Galectin-1 Interacts with the α5β1 Fibronectin Receptor to Restrict Carcinoma Cell Growth via Induction of p21 and p27*

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Surface binding of galectin family members has the potential to link distinct glycan structures to growth regulation. Therefore, we addressed the antiproliferative potential of galectin-1 (Gal-1) in a panel of carcinoma cell lines. We discovered growth inhibition by Gal-1 in epithelial tumor cell lines from different origins and provide evidence that this effect requires functional interaction with the α5β1 integrin. Antiproliferative effects result from inhibition of the Ras-MEK-ERK pathway and consecutive transcriptional induction of p27. We have further identified two Sp1-binding sites in the p27 promoter as crucial for Gal-1 responsiveness. Inhibition of the Ras-MEK-ERK cascade by Gal-1 increased Sp1 transactivation and DNA binding due to reduced threonine phosphorylation of Sp1. Furthermore, Gal-1 induced p21 transcription and selectively increased p27 protein stability. Gal-1-mediated accumulation of p27 and p21 inhibited cyclin-dependent kinase activity and ultimately resulted in G1 cell cycle arrest and growth inhibition. These data define a novel mechanism whereby Gal-1 regulates epithelial tumor cell homeostasis via carbohydrate-dependent interaction with the α5β1 integrin.

Galectin-1 (Gal-1)† belongs to the galectin family of endogenous lectins characterized by their specificity for β-galactosides, conservation of distinct amino acids responsible for ligand contact, and jelly roll-like folding (1–3). It interacts with carbohydrate moieties of extracellular matrix components, membrane glycoproteins, and lipids such as laminin, fibronectin, carcinoembryonic antigen, and ganglioside GM1 (4, 5). Despite the abundance of β-galactosides in cellular glycoconjugates, Gal-1 binding is restricted to a limited and specific set of interaction partners, likely because of an extended binding site that accommodates more than the central galactose (6, 7). As Gal-1 is a homodimer in solution, it is suited for cross-linking and capable of forming lattice-like structures with distinct glycans, a process assumed essential for signaling after clustering of cell surface glycoprotein receptors (8).

Studies on activated T-cells have set a precedent that Gal-1 can serve as growth regulator via induction of apoptosis (9). Its widespread differential expression in malignant cells and their non-transformed counterparts raises the possibility that Gal-1 also functions as a modulator of proliferation in other cell types, such as epithelial cells. Indeed, growth inhibition of tumor cells by Gal-1 has been reported (10, 11). Of note, these growth effects were unaffected by the presence of a glycan inhibitor (9) or not specified with respect to glycan inhibitors (11), whereas the proapoptotic action of galectins on activated T-cells clearly depended on β-galactoside-specific binding (12).

Galectins have been suggested to exert their biological effects in part through interaction with integrins (5, 13), although this interaction is still poorly understood in the case of Gal-1. Integrins provide adhesion to the appropriate extracellular matrix, a central requirement for the proliferation of epithelial cells (14, 15). As heterodimeric transmembrane receptors, integrins recognize and bind extracellular matrix ligands and thereby control the organization of the intracellular actin cytoskeleton (16, 17). In addition to their adhesive function, integrins also initiate and modulate signal transduction cascades. Both ligand occupancy and integrin clustering are critical for the activation of integrins and trigger intracellular signaling cascades (17). Typically, integrins connect to intracellular signaling networks through recruitment, assembly, and activation of other signaling proteins in a bidirectional manner. Thus, integrin signaling converges with other signaling pathways to control central cellular processes. This cooperative signaling from integrins and other environmental cues such as soluble growth factors and cytokines is mandatory for orderly cell cycle progression (18, 19) and involves multiple mechanisms, among them induction of cyclins D and A as well as sequestration and down-regulation of the cyclin-dependent kinase inhibitors (CKI) p21 and p27 (18). Integrins control these events in part via modulation of growth-regulatory signaling pathways such as the canonical Ras-Raf-MEK-ERK cascade (20). Based on (i) the ability of Gal-1 to regulate growth in activated T-cells and (ii) its carbohydrate-specific interaction with cell surface receptors, we explored the ability of Gal-1 to inhibit cell growth in epithelial tumor cell lines of different origin and delineated the underlying signal transduction events in unprecedented detail.

MATERIALS AND METHODS

The following reagents were purchased: RPMI 1640 and Dulbecco’s modified Eagle’s medium from Invitrogen; fetal calf serum, trypsin/EDTA, penicillin, and streptomycin from Biochrom (Berlin, Germany);...
polyvinylidene difluoride membranes and Renaissance chemiluminescence detection reagent from PerkinElmer Life Sciences; [γ-32P]ATP from Amersham Biosciences; Raf-1 Raf-binding domain-agarose from Upstate Biotechnologies (Hamburg, Germany); effective transfection reagent from Qiagen (Hilden, Germany); and fibronectin from EMP Genetech (Denzlingen, Germany). Antibodies used for immunoprecipitation and immunoblotting were from the following sources: mouse antibodies to α5, β1, cyclin E, cyclin D, p21, p27, and Sp1 from BD Transduction Laboratories (Heidelberg, Germany); rabbit antibodies to Cdk2, Cdk4, and Sp3, mouse anti-Cdc25A and anti-phosphotyrosine, and goat anti-Sp1 from Santa Cruz Biotechnology Inc. (Santa Cruz, CA); mouse anti-cyclin A from Upstate Biotechnology (Lake Placid, NY); mouse antibodies to phospho-α5 in cell signaling (Beverly, MA); mouse anti-pan-Ras and anti-p21 from Oncogene (Darmstadt, Germany). All secondary antibodies were from DianoVA (Hamburg, Germany). Antibodies used for flow cytometry and supershift assays were as follows: mouse antibodies to α1, α2, α3, α4, α6, and αv subunits and goat anti-α5β1 from Chemicon (Hofheim, Germany); mouse anti-α5 R-phycocerythrin and anti-β1 R-phycocerythrin from Cymbus Biotechnology (Hampshire, UK); secondary goat anti-mouse fluorescein isothiocyanate-labeled antibody from Sigma. Phorbol 12-myristate 13-acetate and PD 98059 were obtained from Merck Biosciences GmbH (Bad Soden, Germany).

