Galectin-3 regulates RasGRP4-mediated activation of N-Ras and H-Ras

Ruby Shalom-Feuerstein, Ran Levy, Victoria Makovski, Avraham Raz, Yoel Kloog

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Galectin-3 (Gal-3) is a pleiotropic β-galactoside-binding protein expressed at relatively high levels in human neoplasms. Its carbohydrate recognition domain (CRD) contains a hydrophobic pocket that can accommodate the farnesyl moiety of K-Ras. Binding of K-Ras to Gal-3 stabilizes K-Ras in its active (GTP-bound) state. Gal-3, which does not interact with N-Ras, was nevertheless shown to reduce N-Ras-GTP in BT-549 cells by an unknown mechanism that we explored here. First, comparative analysis of various cancer cell lines (glioblastomas, breast cancer cells and ovarian carcinomas) showed a positive correlation between low N-Ras-GTP/high K-Ras-GTP phenotype and Gal-3 expression levels. Next we found that epidermal growth factor-stimulated GTP loading of N-Ras, but not of K-Ras, is blocked in cells expressing high levels of Gal-3. Activation of Ras guanine nucleotide releasing proteins (RasGRPs) by phorbol 12-myristate 13-acetate (PMA) or downregulation of Gal-3 by Gal-3 shRNA increased the levels of N-Ras-GTP in Gal-3 expressing cells. We further show that the N-terminal domain of Gal-3 interacts with and inhibits RasGRP4-mediated GTP loading on N-Ras and H-Ras proteins. Growth of BT-549 cells stably expressing the Gal-3 N-terminal domain was strongly attenuated. Overall, these experiments demonstrate a new control mechanism of Ras activation in cancer cells whereby the Gal-3 N-terminal domain inhibits activation of N-Ras and H-Ras proteins.

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1. Introduction

Ras proteins act as binary switches, alternating between GDP-bound (inactive) and GTP-bound (active) states. Ras is activated by specific guanine nucleotide-exchange factors (RasGEFs) including SOS, RasGRFs and RasGRPs, and is inactivated by specific Ras GTPase-activating proteins (RasGAPs) [1–3]. Ras-GTP is activated by effectsors, which trigger a diversity of intracellular signaling networks that regulate cellular behavior [4–6]. Regulation of Ras signaling is complex and appears to involve combinations of extracellular-signal-regulated activation of Ras by RasGEFs at the plasma membrane and endomembranes [7,8] and interactions of Ras with escort proteins, including galectin-1 (Gal-1) [9–11] and galectin-3 (Gal-3) [12,13], which were originally discovered as β-galactoside-binding proteins.

Gal-3 [12,14,15] and K-Ras [4,6,15,16] are critical participants in the regulation of cell growth, differentiation, migration, and survival. Some of these common regulatory processes appear to be associated with the rather selective binding of Gal-3 to the activated form of K-Ras [12,13], the most frequently mutated Ras isoform of the three prominent H-, N- and K-Ras proteins [16,17]. Gal-3 is unique among the galectins in possessing, besides the carbohydrate recognition domain (CRD), a long proline- and glycine-rich NH2-terminal domain [18–23]. Gal-3, which is highly expressed in human neoplasms [14], is a

pleiotropic protein with activities mediated by sugar–protein interactions through the binding of Gal-3 to extracellular β-galactosides [20–23]. Other activities of Gal-3 appear to involve sugar-independent interactions with intracellular proteins such as β-catenin [15], synexin [24], and K-Ras [12,13]. Interaction of K-Ras with Gal-3 and of H-Ras with Gal-1 [9–11,13], which depend on the farnesyl group of Ras, prolongs the Ras signal. Structural modeling has identified highly homologous putative farnesyl-binding pockets in Gal-3 [23] and in Gal-1 [11,23], residing between the two β-sheet layers of their respective CRDs. H-Ras-GTP/Gal-1 complexes [9–11] and K-Ras/Gal-3 complexes [12,13] appear to acquire a conformation that allows prolongation of the Ras signal by reducing the efficiency of p120RasGAP-facilitated GTP hydrolysis by each of these Ras isoforms [13].

Consistent with the above findings, we recently showed that stable expression of Gal-3 in human breast cancer cells (BT-549/Gal-3) coincides with a marked increase in K-Ras-GTP [12] and that specific inhibitors for Ras or MEK inhibit Gal-3-mediated resistance to apoptosis as well as anchorage-independent growth functions [12]. Thus, Gal-3 appears to confer on BT-549 human breast carcinoma cells several oncogenic functions by binding to wild-type active K-Ras. Interestingly, the increase in K-Ras-GTP in BT-549/Gal-3 cells is coupled with a loss of N-Ras–GTP [12]. It was not clear, however, how Gal-3, which does not interact with N-Ras, would affect activation of this Ras isoform. In the present study we describe a new control mechanism of Ras activation whereby Gal-3 inhibits RasGRP4 activity and thus blocks the RasGRP4-mediated GTP loading of N-Ras and H-Ras. Two separable domains of Gal-3 appear to regulate Ras activity.
2. Materials and methods

