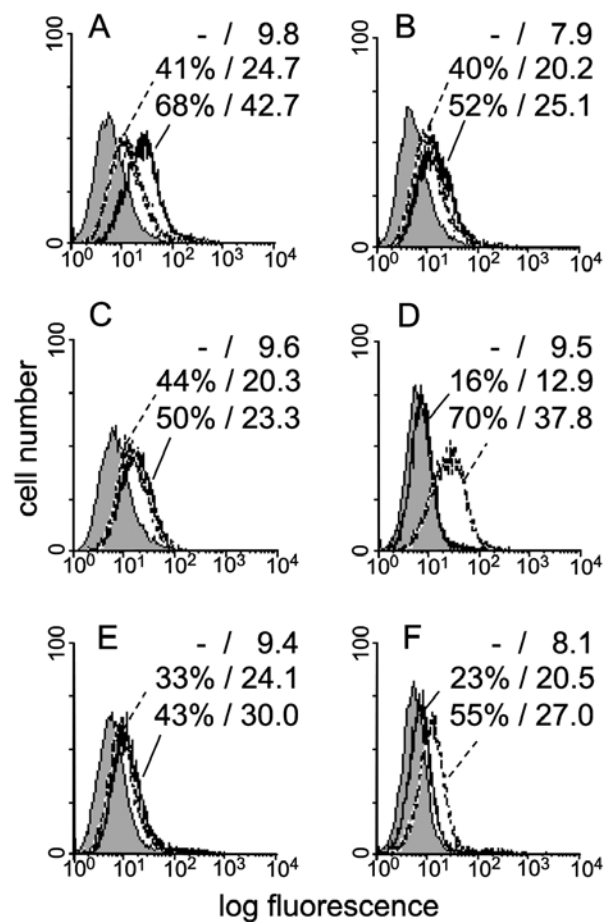


Fig. 3. Cytofluorimetric analysis of cell surface presentation of Gal-1 (solid line) and Gal-3 (dashed line; A-D) as well as Gal-8 (dashed line) and Gal-9 (solid line; E, F) using a two-step procedure with specific non-crossreactive polyclonal rabbit antibodies (10 μ g/ml) and fluorescent second-step (goat anti-rabbit) antibody. A, E: wild-type cells, B: mock-transfected wild-type cells, C: $Rac1^{+}$ cells, D, F: $Rac1^{-}$ cells. Quantitative data on the percentage of positive cells and mean fluorescence intensity are given in each panel. The control value representing marker-independent staining is given as shaded area.

Rac1 silencing on this parameter: Gal-1 was found on the surface of Rac1⁻ cells in a comparatively small quantity (Fig. 3A–D). The Gal-3-dependent signals were rather equal, with a tendency for up-regulation on Rac1⁻ cells (Fig. 3A–D). Whereas the surface presence of Gal-4 and -7 was barely tracked down, Gal-8 and -9 were detectable, and this with differences. Less Gal-9 but more Gal-8 was seen on Rac1⁻ cells (Fig. 3E, F). Viewed from the perspective of a network, Rac1⁻ cells were delineated to have lower Gal-1 and -9 cell surface presence than the other SW620 cell clones, while the level of Gal-8 appeared to be increased. Obviously, a decrease in the Rac1 presence has an impact on several galectins. This effect was seen on the levels of total protein (Western blot) and surface presentation (cytofluorimetry), the latter parameter relevant for glycan-dependent activities in cell death, invasion or migration. In order to trigger respective signalling cascades, suited cell surface glycans that act as counterreceptors for galectins will be essential. This prerequisite prompted us to perform glycophenotyping with human galectins as a tool. Cell positivity was assumed to be a measure of

reactivity to galectin exposure. The panel of probes towards this end was extended from galectins by 14 plant and fungal lectins. They were selected for their capacity to monitor distinct aspects of glycosylation.