Cell Culture—The human hepatoma cell lines HepG2 and SK-Hep-1, colonic carcinoma cell lines HT-29 and Caco-2, breast carcinoma cell line T-47D, and ovarian carcinoma cell line OV90 were obtained from the American Type Culture Collection (ATCC, Manassas, VA) and cultured as recommended. The melanoma cell line SK-Mel-13 was from J. Eberle (Charité-Universitätsmedizin, Berlin, Germany). BON cells were a generous gift from C. M. Townsend (Galveston, TX) and were cultured as recommended. The human fibroblast cell line 5F2 from the American Type Culture Collection (ATCC, Manassas, VA) was used to generate recombinant protein. 

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DNA Constructs and Reporter Gene Plasmids—The following plasmids were used: 5′-deletion luciferase constructs of the human p27 promoter (terms given in parenthesis represent the terms used in the current study) p27PF (−3568–p27-luc), p27ApA1 (−774–p27-luc), p27ApB1 (−571–p27-luc), p27ApC1 (−549–p27-luc), p27ApD1 (−511–p27-luc), p27 ApE1 (−462–p27-luc), p27MB 435 (−435–p27-luc), p27mSp1–1 (p27mSp-I), p27mSp1–2 (p27mSp-II), and p27mCTF (p27mCTF) (30). Constructs used for Gal4 reporter assays were Gal4-luc (five binding sites for the yeast transcription factor Gal4 upstream of a minimal promoter linked to luciferase) and Sp1-Gal4 and Sp3-Gal4 (activator constructs containing the Gal4 DNA-binding domain linked to the Sp1 or Sp3 transcriptional domain). Expression plasmids CMV-Sp1 and CMV-Sp1 mThr453 (mThr739), in which Thr453 and Thr739 were both changed to Ala, have been described (31). The plasmids encoding constitutively active (MEK S222E) and dominant negative (MEK S222A) MEK were kindly provided by R. Seger (Weizmann Institute of Science, Rehovot, Israel) and X. F. Wang (Department of Pharmacology and Cancer Biology, Duke University, Durham, NC).
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**Transactivation Assays**—Transient transfections were performed using the eфfectene transfection reagent, according to the manufacturer’s protocol; luciferase activity was analyzed as previously described (29). To correct for transfection efficiency, a renilla luciferase construct pRL-TK (Promega) was cotransfected. All experiments were performed in hexaplicates and repeated at least three times.

**Electrophoretic Mobility Shift Assay**—Nuclear extracts were prepared using a non-ionic detergent method, and electrophoretic mobility shift assays were performed as described previously (29). In brief, equal amounts of nuclear extracts (5–10 μg) were incubated either with the radiolabeled oligonucleotides comprising the −555/−512 region (agcctcggcggggcgccgcaaccaagttgatcggg) of the human p27 promoter or the CAGA boxes-containing oligonucleotide (tgagacacacagaagacgagc-gac). For competition experiments, nuclear extracts were preincubated with 100 × molar excess of the unlabeled −555/−512 and CAGA boxes-containing oligonucleotides or the following competitor oligonucleotides: Sp1 consensus, attcgatcgggagc gac; Sp1 mutant, attcgatcgggagc gac; −555/−512 oligonucleotides containing site mutations within the distal Sp1 site mSp1, agcctcggcggggcgccgcaaccaagttgatcggg; and the proximal Sp1-binding site mSp2, agcctcggcggggcgccgcaaccaagttgatcggg. For supershift experiments, extracts were preincubated for 30 min with 2 μg of antibodies against Sp1 and Sp3 prior to the addition of radiolabeled probes.

**Statistical Analysis**—Statistical analysis was performed by two-tailed Student’s t-test for paired observations or one-way analysis of variance using GraphPad statistical software (GraphPad Software Inc., San Diego, CA). All data are expressed as mean ± S.E. unless otherwise indicated.

**RESULTS**

**Galectin-1 Inhibits Growth of Epithelial Tumor Cells in a Carbohydrate-dependent Manner**—To study growth-regulatory effects of Gal-1 on transformed epithelial cells, a panel of human carcinoma cell lines was treated with Gal-1 and cell numbers were recorded. Of eight cell lines analyzed, none revealed an increase in cell proliferation. In contrast, six were growth inhibited, and two (Caco-2 and HT-29) showed no response (Fig. 1A). Significant growth inhibition started at 25 μg/ml Gal-1 and was consistently achieved at 50 or 100 μg/ml depending on the cell line investigated. Gal-1 reduced proliferation in a time-dependent manner, as documented by growth curves of HepG2 and BON cells (Fig. 1B).

Coincubation of HepG2 cells with lactose, an inhibitor of carbohydrate-dependent Gal-1 binding, but not with sucrose (used as osmolarity control), abrogated the antiproliferative action of homodimeric Gal-1 (Fig. 1C), suggesting a carbohydrate-dependent mechanism. Because chimeric-type galectin-3, which is mostly monomeric in solution (32), and monomeric prototype galectin-5 had no effects, Gal-1-effected crosslinking of glycans could be essential for growth inhibition (Fig. 1D).