2.1. Plasmids and drugs

pECFP-C3-K-Ras, pECFP-C3-H-Ras [26], pECFP-Gal-3, and pEGFP-Gal-3(1-42) [15] have been described previously. pECFP-C3-N-Ras was prepared by inserting the entire coding sequence of human N-Ras into pECFP-C3 (Invitrogen, Carlsbad, CA), as previously described [26], pBabe-HA-Ras(13-26) [27] was a generous gift from Dr. Channing Der, University of North Carolina at Chapel Hill, pcDNA3-RasGTP2, and pcDNA3-RasGRP3 were a generous gift from Dr. Gary Reuther, University of South Florida, Tampa. The inhibitors CI993, GOX, SBE35, U73, and 20(2) /K were used as vehicle controls.

2.2. Cell culture and transfection

The human breast cancer cell lines BT-549, BT-549 stably expressing Gal-3 (BT-549-Gal-3) [12], rat intestinal epithelial cells stably expressing the empty vector (RIE-1), and RIE-1 cells stably expressing the constitutively active H-Ras(Q61L) or K-Ras(G12V) mutants [28], human embryonic kidney HEK 293 cells [13], and baby hamster kidney (BHK) cells [25] have been previously described. BT-549 cells stably expressing GFP-K-Ras(G12V) plasmid and selection in the presence of G418 (800 μg/ml) Several clones of BT-549 cells expressing GFP-K-Ras(G12V) were selected (BT-549-GFP-K-Ras(G12V) cells) and maintained in complete DMEM containing G418 (400 μg/ml). Glioblastoma (U251, U373, U87, and 20(2)/K), breast cancer cell lines (MCF, SKBR3, and MDA-MB-435) and ovarian carcinoma (CP200, Z80A, and Ovac3) were purchased from ATCC (Manassas, VA). Unless otherwise indicated, cells were plated at a density of 1×10^5 cells per 10 cm^2 dish at a density of 70% to 80% confluence. All media and reagents were dissolved in DMSO. Ethanol and DMSO were thus used as vehicle controls.

2.3. Infections and shRNAs

Viruses were produced by transient triple-transfections of HEK 293 cells using 6 μg retroviral vectors (Open-Biosystems) encoding for specific shRNA against Gal-3 (V2H5-133962 or V2H5-133963) or RasGRP4 (V2H5-118244 or V2H5-227242) in combination with 3 μg pMD2G and 3 μg pEGFP encoding the retroviral envelope and the Gag and Pol proteins, respectively. As a control, we used 6 μg of no-silencing shRNA (Open-Biosystems, RHS7077) or MSCV-PG encoding GFP. Viruses were collected 48 h after transfection. Two milliliters of viral supernatant containing 8 μg/ml polyethylene (HR26H8) was used for infections using very low density cultured cells as indicated. Infected cells were allowed to recover and used at least 72 h after infections for Western blotting, Ras-GTP pull down assay or cell proliferation assays as described below.

2.4. Western immunoblotting

Cells were washed with phosphate-buffered saline (PBS) and lysed with lysis buffer (50 mM Tris, pH 7.6, 20 mM MgCl2, 200 mM NaCl, 0.5% Igepal® CA-630, Sigma, 1 mM DTT, and antiproteases) [10]. Lysates were then subjected to polyacrylamide gel electrophoresis (PAGE) in the presence of sodium dodecyl sulfate (SDS), followed by electrophoresis (PAGE) in the presence of sodium dodecyl sulfate (SDS), followed by Western immunoblotting with a mouse anti-phospho-ERK Ab, and mouse anti-tubulin Ab (AK-15). Mouse anti-pan-Ras Ab (Ab-3) was from Calbiochem; isoform-specific mouse anti-rabbit Ab, mouse anti-phospho-ERK Ab, and mouse anti-phospho-ERK Ab (AK-15) were from Sigma-Aldrich. Mouse anti-GFP Ab, rabbit anti-phospho-JNK Ab, rabbit anti-JNK Ab, and rabbit anti-F38 Ab were from Santa Cruz Biotechnology (Santa Cruz, CA). Peroxidase–anti-mouse IgG, peroxidase–anti-rat IgG, and peroxidase–anti-rabbit IgG were from Jackson ImmunoResearch Laboratories (West Grove, PA). Anti RasGRP4 Ab were from Abnova (Taiwan).

2.5. Co-immunoprecipitation and Ras-GTP assays

Cell lysates containing 5 mg or 1 mg of proteins in the lysis buffer described above were prepared for co-immunoprecipitation assay or for assay of the Ras–binding domain of Raf-1 (RBD), respectively. For the latter assay the concentration of Igepal® CA-630 was reduced to 0.1%. For immunoprecipitation, proteins (5 μg, adjusted to a total volume of 1 ml) was incubated for 4 h at 4 °C with 20 μl of mouse anti-HA Ab or of mouse anti-GFP Ab together with 50 μl of sheep anti-mouse iron beads (Dynal Biotech, Los Angeles, CA). The beads were then collected using a magnetic field, washed three times with 1 ml of lysis buffer, and the proteins were subjected to SDS-PAGE and Western immunoblotting with either 1:500 anti-HA Ab or 1:1000 anti-GFP Ab and then with 1:5000 peroxidase–anti-mouse IgG (Jackson ImmunoResearch). Lysates containing 1 mg protein were used for determination of Ras-GTP by the glutathione S-transferase (GST)-RBD pull-down assay as previously described [10], followed by Western immunoblotting with Ras-isoform-specific Abs as described above.