Glycophenotyping of cell surfaces by human and plant/fungal lectins

Routinely, comparative monitoring started with measuring the concentration dependence of surface staining and the effect of cognate sugar on signal generation, as illustrated for a galectin (Gal-3) and a plant lectin (VAA) in Fig. 4. The presence of haptenic sugar nearly completely blocked binding, ascertaining the assumed carbohydrate dependence of binding. To exclude an osmolarity effect by the sugar presence, parallel experiments with 20 mM mannose (in these cases) were performed, with no effect on staining. As usual in cytofluorimetry, binding data were reported as percentage of positive cells/mean fluorescence intensity, these numbers inserted to each panel of respective figures (Figs. 4–7). For each lectin, assays were carried out simultaneously with aliquots of lectin-containing solutions (at the same concentration) and of cell suspensions, for comparison of at least two lectin concentrations below the saturation level. Sets of scans for the four cell types were thus obtained, as exemplarily documented for VAA in Fig. 5. Routinely, they were collected for the same culture batch at consecutive days and for several preparations of different cell stocks as noted above. In this figure (for VAA), a higher reactivity of Rac1⁻ cells for the galactoside-specific plant lectin was consistently

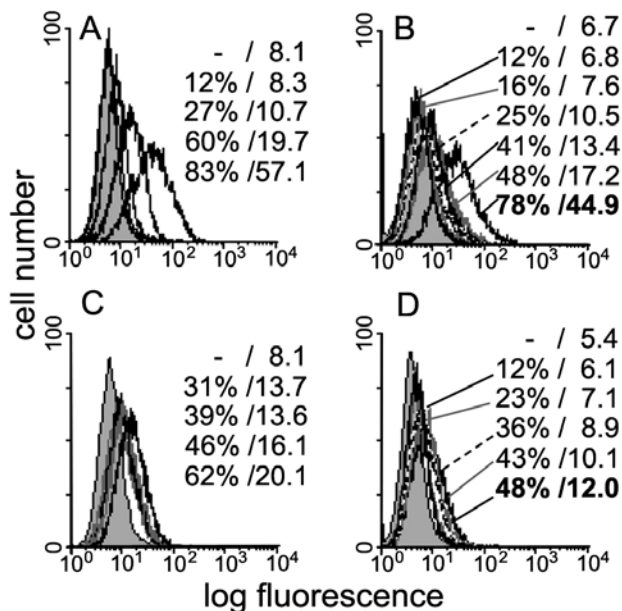


Fig. 4. Cytofluorimetric analysis of cell surface staining by labelled lectins (A, B: Gal-3, C, D: VAA). The control value representing lectin-independent staining is given as shaded area, quantitative data on the percentage of positive cells/mean fluorescence intensity are presented for each scan profile in the order of listing the respective series of concentrations (from top to bottom). A, C: staining parameters of Rac1⁻ cells measured with increasing concentrations of labelled lectin from 0.5 μ g/ml to 1 μ g/ml, 2 μ g/ml and 4 μ g/ml; B: inhibition of Gal-3-dependent staining of wild-type cells by lactose concentrations of 20 mM, 5 mM, 2 mM, 1 mM and 0.5 mM (at 4 μ g/ml Gal-3); D: inhibition of VAA-dependent staining of Rac1⁻ cells by lactose concentrations of 20 mM, 5 mM, 2 mM and 0.5 mM (at 2 μ g/ml VAA). The 100% values (in the absence of inhibitor) are printed in bold (B, D).

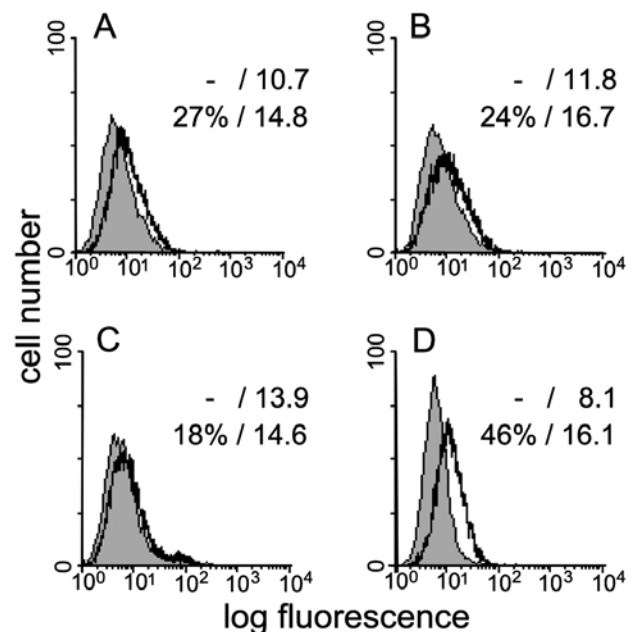


Fig. 5. Comparative cytofluorimetric analysis of surface staining by labelled VAA (at 2 μ g/ml) of wild-type (A), mock-transfected wild-type (B), Rac1⁺ (C) and Rac1⁻ (D) cells (scan profile for Rac1⁻ cells in panel D is part of the concentration series presented in Fig. 4C).

detected than for the other three test lines (please note relatively higher background levels for mean fluorescence intensity in these lines relative to Rac1⁻ cells).