**Expression of α5β1 Integrin Is Required for Gal-1-mediated Growth Inhibition**—Exploring a potential involvement of integrins in Gal-1-mediated growth inhibition, we next characterized the expression of different integrins in Gal-1-responsive (HepG2, BON) and Gal-1-resistant (Caco-2, HT-29) cell lines by flow cytometry (Fig. 2A). All cell lines expressed α1, α2, α3, α4, αv, and β1 integrins. Remarkably, α5 was expressed in HepG2 and BON cells but was not detectable in Caco-2 and HT-29 cells. This putative relationship between α5 expression and Gal-1 responsiveness was further supported by immunoblot analyses of α5 and β1 expression in eight cell lines (Fig. 2B). Again, all Gal-1-responsive cell lines expressed α5 integrin, whereas Gal-1 resistance correlated with lack of α5 expression in HT-29 and Caco-2 cells (see also Fig. 1A).

Based on the hypothesis that Gal-1 might functionally interact with α5β1 integrin and thereby modify its signaling, we examined whether Gal-1-mediated growth inhibition could be prevented by preincubation with the α5β1 ligand fibronectin. Although fibronectin alone did not alter cell proliferation in HepG2 cells, it abrogated Gal-1-mediated inhibition of cell growth (Fig. 2C). Similarly, a neutralizing antibody to α5β1 integrin completely prevented cell growth inhibition by Gal-1 (Fig. 2D). In contrast, preincubation with either laminin or vitronectin or addition of neutralizing antibodies to α3β1 and αvβ1 integrins did not alter Gal-1-mediated growth inhibition (data not shown).

To provide conclusive evidence for a functional role of α5β1 integrin in Gal-1-mediated growth inhibition, both α5-deficient colon cancer cell lines were stably transfected with a cDNA encoding the α5 integrin subunit. Expression of α5 integrin in the transfected cells was confirmed by immunoblotting (Fig. 3A) and flow cytometry (data not shown).
predicted emergence of strong reactivity to Gal-1. Indeed, α5-transfected HT-29 (HT-29/α5) and Caco-2 (Caco-2/α5) cells bound substantially increased amounts of biotinylated Gal-1 (Gal-1-bio), relative to mock-transfected controls (Fig. 3B). Because β1 integrin surface expression remained unchanged (Caco-2/α5) or only slightly increased (HT-29α5) (data not shown), increased Gal-1 binding was unlikely to result from better access to the β1 subunit. In support of α5 integrin being a Gal-1 interaction partner, binding of labeled Gal-1 to HT-29α5 cells was substantially reduced when cells were preincubated with either fibronectin or a neutralizing antibody to α5β1 integrin (Fig. 3B). Functionally, Gal-1 treatment for 96 h resulted in significant growth inhibition in α5-transfected clones, whereas growth of mock-transfected controls was unaltered (Fig. 3C). Conversely, no Gal-1-mediated growth inhibition occurred in a subset of BON cells isolated from the parental population on the basis of low α5 integrin surface presentation, whereas...
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Gal-1 expectedly reduced cell numbers in the parental cultures (Fig. 3D). In contrast, subpopulations selected based on low α3 or low αV integrin expression did not differ from parental cells with respect to Gal-1 responsiveness (Fig. 3D). Taken together, these experiments indicated that expression of cell surface α5β1 integrin was required for Gal-1-mediated growth inhibition.

Gal-1 Delays G1 Cell Cycle Progression—To gain insight into the mechanism of Gal-1-mediated growth inhibition, we analyzed Gal-1-dependent changes in cell cycle distribution by flow cytometry. HT-29α5 cells were used throughout all further studies, because cell cycle synchronization was most reproducibly achieved in this cell system. HT-29α5 cell populations were synchronized in G0/G1 to monitor their progression through the individual cell cycle phases. Synchronization was achieved by serum starvation, which routinely retained ~90 ± 1% of cells in the G0/G1 phase (Fig. 4A). Cells were then stimulated to resume cycling by addition of fetal calf serum, and cell cycle progression was compared between Gal-1-treated cells and time-matched controls for up to 24 h.

Following release from serum starvation, control cell populations exited the G1 phase at 16 h, proceeded through the S phase, and reached G2/M by 24 h. Compared with controls, a significantly increased fraction of Gal-1-treated cells remained in the G1 phase at 16, 20, and 24 h and barely started to enter the S phase at 20 h, suggesting a G1 cell cycle delay (Fig. 4A and B). A very similar retention in the G1 fraction occurred in Gal-1-treated BON and T-47D cell populations at 20 h following release from synchronization (Fig. 4C). Of particular note, Gal-1 did not increase the fraction of cells with subdiploid DNA content, suggesting that the antiproliferative effect did not result from induction of apoptosis.

Gal-1 Inhibits Cdk2 Activity via Induction of p21 and p27—To unravel the molecular mechanisms underlying Gal-1-mediated G1 retention, we next assessed the activity of Cdk that control G1/S cell cycle progression, i.e. Cdk4 and Cdk2. Again, HT-29α5 cells were synchronized and released in the presence or absence of Gal-1. In HT-29α5 cells, basal as well as serum-stimulated Cdk4 activity was low and was not modulated by Gal-1 addition (Fig. 5A). Furthermore, we did not detect a significant Gal-1-dependent reduction of cyclin D1 or Cdk4 expression (Fig. 5A). In sharp contrast, Cdk2 activity consistently increased with G1/S progression, and this induction was substantially suppressed by Gal-1 treatment (Fig. 5B).

To further investigate the mechanism of Cdk2 inhibition by Gal-1, we analyzed main regulatory components of Cdk2 complexes, i.e. cyclin E, cyclin A, and Cdk2 (Fig. 5B). The expression of cyclin E was transiently induced in both control cell populations and Gal-1-treated cultures to a comparable extent. In contrast, the subsequent rise of cyclin A expression, which occurs in a Cdk2/cyclin-E-dependent fashion, was diminished by Gal-1 at 16 and 20 h.