2.6. EGF and PMA stimulation and PKC inhibition

Starting 24 h after transfection of HEK293 cells, the cells were starved of serum for 16 h and then stimulated with 100 ng/ml epidermal growth factor (EGF) for the indicated time periods. The cells were then lysed and subjected to determination of Ras-GTP by the GST-RBD pull-down assay, as described above. In experiments where PMA and phosphokinase C (PKC) inhibitors were used, the PKC inhibitors (3 μM GO or 1 μM Go) were added 20 min after the stimulation of the addition of 40 nM PMA to 30 min. The cells were then lysed and subjected to Western blotting or to PBD pull-down assay as described above.

2.7. Fluorescence confocal microscopy

BHK cells (1×10^5 cells per well in six-well plates) were plated on glass cover slips and then cotransfected with HA-RasGRP4 and GFP or with HA-RasGRP4 and GFP-Gal-3 (1–42). Forty eight hours after transfection, the cotransfectants were incubated for 10 min with vehicle (control) or with 40 nM PMA. The cells were then fixed in PBS containing 4% paraformaldehyde, and permeabilized with 0.5% Triton X-100. Samples were blocked (30 min) with 2% bovine serum albumin and 200 μg/ml goat gamma globulin. Cells were then labeled with anti-HA Ab and then with donkey anti-mouse Cy3-labeled Ab (Jackson ImmunoResearch). Each incubation was followed by three extensive washes. Dual fluorescent digital images were collected on a Zeiss LSM 510 confocal microscope fitted with fluorescein and rhodamine filters. The extent of HA-RasGRP4 translocation to the cell membrane was quantified by blinded screening of the images and scoring of 15–30 cells from each cotransfection for the presence or absence of Cy3 labeling of the plasma membrane. Data represent the percentage of cells with HA-RasGRP4 labeling of the plasma membrane in each treatment.

3. Results

3.1. Levels of N-Ras-GTP in Gal-3 expressing cells are not dependent on K-Ras–Gal-3 interactions

Early studies showed that Gal-3 intersects with active K-Ras, reducing the p120 RasGAP-mediated GTP hydrolysis by K-Ras-GTP, but does not interact with N-Ras or with H-Ras [13]. To determine whether the low level of N-Ras-GTP observed in BT-549-Gal-3 cells [12] depends specifically on the interaction of Gal-3 with K-Ras [13], we examined the effect of constitutively active Ras (which is insensitive to p120 RasGAP) on N-Ras-GTP levels. Expression levels were assayed by Western blotting, Ras-isoform-specific Abs and, levels of active Ras were determined by the GST-RBD pull-down assay followed by immunoblotting with Ras-isoform-specific Abs [12]. In line with earlier reports [29] we found that constitutively active Ras proteins increased the levels of Gal-3 expression. Thus, stable expression of GFP-tagged K-Ras(G12V) in BT-549 cells (Fig. 1A) or of K-Ras(G12V) or both. The decrease in N-Ras-GTP (73%–98%) was significant higher than the decrease in N-Ras protein (21–32%), suggesting the hypothesis that Gal-3 might indirectly regulate N-Ras-GTP levels.
Although Gal-3 interactions with K-Ras-GTP reduce p120 RasGAP-mediated GTP hydrolysis by K-Ras [12,13] this may not account for the observed reciprocal relationships between Gal-3 and N-Ras-GTP in BT-549 and RIE-1 cells. BT-549 and BT-549/GFP-K-Ras(G12V) cells of two distinct clones (C13 and C19) (A) or RIE-1/H-Ras(Q61L), and RIE-1/ K-Ras(G12V) cells (B) were grown as described in Materials and methods and then lysed and N-Ras-GTP, K-Ras-GTP, and H-Ras-GTP in samples of the cell lysates were determined by the GST-RBD pull-down assay followed by immunoblotting with Ras-isoform-specific Abs, as described in Materials and methods. N-Ras, Gal-3, and β-tubulin (loading control) were determined in samples of the lysates by immunoblotting with anti-N-Ras, anti-Gal-3, and anti-tubulin Abs. The percentages of N-Ras-GTP relative to the total levels of N-Ras (mean values±SD, n = 3) were 64±%, 40±% and 45±% respectively, in BT-549, BT-549/C3 and BT549/C19 cells and 84±%, 37±% and 22±%, respectively, in RIE-1, RIE-1/H-Ras(Q61L), and RIE-1/ K-Ras(G12V) cells. In a second set of experiments (C), BT-549/Gal-3 cells were incubated for 24 h with vehicle (Con) or with 30 μM U0126 (U, a MEK inhibitor), 2.5 μM SB203580 (SB, a p38 inhibitor), or 25 μM SP600125 (SP, a JNK inhibitor). Lysates of the cells were then immunoblotted with anti-Gal-3, anti-ERK, anti-phospho-ERK, anti-p38, anti-phospho-p38, anti-JNK, and anti-phospho-JNK Abs. (D) Gal-3 levels in human cancer cell lines correlate with high K-Ras-GTP and low N-Ras-GTP. The indicated cancer cell lines were grown as described in Materials and methods, then lysed, and subjected to the determination of Gal-3, K-Ras, and N-Ras by immunoblotting or of K-Ras-GTP and N-Ras-GTP. Typical immunoblots are shown in the upper panels. Results of the quantitative analysis obtained by densitometry of four separate experiments are shown in the lower panels. Dark bars and bright bars represent calculated percentage of N-Ras and K-Ras activity, respectively. Data are presented as mean values (±SD) of Ras-GTP/total Ras in percentage.