Systematic application of labelled galectins to the four types of SW620 cells traced differences. Like Gal-3, Gal-1 (and also Gal-7) was most reactive with Rac1⁻ and least reactive with Rac1⁺ cells (Fig. 6). In contrast, the binding profiles for Gal-4, -8 and -9 were rather similar (not shown), indicating non-uniform target selection on SW620 cells among galectins. Viewed together with the data presented in Fig. 5 on VAA-dependent staining, increased presentation of β -galactosides reac-

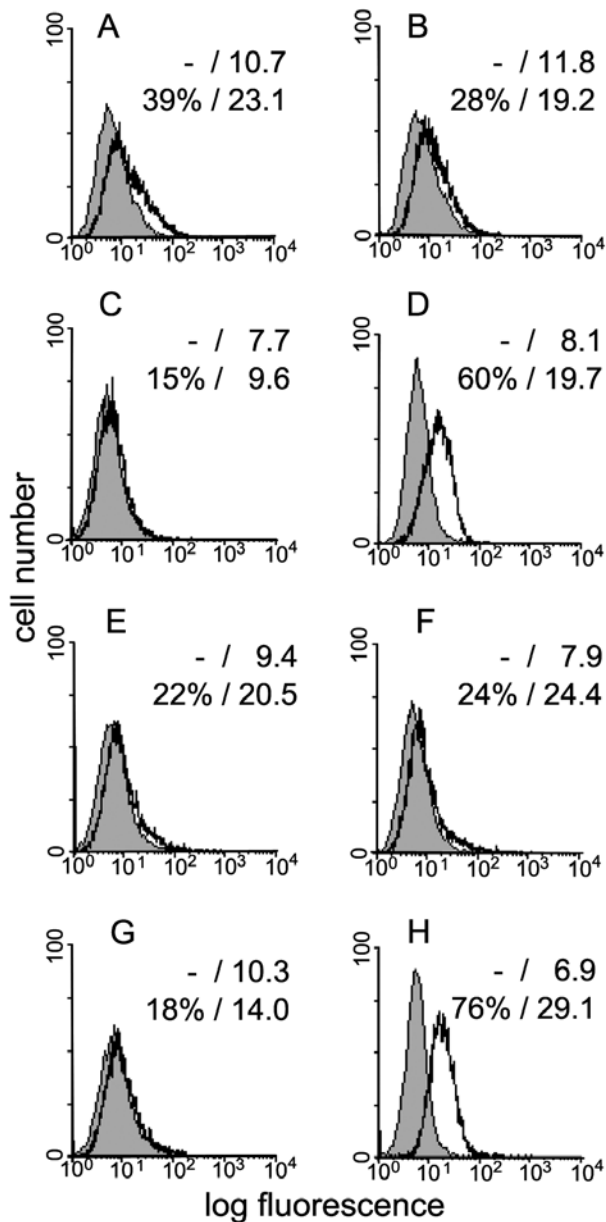


Fig. 6. Comparative cytofluorimetric analysis of surface staining by labelled Gal-3 (A-D; at 2 μ g/ml) and Gal-1 (E-H; at 20 μ g/ml) of wild-type cells (A, E), mock-transfected wild-type (B, F) Rac1⁺ (C, G) and Rac1⁻ (D, H) cells (scan profile for Gal-3-dependent staining of Rac1⁻ cells in panel D is part of the concentration series presented in Fig. 4A).

tive with galectins-1, -3 and -7 characterized Rac1⁻ cells. Which shifts within cell surface glycosylation may explain the increased galectin reactivity and whether other aspects of the glycome were altered was studied by an extension of the panel of probes from human galectins to 14 plant/fungal lectins. In detail, glycan characteristics monitored were the N-glycan core (without/with core fucosylation) by ConA, PSA and LCA, the core substitution by bisecting N-acetylglucosamine (PHA-E), β 1,6-branching (PHA-L), extension of branches by N-acetylglucosamine repeats (LEA), α 2,3/6-sialylation (MAA-I, PSL, SNA), T_n antigen (DBA), mucin-type core 1 O-glycan disaccharide (without/with sialylation) by PNA and jacalin as well as histo-blood group H(O) fucosylation (UEA-I).