We next focused on regulatory molecules capable of inhibiting Cdk2 activity and determined effects of Gal-1 on the expression of the Cdk inhibitors p21 and p27 (Fig. 5B). p21 expression was low in serum-starved cells at the time of release, rapidly increased with serum stimulation (data not shown), and subsequently remained at moderate steady-state levels. Gal-1 treatment increased the cellular p21 content relative to time-matched controls at 12, 16, and 20 h (Fig. 5B). Con-

![Image 4](https://example.com/image4.png)

**FIGURE 4.** Gal-1 delays G1/S phase progression of the cell cycle. **A,** HT-29α5 cells were synchronized in G0/G1 by serum starvation for 36 h and subsequently stimulated to re-enter the cell cycle either in the absence (control) or presence of 200 μg/ml Gal-1. Cell cycle distributions of six independent experiments were obtained by flow cytometry. The fractions of cells in G1, S, and G2/M were compared between Gal-1-treated cells and time-matched controls (A). Representative histograms. **B,** Gal-1-treated cells in G1 were expressed as mean percentage ± S.E. of time-matched control G1 populations (*, p < 0.05; **, p < 0.01). **C,** representative results from analogous cell cycle analyses conducted on BON and T-47D cells at 20 h after release from synchronization.

![Image 5](https://example.com/image5.png)

**FIGURE 5.** Gal-1 treatment inhibits Cdk2 activity via up-regulation of p21 and p27. Autoradiographs from Cdk4 (A) and Cdk2 (B) kinase assays were performed on HT-29α5 cells. Alternating lanes represent Gal-1 (200 μg/ml)-treated cells (+) and vehicle-treated controls (−) at the indicated time points following release from synchronization (0 h). Shown are representative kinase assays from three and four individual experiments. Immunoblot analyses for detection of Cdk4 and Cdk2 complex components were conducted on aliquots of whole cell lysates utilized in the respective kinase assays. C, immunoblot analyses for the CKIs p21 and p27 in Cdk2 immunoprecipitates of whole cell lysates obtained from HT-29α5 cells. An additional immunodetection was carried out for Cdk2 as control to ascertain the quality of immunoprecipitation.
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versely, p27 expression was high in serum-starved cultures and subsequently declined as control cells re-entered the cell cycle. Gal-1 treatment substantially increased the cellular p27 content relative to untreated controls (Fig. 5B). In contrast, the expression of other cell cycle-regulatory proteins such as Cdc25A phosphatase and Cdk7 (data not shown) remained unchanged, excluding unspecific effects of either Gal-1 treatment or the synchronization procedure.

The relevance of p21 and p27 for Cdk2-dependent G1 inhibition is based on their ability to bind to and inactivate Cdk2/cyclin complexes. Therefore, we determined the composition of immunoprecipitated Cdk2 complexes in cells treated as described above (Fig. 5C). Comparable amounts of Cdk2 were detected in all samples, confirming that equal quantities of Cdk2 had been precipitated. However, Cdk2 complexes from Gal-1-treated cultures contained markedly elevated amounts of p21 and p27 compared with their time-matched controls. Thus, Cdk2 inhibition resulted from increased association with p21 and p27 CKIs.

Gal-1 Increases p21 and p27 Transcription and p27 Protein Stability—Cellular CKI content is tightly controlled via regulation of both CKI transcription and CKI degradation. To elucidate which of these mech-
of Sp1 and Sp3 antibodies almost completely supershifted both complexes (lane 8). Transient transfection assays demonstrated that both GC boxes within the −549/−511 element of the human p27 promoter were required for responsiveness to Gal-1 treatment. In line with these observations, oligonucleotides containing functionally inactivated GC boxes did not inhibit Sp1 and/or Sp3 complex formation in competition experiments (lanes 11 and 12). Finally, treatment of HT-29a5 cells with Gal-1 for various periods of time revealed that Gal-1 time dependently induced the binding of Sp1 and Sp3 to the −555/−512 element of the p27 promoter (Fig. 8B, lanes 16–22). This increase in DNA binding was specific and selective, as Gal-1 treatment did not change the relative amounts of Smad transcription factors bound to the 32P-labeled CAGA boxes-containing oligonucleotide (Fig. 8B, lanes 23–25). Furthermore, Gal-1 had no effect on Sp1 and Sp3 DNA binding to the −555/−512 element of the p27 promoter in parental HT-29 cells lacking expression of α5 integrin (Fig. 8A, lanes 13–15).

Gal-1 Stimulates Sp1- and Sp3-transactivating Capacity—To assess whether enhanced DNA binding of Sp1 and Sp3 resulted from a Gal-1-induced change of cellular or nuclear Sp1 and Sp3 content, we determined protein levels of both transcription factors in whole cell lysates and nuclear extracts by immunoblotting. Compared with untreated controls, Gal-1 treatment of HT-29a5 cells up to 24 h did not change the Sp1 and/or Sp3 content of whole cell or nuclear protein lysates (Fig. 9A).

Therefore, we explored whether Gal-1 influenced the transactivation capacity of Sp1 and Sp3, using appropriate Gal4 reporter assay systems. For these experiments, we cotransfected constructs containing the transactivation domain of the Sp1 or Sp3 protein linked to the Gal4 DNA-binding domain along with a luciferase construct containing five Gal4-binding sites as a reporter (Fig. 9B). Gal-1 treatment significantly induced the transactivation capacity of both Sp1-Gal4 and Sp3-Gal4. These data confirmed the regulation of Sp1 and Sp3 by Gal-1 and suggest that, in addition to the enhanced binding of both transcription factors shown in gel shift assays, an induction of their transactivation capacity represents a regulatory mechanism through which Gal-1 stimulates Sp1/Sp3-dependent p27 transcription.