Fig. 1. Negative regulation of N-Ras GTP loading by Gal-3 is independent of Gal-3/K-Ras interactions or on K-Ras signals. (A–C) Constitutively active K-Ras and H-Ras upregulate Gal-3 and downregulate N-Ras-GTP in BT-549 and RIE-1 cells. BT-549 and BT-549/GFP-K-Ras(G12V) cells of two distinct clones (C13 and C19) (A) or RIE-1/H-Ras(Q61L), and RIE-1/ K-Ras(G12V) cells (B) were grown as described in Materials and methods and then lysed, N-Ras-GTP, K-Ras-GTP, and H-Ras-GTP in samples of the cell lysates were determined by the GST-RBD pull-down assay followed by immunoblotting with Ras-isoform-specific Abs, as described in Materials and methods. N-Ras, Gal-3, and β-tubulin (loading control) were determined in samples of the lysates by immunoblotting with anti-N-Ras, anti-Gal-3, and anti-tubulin Abs. The percentages of N-Ras-GTP relative to the total levels of N-Ras (mean values±SD, n = 3) were 64±%, 40±% and 45±%, respectively, in BT-549, BT-549/C3 and BT549/C19 cells and 84±%, 37±% and 22±%, respectively, in RIE-1, RIE-1/H-Ras(Q61L), and RIE-1/ K-Ras(G12V) cells. In a second set of experiments (C), BT-549/Gal-3 cells were incubated for 24 h with vehicle (Con) or with 30 μM U0126 (U, a MEK inhibitor), 2.5 μM SB203580 (SB, a p38 inhibitor), or 25 μM SP600125 (SP, a JNK inhibitor). Lysates of the cells were then immunoblotted with anti-Gal-3, anti-ERK, anti-phospho-ERK, anti-p38, anti-phospho-p38, anti-JNK, and anti-phospho-JNK Abs. (D) Gal-3 levels in human cancer cell lines correlate with high K-Ras-GTP and low N-Ras-GTP. The indicated cancer cell lines were grown as described in Materials and methods, then lysed, and subjected to the determination of Gal-3, K-Ras, and N-Ras by immunoblotting or of K-Ras-GTP and N-Ras-GTP. Typical immunoblots are shown in the upper panels. Results of the quantitative analysis obtained by densitometry of four separate experiments are shown in the lower panels. Dark bars and bright bars represent calculated percentage of N-Ras and K-Ras activity, respectively. Data are presented as mean values (±SD) of Ras-GTP/total Ras in percentage.

3.2. Levels of Gal-3 expression in human cancer cell lines positively correlate with N-Ras/K-Ras usage

To substantiate the possibility that Gal-3 can regulate N-Ras/K-Ras-isoform usage it was necessary to determine the amount of active N-Ras and active K-Ras without the forced expression of Gal-3 or of
constitutively active Ras isoforms. We therefore examined whether variations in the amounts of active N-Ras and active K-Ras in human cancer cell lines correlate with expression of Gal-3. We screened human cancer cell lines that do not harbor oncogenic Ras. Comparative analysis of four glioblastoma (GBM) cell lines (U373, U251, U87 and 20–20), three ovarian carcinoma cell lines (CP200, 2780A and Ovcar3), and four breast cancer cell lines (MCF7, SKBR3, MDA-MB-35 and BT-549) yielded, with only few exceptions, a fairly general pattern of positive correlation between Gal-3 expression and K-Ras- or N-Ras-isofrom usage within each group of cancer cell lines (Fig. 1D); the larger the amounts of Gal-3, the lower the amounts of N-Ras-GTP (and of N-Ras) and the higher the amounts of K-Ras-GTP. Because the ratios of GTP-bound and total K-Ras or total N-Ras showed the same pattern of correlation (Fig. 1D), these correlations seem to be associated mainly, but not only, with the basal state of activation of each of the Ras isoforms, and less with variations in the protein levels.