The majority of the probes bound to the cells in a comparable manner. Tests were routinely run at different lectin concentrations to protect us against missing a difference. These were reproducibly apparent in the comparative measurements for three lectins. As documented in Fig. 7A-J, the SW620 cell types differed in α 2,6-sialylation of N-glycans (independently detected by two probes (PSL, SNA) and excluding a contribution of clustered T_n antigen to SNA-dependent staining by measuring a low level of DBA reactivity in all four cell types; please see Table 1 for details on glycan specificities of the mentioned lectins) but not α 2,3-sialylation (monitored by MAA-I, also by Gal-8 mentioned above). As a second discriminatory feature, the extent of presentation of non-sialylated core 1 (PNA-reactive) disaccharide was disparate. The extent of jacalin staining, an indicator for additional presence of sialylated core 1 O-glycans (Table 1), was uniform, shown for Rac1⁺ and Rac1⁻ cells in Fig. 7K, L. Evidently, the presence of jacalin-reactive core 1 O-glycans (the disaccharide and its α 2,3-sialylated form) remained constant. Instead of a lack of effect or a general change in the glycome, modulation of the Rac1 level by transfection led to alterations of distinct structural features, i.e. α 2,6-sialylation of N-glycans and core 1 O-glycan disaccharide presence.

Discussion

Our study was designed to answer the questions whether modulating the Rac1 level in SW620 cells will translate into effects on i) the protein presence for adhesion/growth-regulatory Gal-1, taking the next step after detecting the wide difference between Rac1⁺ and Rac1⁻ cells by mRNA-based array analysis (Gómez del Pulgar et al., 2007), and for other members of this family, ii) the surface presentation of the galectins and iii) cell surface glycosylation. Indeed, marked down-regulation of Gal-1-specific mRNA presence, confirmed by RT-PCR data, accounted for the lowered protein level measured by Western blotting. The obtained protein spots in two-dimensional gel electrophoretic analysis formed the common pattern of variants with differences in the isoelectric point known from human Gal-1 (Avellana-Adalid et al., 1992). No evidence for phosphorylation, Gal-1