Galectin-1 Inhibits Threonine Phosphorylation of Sp1—The transactivating properties of Sp1 are modulated by several posttranslational modifications, including phosphorylation on tyrosine, serine, and threonine residues. To assess whether Gal-1-mediated stimulation of Sp1 transactivation was because of changes in the phosphorylation status, Sp1 was immunoprecipitated from Gal-1-treated cells, and the abundance of phosphotyrosine, -serine, and -threonine was determined by immunoblotting using phospho-specific antibodies (Fig. 9C). To ensure that

**FIGURE 7.** Identification and functional analysis of the Gal-1-responsive element of the p27 promoter. A, HT-29a5 cells (upper panel) or parental HT-29 cells (lower panel) were transiently transfected with sequential 5′-deletion reporter constructs of the human p27 promoter and subsequently treated with vehicle or 200 μg/ml Gal-1. Results represent luciferase activity as mean percentage ± S.E. of vehicle-treated 3568-p27-luc obtained from at least four experiments, each conducted six times (**, p < 0.01; ***, p < 0.001). B, schematic presentation of the Gal-1-responsive element −549/−511 of the p27 promoter indicating two GC-rich boxes as putative binding sites for transcription factors Sp1 and/or Sp3 and a CCAAT box. Nucleotide positions of putative binding sites are numbered according to their positions relative to the translation start site. C, HT-29a5 cells were transiently transfected with the indicated p27-luciferase reporter gene constructs encoding inactivated transcription factor-binding sites (substituted nucleotides are shown in bold letters) and treated with 200 μg/ml Gal-1 or vehicle for 48 h. Data represent the luciferase activities as the mean fold increase ± S.E. of respective vehicle-treated controls obtained from four experiments, each conducted six times (***, p < 0.001).
equal amounts of Sp1 had been precipitated, the blots were subjected to immunoblotting with an Sp1 antibody. The levels of tyrosine and serine phosphorylation of Sp1 remained unchanged up to 18 h of Gal-1 exposure. In contrast, the relative abundance of phosphothreonine was substantially reduced by Gal-1, suggesting that a Gal-1-mediated decrease in threonine phosphorylation of Sp1 accounted for the observed increase of Sp1 transactivation.

To establish the functional relevance of Gal-1-induced reduction of Sp1 threonine phosphorylation with respect to p27 regulation, the full-length p27 reporter construct was cotransfected with either wild-type Sp1 (CMV-Sp1) or Sp1 harboring a mutation at the threonine phosphorylation site (CMV-Sp1.mThr453/mThr739), and the ensuing effects of Gal-1 treatment on p27 promoter activity were determined. Upon cotransfection of wild-type Sp1, Gal-1 treatment reproduced the stimulation of the p27 promoter that was observed with endogenous Sp1. As predicted from our hypothesis, cotransfection of the threonine phosphorylation-deficient mutant per se stimulated p27 promoter activity when compared with wild-type Sp1. Importantly, p27 promoter activity had lost Gal-1 responsiveness under these conditions, supporting the functional involvement of Sp1 threonine phosphorylation in p27 regulation by Gal-1.

Gal-1 Inhibits the Ras-MEK-ERK Signaling Pathway—To link the Sp1-dependent induction of p27 to upstream signaling events initiated by Gal-1, we focused on signals that are (i) elicited or modulated by the α5β1 fibronectin receptor and (ii) capable of modifying Sp1 transcriptional activity via threonine phosphorylation. As ERK1/2 have been proposed to directly phosphorylate Sp1 on threonine residues, this profile applies to the ERK signaling module, which is also critically implicated in G1/S cell cycle progression.

Initially, ERK phosphorylation was determined using a phospho-specific ERK antibody as an indicator of kinase activity (Fig. 10A, upper panel). When synchronized cultures were analyzed, activity was low in serum-starved cells but substantially increased upon serum stimulation in control cells. In contrast, Gal-1 treatment suppressed ERK activity to the level of serum-starved controls at all time points analyzed. Gal-1-induced differences in ERK activity were due to activity rather than...
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**Gal-1 inhibits threonine phosphorylation of Sp1 and increases its transactivating capacity.**

A. Sp1 and Sp3 protein levels in whole cell lysates and nuclear extracts from Gal-1-treated HT-29 cells were analyzed by immunoblotting at the indicated time points. The Sp3 antibody binds two Sp3 isoforms, p84 and p91. B. HT-29 cells were transiently transfected with the Gal4-luc plasmid, which contains five Gal4-binding sites in front of a minimal promoter fused to the luciferase gene and constructs containing either the Sp1 (Sp1-Gal4) or Sp3 (Sp3-Gal4) transactivation domain fused to a Gal4-DNA-binding domain. Cells were subsequently incubated with 200 μg/ml Gal-1 or vehicle for 48 h, and luciferase activity was expressed as relative light units (RLU). Bars represent the mean ± S.E. of four experiments, each performed six times (*, p < 0.05). C. HT-29 cells were treated with 200 μg/ml Gal-1 for the indicated periods of time, nuclear extracts were prepared, and Sp1 was immunoprecipitated. Immunoblots with the indicated antibodies were performed to determine Gal-1-mediated changes in Sp1 phosphorylation of threonine, serine, and tyrosine residues. To ensure comparable precipitation of Sp1, membranes were subsequently stripped and reprobed with a Sp1-specific antibody. Shown are representative immunoblots of three independent experiments. D. HT-29 cells were cotransfected with plasmids encoding the 3568-p27-luc reporter construct and either wild-type Sp1 (CMV-Sp1) or mutant Sp1.mThr453/mThr739 (CMV-Sp1mut) and then treated with Gal-1 (200 μg/ml) or vehicle for 48 h before luciferase activity was determined. Data represent the luciferase activities as the mean fold increase ± S.E. of vehicle-treated wild-type Sp1-transfected cells obtained from three experiments, each conducted six times (*, p < 0.05, ns, not significant; **, p < 0.01).