The relatively low level of expression of N-Ras in Gal-3 expressing cells did not appear to be the main reason for lack of N-Ras activation in such cells. This was evident in experiments in which BT-549/Gal-3 cells, RIE-1/H-Ras(Q61L), 20/20 and Ovcar3 cells were stimulated with the phorbol ester PMA which can activate RasGRPs. Stimulation of the cells with 40 nM PMA (20 min) induced a clear increase in N-Ras-GTP without an effect on the total levels of N-Ras (Fig. 2A–C). Apparently, the PMA treatment caused N-Ras-GTP levels in BT-549/Gal-3 cells to recover completely reaching levels recorded in the parental cells (Fig. 2A).

3.3. Gal-3 expression blocks EGF-stimulated activation of N-Ras but not of K-Ras

To further support the conclusion that the effects of Gal-3 on K-Ras and on N-Ras are the main reason for the lack of N-Ras activation in such cells. This was evident in experiments in which BT-549/Gal-3 cells, which express low levels of Gal-3–12], we found that EGFR induced a typical transient increase in N-Ras–GTP that peaked at 5–10 min and faded within 30 min (Fig. 3A), K-Ras-GTP levels did not increase upon stimulation with EGF (Fig. 3A), supporting the importance of Gal-3 for K-Ras activity in these cells (Fig. 1D)–12]. In BT-549/Gal-3 cells which express high levels of Gal-3 EGF indeed induced strong activation of K-Ras, which peaked at 5–10 min and faded within 30 min (Fig. 3B). Loading of N-Ras-GTP, however, was completely blocked in the EGF-stimulated BT-549/Gal-3 cells (Fig. 3B). Similar results were obtained in an analogous experiment performed with the RIE-1 and with the H-Ras- or K-Ras-transformed RIE-1 cell lines. Here too, EGFR induced a time-dependent increase in N-Ras-GTP loading in the RIE-1 cells whereas the GTP loading of N-Ras in RIE-1/K-Ras(G12V) or in RIE-1/H-Ras(Q61L) cells was completely blocked (Fig. 3C).

The complete inhibition of GTP loading of N-Ras in the very same cells in which GTP loading of K-Ras was highly effective (BT-549/Gal-3) or in cells that constitutively express active H-Ras or K-Ras (RIE-1/ Rasi cells), all of which strongly expressed Gal-3, suggested that Gal-3 might inhibit a RasGEF that induces the exchange of GTP for GDP in N-Ras. Activation of an N-Ras-specific GAP by Gal-3 could nonetheless also decrease N-Ras-GTP levels. The observed rescue of the low N-Ras-GTP phenotype by PMA in Gal-3 expressing cells (Fig. 2) hinted to the possible involvement of a PMA-sensitive RasGEF.

3.4. Phorbol ester rescues the low N-Ras-GTP phenotype in Gal-3 expressing cells independently of PKC

Three families of RasGEFs have been described: the SOS family (SOS1 and SOS2), the RasGRF family (RasGRF1 and RasGRF2), and the RasGRP family (RasGRP 1–4)–2,3]. The pharmacological experiments showing that PMA rescued the low N-Ras-GTP phenotype in Gal-3 expressing cells (Fig. 2) have pointed to the possible involvement of a RasGRP since RasGRPs are activated by diacylglycerol (DAG) or by phorbol esters that bind to their C1 domain–2,3]. We found, nonetheless, that rescue of the low N-Ras-GTP phenotype in Gal-3-expressing cells by PMA was not blocked by the PKC inhibitors GF109203X or Go6976 (Fig. 2A–C), suggesting that the effect was mediated by PMA-induced activation of RasGRPs, not by PMA-mediated activation of PKC. This possibility was supported by the finding that the PKC inhibitors themselves did not reduce the high-level N-Ras-GTP phenotype in parental BT-549 and RIE-1 cells (Fig. 2A, B). Because the activity of RasGRP1 and RasGRP3, but not of RasGRP2 or RasGRP4, depends on PKC–2,3], the effect of PMA could apparently be mediated by activation of RasGRP2 or RasGRP4. These experiments, however, did not rule out the possibility that the low N-Ras-GTP phenotype in Gal-3-expressing cells is also mediated by Gal-3 inhibition of the activity of SOS1/2 or RasGRFs activity.

**Fig. 2.** PMA, independent of PKC, rescues the low N-Ras-GTP phenotype of Gal-3 expressing cells. (A–C) The indicated cells were incubated for 20 min with or without 3 μM 109203X, or 1 μM Go6976 then treated for 30 min with or without 40 nM PMA, and lysed. (D) in separate experiments, BT-549 and RIE-1 cells were incubated for 20 min with or without Ca2+/calmodulin inhibitors W12 or W13 (each 15 μM). The cells were then lysed, and N-Ras-GTP and N-Ras expression in the lysates was determined as described in Fig. 1. Upper panels: typical immunobLOTS. Lower panels: quantitative analysis of the results obtained by densitometry (arbitrary units, AU) in three separate experiments (means±SD).
Nonetheless, the Ca\textsuperscript{2+}/calmodulin inhibitors W12 and W13 failed to downregulate the relatively high levels of N-Ras-GTP in the parental BT-549 and RIE-1 cells (Fig. 2D); since the activity of RasGRFs depends on Ca\textsuperscript{2+}/calmodulin [2,3], this finding suggests that the high levels of N-Ras-GTP recorded in these cells are independent of RasGRFs.