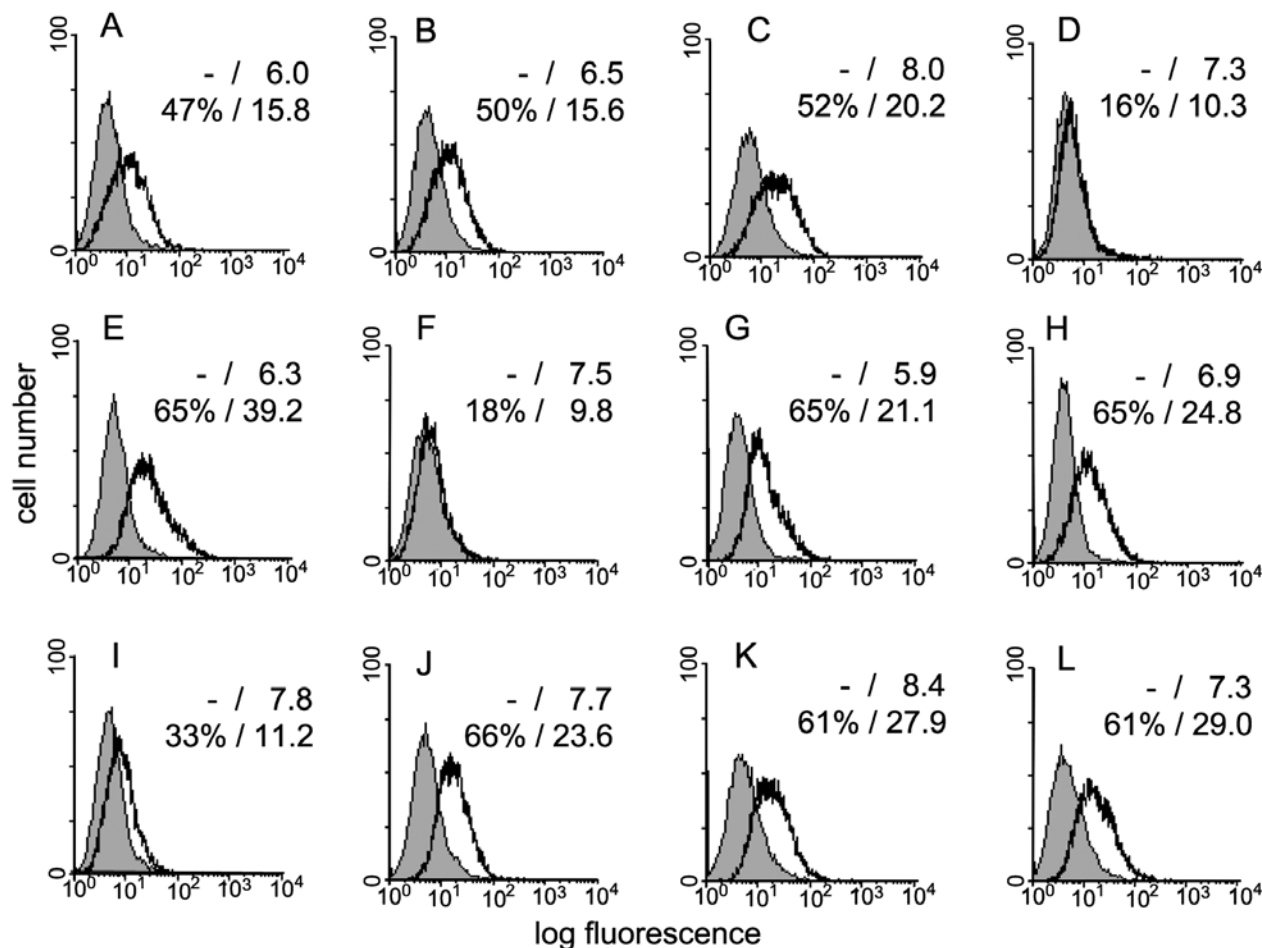


Fig. 7. Comparative cytofluorimetric analysis of surface staining by labelled PSL (A-D; at 0.25 $\mu\text{g/ml}$), SNA (E, F; at 5 $\mu\text{g/ml}$), MAA-I (G, H; at 20 $\mu\text{g/ml}$), PNA (I, J; at 10 $\mu\text{g/ml}$) and jacalin (K, L; at 5 $\mu\text{g/ml}$) for wild-type (A, G), mock-transfected wild-type (B), Rac1⁺ (C, E, I, K) and Rac1⁻ (D, F, H, J, L) cells.

Table 1. Plant/fungal agglutinins of special relevance for glycophenotyping of SW620 wild-type cells and their transfectants

| Species | Abbreviation | Monosaccharide specificity | Potent glycan ligands |
|---|---------------|----------------------------|---|
| <i>Viscum album</i> (mistletoe) | VAA | Gal | Gal β 2(3)Gal, Gal α 3(4)Gal, Gal β 3(4)GlcNAc without/with α 2,6-sialylation, Fuca2Gal |
| <i>Polyporus squamosus</i> (polypore mushroom) | PSL | ^a | Neu5Ac α 6Gal β 4Glc(NAc) (over 300-fold more active than LacNAc, not reactive with free Neu5Ac); 6'-sulphation tolerated; 6'-sialyl T _n not reactive |
| <i>Sambucus nigra</i> (elderberry) | SNA | Gal/GalNAc | Neu5Ac/Gc α 6Gal/GalNAc ^b ; 9'-O-acetylation tolerated; clustered T _n antigen |
| <i>Dolichos biflorus</i> (horse gram) | DBA | GalNAc | GalNAc α 3GalNAc α 3Gal β 4Gal β 4Glc; clustered T _n antigen |
| <i>Maackia amurensis</i> agglutinin-I (leukoagglutinin) | MAA-I | ^a | Neu5Ac/Gc α 3Gal β 4GlcNAc/Glc ^c ; 3'-sulphation instead of sialylation and 9'-O-acetylation tolerated |
| <i>Arachis hypogaea</i> (peanut) | PNA | Gal | Gal β 3GalNAc α / β |
| <i>Artocarpus integrifolia</i> (jack fruit) | jacalin (JAC) | Gal/GalNAc | Gal β 3GalNAc α ; sialylation of T/T _n antigens tolerated |

^a no monosaccharide known as ligand; ^b binding of type I LacNAc (Gal β 3GlcNAc) core preferred, 6'-sulphation of GlcNAc in α 2,6-sialylated LacNAc (type I/II) enhances affinity (Chokhawala et al., 2008); ^c binding specific for type II LacNAc (Gal β 4GlcNAc) core (Knibbs et al., 1991)

being a substrate for protein kinase CK1 (Kübler et al., 2014), was obtained. Regarding this type of post-translational galectin modification, Gal-3 proved to be the only positive case. Its extent of phosphorylation, which

is connected to recognition processes within nuclear export and association with distinct glycoproteins (Hamann et al., 1991; Díez-Revuelta et al., 2010), was reduced in Rac1⁻ cells.