Figure 9. Galectin-1 inhibits threonine phosphorylation of Sp1 and increases its transactivating capacity.

regulation of expression, because immunoblots using a regular ERK1/2 antibody documented equal cellular ERK content. Furthermore, ERK1/2 activity remained entirely unchanged when α5-deficient HT-29 cells (mock controls) were exposed to Gal-1 treatment (data not shown), suggesting that the modulation of ERK activity was due to interaction of Gal-1 with glycans of the α5β1 fibronectin receptor. In line with the inhibition of ERK1/2 activity, Gal-1 also reduced the activity of the immediate upstream ERK1/2 kinase MEK, as determined based on a reduction in the extent of phosphorylation (Fig. 10A).

To further clarify whether the Gal-1-dependent inhibition of MEK and ERK activity resulted from impaired proximal signaling input, we examined the activity of the small GTPase Ras, which represents a prototype upstream activator of ERK signaling and is also regulated by MEK-ERK signal transduction pathway. To further corroborate the functional relevance of Ras, MEK, and ERK inhibition for Gal-1-mediated p27 induction, the ability of constitutively active Ras (K-RasV12) and MEK (MEK S222E) variants to prevent the Gal-1-mediated induction of the p27 luciferase reporter construct was tested (Fig. 10E). Importantly, cotransfection of either K-RasV12 or MEK S222E constructs counteracted Gal-1-dependent p27 promoter activation but had no measurable effect on basal promoter activity. Conversely, transfection of a dominant negative MEK variant (MEK S222A), as well as the presence of PD 98059, mimicked Gal-1-mediated p27 promoter induction (Fig. 10E).

In a second approach, we explored whether inhibition of the Ras-MEK-ERK pathway also accounted for Gal-1-mediated induction of Sp1-transactivating capacity. Again, a construct containing the transactivation domain of the Sp1 protein linked to the Gal4 DNA-binding domain (Sp1-Gal4) and a luciferase construct containing five Gal4-binding sites as a reporter (Gal4-luc) were utilized (Fig. 10F). Cotransfection of either K-RasV12 or MEK S222E constructs completely blocked the Gal-1-mediated increase of Sp1 transactivation capacity. Taken together, these experiments establish Gal-1-dependent inhibition of Ras, MEK, and ERK as functionally required signaling events that link Gal-1 binding to the α5β1 fibronectin receptor on the cell
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FIGURE 10. Gal-1-stimulated p27 gene transcription requires inhibition of the Ras-MEK-ERK signal transduction pathway. A, HT-29x5 cells were treated with 200 µg/ml Gal-1 or vehicle following release from synchronization, and whole cell lysates were prepared. The extent of MEK and ERK1/2 phosphorylation was detected by immunoblotting using phospho-MEK- and phospho-ERK1/2-specific antibodies. To visualize protein amounts loaded, blots were stripped and reprobed with a non-phospho-ERK1/2-specific antibody. B, to determine Ras activity, GTP-bound active Ras was selectively precipitated in Raf-binding domain pulldown assays and subsequently detected by immunoblotting using an antibody that recognizes H-Ras, N-Ras, and K-Ras homologues. Shown are representative immunoblots of at least five independent experiments, each yielding similar results. The lower panel represents an immunoblot analysis of Ras content in the lysates before Raf-binding domain pulldown. C, HT-29x5 cells were treated with 200 µg/ml Gal-1 or the indicated concentrations of PD 98058, and then ERK1/2 phosphorylation (upper row) or expression (lower row) was detected by immunoblotting as above. D, HT-29x5 cells were treated with 20 or 50 µM PD 98058 for 16 h, and Sp1 was immunoprecipitated from nuclear extracts. Immunodetection of phosphothreonine was performed, and the blot was reprobed with a Sp1-specific antibody (upper panel). Nuclear extracts from HT-29x5 cells treated with vehicle, 200 µg/ml Gal-1, or 25 µM PD 98058 were incubated with the 32P-labeled 5’-SSS-5’-112 oligonucleotide, and the presence of Sp1- and Sp3-DNA complexes was detected in gel shift assays (middle panel). HT-29x5 cells were treated with 20 µM PD 98059 or vehicle following release from serum starvation, and protein levels of p27 in whole cell lysates were detected by immunoblotting (lower panel). Synchronized HT-29x5 cells were treated with either 200 µg/ml Gal-1 or the indicated concentrations of PD 98059 for 16 h, and the cell cycle distribution was determined (graph). Shown is the percentage of cells in the G1 phase, n = 3, E, HT-29x5 cells were cotransfected with the 3568-p27-luc plasmid and constructs encoding constitutive active Ras (K-RasV12), constitutively active MEK (MEK S222E), as well as dominant negative MEK (MEK S222A) prior to treatment with 200 µg/ml Gal-1 or MEK S222E, vehicle, F, cotransfection of HT-29x5 cells with Gal4-luc and Sp1-Gal4 plasmids, along with constructs encoding constitutively active Ras (K-RasV12) or constitutively active MEK (MEK S222E) prior to treatment with 200 µg/ml Gal-1. E and F, results were obtained from four independent experiments and represent luciferase activity as mean fold ± S.E. of vehicle-treated 3568-p27-luc (E) or Gal4-luc (F) (*, p < 0.05, ***, p < 0.001).

surface to Sp1-dependent transcriptional induction of p27 and subsequent cell cycle inhibition.

DISCUSSION

Gal-1 is a carbohydrate-dependent inducer of apoptosis in activated T-cells. The current study has extended the biological function of Gal-1 to growth control in human epithelial cancer cells, where Gal-1 acts as a modifier of integrin-dependent cell cycle regulation. In addition, we have mapped the chain of signaling events responsible for this type of Gal-1-mediated growth inhibition. Specifically, we have provided evidence that Gal-1 (i) binds to epithelial tumor cells via its lectin domain in an α5β1-dependent manner, (ii) inhibits the Ras-MEK-ERK signaling cascade, which integrates the mitogenic response to various stimuli, (iii) relieves ERK-dependent suppression of Sp1 transactivation capacity, (iv) induces Sp1-dependent p27 (and p21) gene transcription and protein stability, and (v) inhibits Cdk2 activity and subsequent G1/S cell cycle progression, which ultimately leads to growth inhibition (summarized in Fig. 11).