3.5. Gal-3 inhibits the RasGRP4-mediated increase in N-Ras-GTP

To substantiate the hypothesis that Gal-3 inhibits RasGRP4-mediated GTP loading of N-Ras, we first used HEK293 cells that were cotransfected with RasGRP2 and a control vector, or with RasGRP2 and GFP-N-Ras or GFP-H-Ras. After 48 h no RasGRP2-mediated increase in GFP-N-Ras or GFP-H-Ras was detectable (not shown), possibly because of the low Ras-exchange activity of RasGRP2, as reported previously [27]. We then examined the activity of GRP4. This was done by cotransfecting HEK293 cells with GFP-N-Ras and a control vector, or with GFP-N-Ras, RasGRP4 and empty vector, or with GFP-N-Ras, RasGRP4 and Gal-3. On examining GFP-N-Ras-GTP levels 48 h after the transfections, we found that RasGRP4 had induced GTP loading of GFP-N-Ras, as indicated by the significantly higher levels of GFP-N-Ras-RasGRP4 cotransfectants than in the GFP-N-Ras/empty vector cotransfectants (Fig. 4A). GFP-N-Ras-GTP levels recorded in the GFP-N-Ras/ RasGRP4/Gal-3 cotransfectants, however, were much lower than those recorded in the GFP-N-Ras/RasGRP4 cotransfectants (Fig. 4A). These results showed that Gal-3 can inhibit the RasGRP4-mediated GTP loading of GFP-N-Ras, and suggested that Gal-3 might interact directly with RasGRP4 to block its guanine nucleotide-exchange activity. Separate co-immunoprecipitation experiments using HEK293 cells cotransfected with Gal-3 and HA-RasGRP4 confirmed that Gal-3 interacts with HA-RasGRP4 (Fig. 4B).

In view of previous findings that H-Ras serves as a substrate for RasGRP4 [27], it was reasonable to assume that Gal-3 would also inhibit RasGRP4-mediated H-Ras-GTP loading. Experiments with HEK293 cells cotransfected with GFP-H-Ras and HA-RasGRP4 confirmed the earlier findings: HA-RasGRP4 induced an increase in GFP-H-Ras-GTP (Fig. 4A). Consistent with our assumption, Gal-3indeed inhibited the HA-RasGRP4-mediated GTP loading of GFP-H-Ras (Fig. 4A). Similar experiments performed with GFP-K-Ras/HA-RasGRP4 cotransfectants showed that HA-RasGRP4 induced only a small, non-significant increase in GFP-K-Ras-GTP (Fig. 4A). Thus, K-Ras appears to be a poor substrate for RasGRP4. The observed rank order of potency of HA-RasGRP4-induced GTP loading of the various Ras substrates was clearly GFP-H-Ras>GFP-N-Ras>>>GFP-K-Ras (Fig. 4A). Taken together, these experiments showed that H-Ras and N-Ras are preferable substrates for RasGRP4, which is for itself inhibited by Gal-3. These observations are in line with our pharmacological data suggesting that selective inhibition of the DAG-dependent/PKC-independent exchange factor RasGRP4 by Gal-3, not inhibition of the PKC-dependent RasGRP1/3, contributes to the lack of N-Ras activation in cells that express high levels of Gal-3. Consistent with this conclusion we found that RasGRP4 is expressed in Ovcar3 and in 20–20 GBM cells which exhibit relatively high levels of Gal-3 and low levels of N-Ras-GTP (Fig. 4D).

Several lines of evidence supported this conclusion. First, downregulation of Gal-3 by two distinct Gal-3 shRNAs in 20–20 GBM cells were accompanied by an increase in the levels of N-Ras-GTP (Fig. 4C). Non-silencing shRNA had no effect on Gal-3 or on N-Ras-GTP levels (Fig. 4C). Similar results were obtained in RIE-1/H-Ras(Q61L) (Fig. 4C). Second, we found that downregulation of RasGRP4 by two distinct RasGRP4 shRNAs in BT-549 caused a decrease in N-Ras-GTP (Fig. 4D). Non-silencing shRNA had no effect on RasGRP4 or N-Ras-GTP levels (Fig. 4D). Third, we found that activity of the PKC-dependent RasGRP3, which itself induced strong activation of GFP-N-Ras in GFP-N-Ras/RasGRP3 HEK293 cotransfected cells, was not inhibited by Gal-3 expression (Fig. 4E).