Flow cytometry took the characterization of effects to the level of the cell surface, where Gal-1 was much less present in Rac1⁻ cells than in the other three cell forms. Beyond Gal-1, a decrease of the protein level was also detected for Gal-7 and -9 (as seen in RT-PCR analyses), and cell surface availability also lowered for Gal-9 but increased for Gal-8. This galectin is known for reducing migration of colon cancer cells with low growth rate *in vivo* (HCT-15, CoLo201) (Nagy et al., 2002). Overall, the presented set of results underscores the importance of extending measurements from individual family members to other constituents of the network. In fact, the potential for functional antagonism/synergy and for compensatory regulation upon an induced loss, an idea inspiring a promising research route, calls for such fingerprinting, especially if not all galectin genes are represented on the arrays. Immunohistochemically, galectin fingerprinting had proved characteristic expression profiles for individual galectins and indicated diagnostic applicability, recently documented in head and neck tumours with focus on squamous epithelium (Čada et al., 2009; Cludts et al., 2009; Saussez et al., 2009; Rimmelink et al., 2011; Fik et al., 2013). Cell biologically, the tempting hypothesis for functional antagonism in growth regulation had been substantiated for Gal-1 and Gal-3, the chimera-type protein competing with Gal-1 for counterreceptor binding (Kopitz et al., 2001; Sanchez-Ruderisch et al., 2010).

Precedents for transcriptional regulation of *gal1* or *gal7* (also called p53-induced gene 1), respectively, are given by the tumour suppressors p16^{INK4a} and p53 (Polyak et al., 1997; André et al., 2007; Barkan et al., 2013). Of note, the orchestration of galectin expression by p16^{INK4a} in pancreatic adenocarcinoma (Capan-1) cells, one factor to increase susceptibility for anoikis induction, entailed reduction of stability of the anti-apoptotic Gal-3 protein (Sanchez-Ruderisch et al., 2010). Five lines of evidence have prompted us to proceed from looking at the galectin network to monitoring the cell surface glycophenotype in this cell system:

- i) the tumor suppressor p16^{INK4a}, acting as master regulator, not only reprograms the galectin availability (protein presence, cell surface presentation) but also directs remodelling of cell surface glycosylation by lowering sialic acid biosynthesis to make cells more reactive to Gal-1, hereby furthering its capacity to induce anoikis by $\alpha_5\beta_1$ -integrin cross-linking and caspase-8 activation (André et al., 2007; Amano et al., 2012).
- ii) ectopic expression of the Rho GTPase oncogene *ras* potently induces $\alpha_2,6$ -sialyltransferase expression to enhance $\alpha_2,6$ -sialylation in fibroblasts and HD3 colonocytes (Bolscher et al., 1988; Le Marer et al., 1992; Vandamme et al., 1992; Seales et al., 2003; Dalziel et al., 2004).
- iii) reconstitution of the status of three distinct tumour suppressors in a microsatellite-unstable colon cancer cell line (HCT116), up-regulation of caveolin-1, a cell-context-dependent oncogene/suppressor, and

Wnt signalling activation are documented to affect distinct aspects of the glycophenotype, prominently among them $\alpha_2,6$ -sialylation (Patsos et al., 2009; Sengupta et al., 2010; Yu et al., 2012).

- iv) ensuing changes of the glycophenotype such as altered $\alpha_2,6$ -sialylation of N-glycans (here β_1 -integrin has been shown to be among the targets, with implications for its *cis/trans* activities; for review, please see Gu et al., 2009) make their presence felt in cell biological assays, e.g. for colony formation, invasiveness, matrix attachment, and motility of colon cancer and epithelial (SW48) cells (Dall'Olio et al., 1991; Zhu et al., 2001; Seales et al., 2005; Shaikh et al., 2008).
- v) cell membrane staining of clinical specimens of advanced head and neck (oropharyngeal and laryngeal) cancer (stages III/IV) with labelled Gal-3 proved to be an independent (favourable) prognostic indicator ($P = 0.026$; Plzák et al., 2004).