In detail, we have reported that Gal-1 inhibits growth in carcinoma cell lines of different origin, suggesting that the growth-suppressive function of Gal-1 remains conserved across transformed epithelial cells derived from different tissues. Of note, two cell lines were entirely resistant to Gal-1 action and thereby allowed the identification of the fibronectin receptor α5β1 as a critical mediator of Gal-1 responsiveness. Our results in α5-transfected cell lines and their α5-deficient counterparts indicated that α5 confers the ability of Gal-1 cell surface binding. Importantly, modified Gal-1 responsiveness occurred without overt changes of β1 integrin surface expression but required the presence of the α5 subunit. In view of the previously reported binding of Gal-1 to β1 integrin subunits (13), Gal-1 likely contacts both integrin subunits with the α subunit conveying specificity to the interaction in the carcinoma cell lines we investigated. This is an intriguing demonstration for selective interaction of Gal-1 with distinct target structures on epithelial tumor cells, despite the ubiquitous abundance of β-galactosides on the cell surface. Structural research will have to resolve how Gal-1 recognizes distinct target sites in complex glycans, as was accomplished recently for ganglioside Ga3, the main binding partner for growth regulation on neuroblastoma cells (7).

Both Gal-1 binding and subsequent growth inhibition were abrogated by coinubcation with fibronectin or a neutralizing α5β1 antibody, implying a functional interaction of Gal-1 with the α5β1 fibronectin receptor. In previous reports, Gal-1 was demonstrated to interact with αβ1 or α1β1 integrin in mesenchymal cells and β1 integrin in T-cells, suggesting that cell type-specific expression patterns of integrins and
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FIGURE 11. Scheme of Gal-1-mediated growth inhibition via transcriptional induction of the p27 gene promoter. Functional interaction of Gal-1 with the fibronectin receptor α5β1 inhibits the Ras-MEK-ERK signaling pathway, resulting in reduced threonine phosphorylation of Sp1, increased Sp1 transcription and DNA binding, and consecutive induction of p27 gene transcription. Accumulation of p27 inhibits Cdk2 activity and ultimately results in G1 cell cycle arrest and growth inhibition. Gal-1-mediated effects on most steps within this cascade can be either blocked (I-) or mimicked (II+) as indicated. For reasons of simplicity, Gal-1-induced p21 transcription is not included in the scheme.

In addition to stabilization, however, we have provided a second mechanism of p27 up-regulation by Gal-1, i.e. Gal-1-stimulated p27 gene transcription. Transcriptional control of p27 expression represents a novel finding within the context of Gal-1-mediated biological effects. Fittingly, a recent survey of genes differentially expressed in human umbilical vein endothelial cells upon fibronectin engagement of α5β1 integrin revealed a >3-fold reduction of p27 mRNA expression (51). These data are in intriguing agreement with the functional antagonism between Gal-1 and fibronectin observed in the current study, which results in transcriptional activation of p27.

According to this concept, the p27 promoter should be responsive to Gal-1 stimulation. This is indeed the case. Using a combination of progressive 5′-deletion analysis and systematic mutagenesis of the p27 promoter, we identified the −549/−511 region as the Gal-1-responsive element. This region harbors two GC-rich sequences that serve as consensus sites for Sp-like transcription factors. Both Sp consensus sites are required for Gal-1 responsiveness of the p27 promoter, suggesting a coordinate function. Gal-1 treatment stimulated Sp1 and Sp3 binding to this cis-regulatory element and increased Sp1/3 transcriptional capacity. Both processes were regulated in an ERK-dependent manner, such that ERK inhibition permits increased Sp1-dependent p27 transcription. Enhanced Sp1 activity did not result from increased nuclear Sp1 concentration or from changes in the Sp1/Sp3 ratio, which have both been shown to affect Sp1-dependent gene transcription (52, 53).

Their glycans contribute to differential effects of Gal-1 in mesenchymal, lymphoid, and epithelial cancer cells (35–37). In the case of human smooth muscle cells Gal-1 binding did not cross-link β1 integrin but nonetheless led to a transient increase in tyrosine phosphorylation of two cytoskeleton-associated proteins and to modulation of cell attachment, possibly due to an effect of Gal-1 on β1 conformation (13). Cytoskeletal reorganization also occurs following interaction of the tandem repeat-type lectin galectin-8 with Cytoskeletal reorganizationalsooccursfollowinginteractionofthetan-

G1-1 integrin revealed a 3-fold reduction of p27 mRNA expression (51). These data are in intriguing agreement with the functional antagonism between Gal-1 and fibronectin observed in the current study, which results in transcriptional activation of p27.

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In this context, posttranslational modifications such as O-GlcNAcylation and phosphorylation on serine and threonine (or tyrosine) residues provide dynamic and bidirectional control of Sp1 transcriptional capacity (53–55). In the current study, Gal-1 treatment did not stimulate serine or tyrosine phosphorylation to increase Sp1-dependent p27 transcription but rather reduced Sp1 phosphorylation at threonine residues. As threonine phosphorylation has been shown (i) to occur in response to ERK1/2 activation (56) and (ii) to restrain Sp1 transcriptional and DNA binding capacity (57, 58), the Gal-1-mediated reduction of Sp1 phosphothreonine content provides a plausible molecular link between ERK inhibition and transcriptional induction of p27. Furthermore, activation of Sp1 could also offer a molecular link to the transcriptional induction of p21 that occurred in response to Gal-1 treatment. In fact, the p21 promoter represents an established prototype target of Sp1-dependent gene regulation (59).