3.6. The N-terminal domain of Gal-3 inhibits RasGRP4-mediated increase in GFP-N-Ras-GTP

Gal-3 does not interact with N-Ras but interacts with K-Ras-GTP through the C-terminal CRD domain of Gal-3 which is separate to the N-terminal 1–42 region. We thus wondered whether the observed RasGRP4-mediated N-Ras GTP loading might involve interaction of RasGRP4 with the N-terminal domain of Gal-3. To test this hypothesis...
we examined the effect of the GFP-tagged N-terminal tail of Gal-3 (GFP-Gal-3(1–42)) on RasGRP4-mediated GTP loading of N-Ras. In these experiments HEK293 cells were cotransfected with N-Ras, HA-RasGRP4, and GFP-Gal-3(1–42) or GFP, and the levels of N-Ras-GTP in the cotransfectants were then determined. The results showed that N-Ras-GTP was strongly reduced in the GFP-N-Ras/HA-RasGRP4/GFP-Gal-3(1–42) cotransfectants (Fig. 4A). Similar results were obtained when we examined HA-RasGRP4-mediated H-Ras-GTP loading in the presence of GFP-Gal-3(1–42) (Fig. 4A). GFP-Gal-3(1–42) did not inhibit the RasGRP3-induced GTP loading of N-Ras (Fig. 4D), suggesting that the effect was specific to RasGRP4. Co-immunoprecipitation experiments using GFP-Gal-3(1–42)/HA-RasGRP4 cotransfectants confirmed that that GFP-Gal-3(1–42) interacts with HA-RasGRP4 (Fig. 4B). It thus appears that the N-terminal domain of Gal-3 can bind to RasGRP4 and that this domain is sufficient for inhibition of RasGRP4 activity.

Previous experiments have shown that PMA enhances RasGRP4 activation and its translocation to the membrane [27]. Thus, the observed inhibition of RasGRP4 activity by Gal-3 suggested that Gal-3 might actually inhibit its membrane translocation. This possibility was examined by fluorescence confocal microscopy in BHK cells that were transfected with HA-RasGRP4 and GFP or cotransfected with HA-RasGRP4 and GFP-Gal-3(1–42), and then stimulated with PMA (40 nM, 10 min). HA-RasGRP4 was labeled with anti-HA mouse Ab and then with donkey anti-mouse Cy3-labeled Ab, and dual fluorescent confocal images were collected (green, GFP; red HA-RasGRP4). Typical images demonstrate that PMA induced strong translocation of RasGRP4 to the plasma membrane (Fig. 5), consistent with reported observations [27]. The PMA-induced translocation of RasGRP4 was strongly attenuated by GFP-Gal-3(1–42) (Fig. 5); whereas 79% of the HA-RasGRP4-transfected cells exhibited PMA-induced translocation of HA-RasGRP4, only 33% exhibited PMA-induced translocation in the HA-RasGRP4/GFP-Gal-3(1–42) cotransfectants.

3.7. Gal-3(1–42) reverses the transformed phenotype of BT-549 cells

The marked inhibition of RasGRP4-mediated N-Ras GTP loading by Gal-3(1–42) prompted us to examine whether Gal-3(1–42) can affect the transformed phenotype of BT-549 cells. We employed two distinct clones of BT-549 cells stably expressing GFP-Gal-3(1–42) and found, as expected, that GFP-Gal-3(1–42) caused a marked reduction in the
levels of N-Ras-GTP (Fig. 6A). Concomitantly, a clear change in cell morphology was observed (Fig. 6A) accompanied by a marked reduction in growth rate (60% reduction, Fig. 6B).

We also examined sensitivity of BT-549/GFP-Gal-3(1–42) to cytotoxic drugs in comparison with the sensitivity of the parental BT-549 to such drugs. The cells were exposed to 10 μM adriamycin for 24 h or to 500 nM staurosporine for 8 h then subjected to FACS analysis. In line with previous experiments [12], adriamycin and staurosporine induced an increase in the sub-G1 population of cells (indicative of apoptotic cell death) in BT-549 cells (Fig. 6C). The increase in sub-G1 population observed in all BT-549/GFP-Gal-3(1–42) cell lines was clearly higher than that observed in BT-549 cells (Fig. 6C) suggesting that GFP-Gal-3(1–42) had rendered the cells more sensitive to apoptosis, namely had partially reversed their transformed phenotype.

4. Discussion

The results presented here point to a new control mechanism showing that Gal-3 protein can regulate the RasGRP4-mediated GTP loading of N-Ras or H-Ras in cancer cells. It appears that Gal-3 does not affect RasGRFs and SOS1/2, although this possibility has not yet been unequivocally excluded. Whether or not Gal-3 can affect the activity of GRP2 might be less important for N- and H-Ras, because this exchange factor appears to act more potently as an exchange factor of Rap1 than of Ras proteins [3,27]. On the basis of our results and of earlier studies [12,13,25], we propose a model that can explain the shift from N-Ras to K-Ras usage in cells expressing high levels of Gal-3 (Fig. 6D). In our model, two separable domains of Gal-3 act independently on K-Ras and on RasGRP4 to promote, respectively, accumulation of active K-Ras [12,13] and inhibition of N-Ras or H-Ras activation. One domain is the CRD, which can accommodate the farnesyl moiety of K-Ras [25] and strengthen the direct binding of Gal-3 to K-Ras-GTP [13]. The other domain is the N-terminal tail of Gal-3, which interacts with RasGRP4 and inhibits its activity. The model can explain the Gal-3-associated high K-Ras/GTP/low N-Ras-GTP phenotype observed in BT-549/Gal-3 cells and in human cancer cell lines such as GBM, breast cancer cells, and ovarian carcinomas. Consistent with the model, we found that downregulation of Gal-3 by Gal-3 shRNA, or that phorbol 12-myristate 13-acetate (PMA) activating Ras guanine nucleotide releasing proteins (RasGRPs) independently of protein kinase C, both, increased N-Ras-GTP levels in Gal-3 expressing cells. In accordance downregulation of RasGRP4 by RasGRP4 shRNA reduced the levels of N-Ras-GTP. Because PMA and DAG both enhance activation of RasGRP4 by binding to the C1 domain of RasGRP4 [27], it is possible that the N-terminal tail of Gal-3 interacts with the C1 domain. Our results thus assign to the N-terminal domain of Gal-3, whose significance in cell transformation has been well documented [19,30], a novel regulatory function.