Our decision to focus on cell surface glycophenotyping by flow cytometry but not RT-PCR or microarray monitoring of glycogene expression was based on the fact that SW620 cells are an intriguing case for the scenario that “an average level of enzyme activity (here $\alpha_2,6$ -sialyltransferase) is supported by extremely weak mRNA expression” (Dall'Olio et al., 1999). Moreover, the accessibility of glycan determinants to the probes indicates bio-availability, a prerequisite to serving as ligand in biorecognition.

The cytofluorimetric monitoring revealed a conspicuous increase in cell reactivity to Gal-1, -3 and -7 in Rac1⁻ relative to Rac1⁺ cells. On average, especially the percentage of positive cells went up in comparative measurements. However, cells did not become more reactive to all tested galectins. Interestingly, the tandem-repeat-type Gal-4, -8 and -9 bound to cells of the same preparation, assays run in parallel, in similar extents. This result underscores intra-family differences, which preclude simple extrapolations. More than one biochemical route can explain this result. In principle, either the reduced Gal-1 presence may unmask sites, or a glycan remodelling may let cells reach the same end point. Such shifts in the glycome can be revealed by the same technical approach. Among the glycan characteristics examined by the 14 plant/fungal lectins, a decrease of $\alpha_2,6$ -sialylation (but not $\alpha_2,3$ -sialylation) of N-glycans and an increase of non-sialylated core 1 disaccharide presentation (reactive with PNA) were detected. Although the molecular mechanisms leading to manifestation of these alterations are presently undefined (regulatory processes at the level of $\alpha_2,6$ -sialyltransferase-I, of enzyme(s) in the pathway of sialic acid biosynthesis or of both are candidates for remodelling this aspect of N-glycosylation; affecting sialidase, core 1-specific $\alpha_2,3$ -sialyltransferases, *de novo* synthesis of the core 1 disaccharide or a combination thereof are possibilities for remodelling this aspect of O-glycosylation), it is in this context salient to note that structural changes in both respects can favour binding of

Gal-1, -3 and -7 (Wasano and Hirakawa, 1997; Ahmad et al., 2004; André et al., 2007; Krzeminski et al., 2011). Evidently, dynamic restructuring within the glycome can at least contribute to the effect measured by applying labelled Gal-1, -3 and -7.

Looking at the data of glycophenotyping with PNA/jacalin, rather constant jacalin reactivity, as documented in Fig. 7K, L, appears to argue in favour of a shift in the population of core 1 O-glycans from the sialylated trisaccharide to the non-sialylated disaccharide by a sialidase-dependent reaction or by reduced core 1 disaccharide α 2,3-sialylation during *de novo* synthesis. In both instances, the sum of jacalin-reactive core 1 di- and trisaccharides stays constant. In turn, the extent of PNA-reactive disaccharide increases. Bringing modulation of sialidase activity into play, it is known to be the cause for enhanced Gal-1 binding on activated effector T cells and on neuroblastoma (SK-N-MC) cells via stimulated conversion of ganglioside GD1a to the Gal-1 counter-receptor GM1 (Kopitz et al., 1998; Wang et al., 2009; Wu et al., 2011; Ledeen et al., 2012). Case studies on activated T cells with PNA^{high} phenotype furnish precedents for the *de novo* route (see Amado et al., 2004 and references cited). Cell biologically, the increased PNA reactivity was achieved in colon adenocarcinoma (HT-29) cells by over-expressing sialidase NEU1, β_4 -integrin as major target glycoprotein of PNA in lectin blots, and this parameter change went along with suppressed cell migration, invasion and adhesion *in vitro* (Uemura et al., 2009). The selective nature of these two Rac1-dependent changes on N- and O-glycosylation is underlined by the series of monitored features found to be unaffected such as the N-glycan core substitutions.

In summary, the presented data disclosed a marked influence of the Rac1 expression level on distinct galectins and particular characteristics of the cell surface glycophenotype. Thus, affecting galectin/glycan-mediated interactions and signalling as well as enabling further functional consequences by changes of glycosylation should now be added to the potential effector mechanisms of Rac1, giving further work a clear direction.

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