In summary, we established in the current study a novel antiproliferative mode of action for Gal-1 in epithelial tumor cells. It is based on specific interference with mitogenic erativemode of action for Gal-1 in epithelial tumor cells. It is based on Sp1-dependent generegulation (59). In addition, activation of Sp1 could also offer a molecular link to the transcriptional response to ERK1/2 activation (56) and (ii) to restrain Sp1 transcription (56). In the current study, Gal-1 treatment did not stimulate the phosphorylation of serine and threonine (or tyrosine) residues. As both Gal-1 and related tissues, our results may also delineate a pathway that controls epithelial progression and thereby favors epithelial tumor cell growth. Conversely, use of Gal-1 or Gal-1-mimetic compounds could provide an attractive novel biotherapeutic approach in cancers that have conserved expression of this fibronectin receptor.

REFERENCES
1. Gabius, H.-J. (1997) Eur. J. Biochem. 243, 543–576
2. Cooper, D. N. (2002) Biochim. Biophys. Acta 1572, 209–231
3. Lopez-Lucendo, M. F., Sols, D., Andres, S., Hirabayashi, J., Kasai, K., Kaltner, H., Gabius, H.-J., and Romero, A. (2004) J. Mol. Biol. 343, 957–970
4. Andre, S., Kojima, S., Yamazaki, N., Fink, C., Kaltner, H., Kayser, K., and Gabius, H.-J. (1999) J. Cancer Res. Clin. Oncol. 125, 461–474
5. Hughes, R. C. (2001) Biochemistry (Paris) 83, 667–676
6. Kopitz, J., von Reitzenstein, C., Burchert, M., Cantz, M., and Gabius, H.-J. (1998) J. Biol. Chem. 273, 11205–11211
7. Siebert, H. C., Andre, S., Lu, S. Y., Frank, M., Kaltner, H., van Kuik, J. A., Korchaagina, E. Y., Bovin, N., Takhorshi, E., Capite, R., Vliegenthart, J. F., de Fougerolles, A. R., Koteliansky, V., and Rosewicz, S. (2000) Eur. J. Biochem. 267, 377–390
8. von Marschall, Z., Scholz, A., Cramer, T., Schafer, G., Schirner, M., Oberg, K., Wiedenmann, B., Hocker, M., and Rosewicz, S. (1993) J. Natl. Cancer Inst. 85, 539–549
9. Hata, Y., Duh, E., Zhang, K., Robinson, G. S., and Aiello, L. P. (1998) J. Biol. Chem. 273, 14762–14773
10. Ye, X., and Liu, S. F. (2002) Int. J. Cancer 98, 1147–1149
11. Faucett, M. C. (1999) J. Immunol. 162, 799–806
12. Maeda, N., Kawaeda, N., Seki, S., Arakawa, T., Ikeda, K., Iwao, H., Okumya, H., Hiramahiro, Y., Kajibesi, K., and Yoshizato, K. (2003) J. Biol. Chem. 278, 18938–18944
13. Zhang, Y., Yamamoto, K., and Hatanaka, M. (2000) Exp. Hematol. 28, 1147–1157
14. Schuppan, D., and Oesch, P. (2000) Hepatology 32, 289–291
15. Gabius, H.-J., Siebert, H. C., Andre, S., Jime`nez-Barbero, J., and Rüdiger, H. (2004) Biochem. Biophys. Res. Commun. 310, 1003–1006
16. Ruoslahti, E. (1999) J. Biol. Chem. 274, 289–291
17. Minami, S., Ohtani-Fujita, N., Igata, E., Tamaki, T., and Sakai, T. (1997) FEBS Lett. 416, 1–6
18. Gu, M., Wang, W., Song, W. K., Cooper, D. N., and Kaufman, S. J. (1994) J. Cell Sci. 107, 175–181
19. Moiseeva, E. P., Spring, E. L., Baron, J. H., and de Bono, D. P. (1999) J. Vasc. Res. 36, 47–58
20. Sturm, A., Lensch, M., Andre, S., Kaltner, H., Wiedenmann, B., Rosewicz, S., Dignass, A. U., and Gabius, H.-J. (2004) J. Immunol. 173, 3825–3837
21. Hadari, Y. R., Arbel-Goren, R., Levy, N., Amsterdam, A., Alon, R., Zuck, R., and Zick, Y. (2000) J. Cell Sci. 113, 2385–2397
22. Levy, Y., Ronen, D., Bershadsky, A. D., and Zick, Y. (2003) J. Biol. Chem. 278, 14353–14422
23. Andre, S., Kaltner, H., Lensch, M., Russwurm, R., Siebert, H. C., Fallehreit, C., Takhorshi, E., Heck, A. J., von Knebel-Dütscher, M., Gabius, H.-J., and Kopitz, J. (2005) Int. J. Cancer 114, 56–57
24. Zhang, Z., Vuori, K., Reed, J. C., and Ruoslahti, E. (1995) Proc. Natl. Acad. Sci. U. S. A. 92, 6161–6165
25. Kopitz, J., von Reitzenstein, C., Burchert, M., Cantz, M., and Gabius, H.-J. (1998) J. Biol. Chem. 273, 11205–11211
26. Siebert, H. C., Andre, S., Lu, S. Y., Frank, M., Kaltner, H., van Kuik, J. A., Korchaagina, E. Y., Bovin, N., Takhorshi, E., Capite, R., Vliegenthart, J. F., de Lieth, C. W., de Fougerolles, A. R., Koteliansky, V., and Rosewicz, S. (2000) Eur. J. Biochem. 267, 377–390
27. von Marschall, Z., Scholz, A., Cramer, T., Schafer, G., Schirner, M., Oberg, K., Wiedenmann, B., Hocker, M., and Rosewicz, S. (1993) J. Natl. Cancer Inst. 85, 539–549