It appears then that cancer cells expressing high levels of Gal-3 with their chronically active K-Ras are phenocopies of the K-Ras mutation, which is the most common oncogenic Ras mutation in human tumors [16,17]. This emphasizes the important contribution of K-Ras [16,17] and of Gal-3 [14] to human malignancies. Nonetheless, it is not unlikely that activated N-Ras or H-Ras would predominate in cancer cells that do not harbor oncogenic K-Ras, or in cells that express low levels of Gal-3, or both. In such cells RasGRP4, which would be free of Gal-3 inhibition, might be a critical transforming factor. Consistent with this possibility we showed that reducing RasGRP4 activity in BT-549 by Gal-3(1–42) partially reversed their transformed phenotype (Fig. 6). Earlier experiments indeed demonstrated that RasGRP4 and RasGRP1 are potent inducers of cell transformation, whereas RasGRP2 and RasGRP3 are weak inducers [2,3]. An important question that remains to be answered is whether or not the specific pattern of Ras-isoform usage has a distinctive impact on the biology of cancer cells, and if so, what its outcome is. The case of BT-549/Gal-3 cells suggests that high levels of Gal-3 and K-Ras-GTP with concomitantly low levels of N-Ras-GTP promote a more aggressive phenotype. For example, BT-549/Gal-3 cells are more resistant to apoptosis than BT-549 cells, and they form tumors in nude mice whereas BT-549 cells do not. Similarly, thyroid tumors that express high Gal-3 levels are far more aggressive and invasive than those expressing low levels of Gal-3 [14,31]. Other studies have shown that K-Ras is a more potent inducer of cell migration than H-Ras or N-Ras [32], while yet others have demonstrated that oncogenic H-Ras is more potent than oncogenic K-Ras or N-Ras in inducing cell proliferation and focus formation [33]. The later, however, refers to mouse fibroblasts that depend on Raf-Mek-Erk pathway and may then not be relevant to human carcinomas. Nonetheless, it is possible that signals originating from active K-Ras contribute more critically to cancer cell migration and invasion, whereas those that originate from H-Ras or N-Ras might be more important for cell-cycle progression.

It is more reasonable to assume that the impact of Ras-isoform usage would be determined not only by the active Ras protein but also by the specific cellular context of the given type of cancer or normal cell and by the cellular localities of the Ras isoform and the RasGEFs. Clearly, factors other than Gal-3 can determine Ras-isoform activation and signaling. It was shown, for example, that activated K-Ras can...
increase N-Ras-GTP in colorectal cell lines, possibly by inducing an increase in interactions between N-Ras and gelsolin [34].

Other studies have shown that Gal-1 increases H-Ras activation and signaling to ERK at the expense of PI3K and RalGEF, and enhances H-Ras transformation [9]. Perhaps even more intriguing are the observations that under certain circumstances oncogenic K-Ras can act as a mitochondrial pro-apoptotic factor [35], while under other conditions K-Ras(G12V) can act as a survival factor [28]. Also, other studies have shown that RasGRP1 and RasGRP3 can specifically activate H-Ras in the Golgi [2,36]. Finally, it is interesting to point out that expression of mouse and rat RasGRP4 proteins is restricted to mast cells [37] where RasGRP4 functions as an H-Ras exchange factor involved in airway reactivity and allergic inflammation [37]. In this context other studies have shown that mast cells from Gal-3 deficient mice exhibit impaired histamine and IL-4 release and reduced levels of JNK [38]. Our results suggest that the levels of expression of Gal-3 may determine Ras isoform usage in mast cells.

In conclusion, this study presents a novel dual-control mechanism of Ras proteins whereby Gal-3 inhibits RasGRP4-mediated activation of N/H-Ras through its N-terminal domain and stabilizes K-Ras-GTP through its CRD domain. Future experiments focusing on the levels of expression and of Gal-3, and on various RasGEFs and their Ras isoform GTP-loading activities, will shed more light on the importance of Ras-isoform usage for the survival, migration, and proliferation of cancer cells.
cells. Such knowledge should provide guidelines for the design of specific and selective Ras inhibitors as potential anti-tumor drugs.

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