Galectin-1 augments Ras activation and diverts Ras signals to Raf-1 at the expense of phosphoinositide 3-kinase

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Running Title:
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SUMMARY

Ras proteins activate diverse effector molecules. Depending on the cellular context, Ras activation may have different biological consequences—induction of cell proliferation, senescence, survival, or death. Augmentation and selective activation of particular effector molecules may underlie various Ras actions. In fact, Ras effector-loop mutants interacting with distinctive effectors provide evidence for such selectivity. Interactions of active Ras with escort proteins, such as galectin-1, could also direct Ras selectivity. Here we show that in comparison with Ras transfectants, H-Ras/galectin-1 or K-Ras4B/galectin-1 co-transfectants exhibit enhanced and prolonged epidermal growth factor (EGF)-stimulated increases in Ras-GTP, Raf-1 activity, and active extracellular signal-regulated kinase. Galectin-1 antisense RNA inhibited these EGF responses. Conversely, Ras and galectin-1 co-transfection inhibited the EGF-stimulated increase in phosphoinositide 3-kinase (PI3-K) activity. Galectin-1 transfection also inhibited Ras(G12V)-induced PI3-K, but not Raf-1 activity. Galectin-1 co-immunoprecipitated with Ras(G12V) or with Ras(G12V/T35S) that activate Raf-1, but not with Ras (G12V/Y40C) that activates PI3-K. Thus, galectin-1 binds active Ras and diverts its signal to Raf-1 at the expense of PI3-K. This demonstrates a novel mechanism controlling the duration and selectivity of the Ras signal. Ras gains selectivity when it is associated with galectin-1, mimicking the selectivity of Ras(T35S) which activates Raf-1 but not PI3-K.
INTRODUCTION

The active GTP-bound forms of Ras proteins activate diverse effector molecules such as Raf-1, phosphoinositide 3-kinase (PI3-K)\(^1\) and Ral-guanine nucleotide dissociation stimulator (Ral-GDS) (1-6). Depending on the cellular context, Ras activation may have different biological consequences—induction of cell proliferation, senescence, survival, or death (3,7,8). The molecular mechanisms allowing active Ras to elicit diverse, sometimes contradictory, biological effects are not well understood. Selective activation of particular effector molecules may underlie various Ras actions. In fact, Ras effector-loop mutants interacting with distinctive effectors provide evidence for such selectivity (5,9-12). Interactions of active Ras with binding partners, such as galectin-1 (13), could also direct Ras selectivity. As noted above, Ras signal selectivity may produce different effects on different cells.

We hypothesized that interactions of active Ras with escort proteins could direct selectivity of Ras signals. In a recent study we found that galectin-1 is such an escort; it is associated with the constitutively active (GTP bound) H-Ras (G12V) mutant (13). Galectin-1 belongs to a family of β-galactoside-binding proteins which act both within cells via sugar-independent interactions with other proteins and outside of cells via sugar-dependent interactions with glycan chains of cellular glycoconjugates after their secretion which is independent of a classical signal sequence (14-19). Our own findings suggested that active Ras is a binding partner of galectin-1 and that this partnership is required for the membrane anchorage of active...
H-Ras, but not its wild type counterpart (13). We proposed that active H-Ras is stabilized in the cell membrane when in complex with galectin-1 and that this would have an impact on Ras signals. Consistent with this proposal was the observation that over-expression of galectin-1 in HEK 293 cells upregulated basal extracellular signal-regulated kinase (ERK) activity under conditions where extra-cellular galectin functions were blocked (13). These early experiments defined a role for galectin-1 as an escort protein of active H-Ras, and we wondered whether it affects Ras’ choice of target proteins.

EXPERIMENTAL PROCEDURES

*Plasmid constructs*—We prepared green fluorescent protein (GFP)-H-Ras and GFP-K-Ras in pEGFP-C3 by inserting the respective full-length cDNAs of the human Ras isoforms into the PstI/BamHI site of pEGFP-C3 (Clontech) as detailed previously (13,20). We cloned rat galectin-1 cDNA and inserted it into the KpnI and HindIII sites of pcDNA3 (sense orientation) or into the NotI site (antisense orientation) as detailed earlier (13). The plasmids pDCR-H-Ras(G12V), pDCR-H-Ras (G12V/T35S), pDCR-H-Ras (G12V/Y40C), pCGN-K-Ras(G12V), pCGN-K-Ras (wt) and H-Ras(wt) restricted into pcDNA3 were a gift from A. D. Cox. All DNA sequences were confirmed by DNA sequencing. Raf-1(wt) cDNA in pUSEamp vector was purchased from Upstate Biotechnology. Glutathione -S-transferase fused to the A85K mutant of the Ras binding domain of c-Raf-1 (21) in pGEX-2TH was a gift from A. Burgess.

*Cell culture and transfection procedures*—We maintained cells in DMEM with
10% fetal calf serum (13). We transfected 1.5x10^6 Cos-7 cells with total amount of 1.5 μg DNA by dextran (Pharmacia) or 8 x 10^5 HEK 293 cells with total amount of 6 μg DNA by calcium phosphate (Sigma). The cells were harvested 48 h after transfections using lysis buffers containing protease and phosphatase inhibitors (13). Lysates were normalized for protein and subjected to the specified assays and quantification as detailed bellow. Data were expressed as means± SD and differences between means were examined in Student’s t Test.

**Co-immunoprecipitation and Western immunoblotting** –We co-transfected Cos-7 cells with 0.75 μg plasmid DNA coding for galectin-1 along with 0.75 μg plasmid DNA coding for each H-Ras(G12V), H-Ras(G12V/T35S), H-Ras(G12V/Y40C), H-Ras(wt), K-Ras(G12V) or K-Ras(wt), and lysed them 48 h later. Lysates containing 7 mg protein were subjected to immunoprecipitation (IP) with 3 μg mouse pan-Ras Ab (Ab-3, Calbiochem) followed by SDS-PAGE and Western immunoblotting (IB) (13,22) with either 1:2000 anti-Ras Ab and 1:7500 peroxidase-goat anti-mouse IgG (Jackson Laboratories) or 1:500 anti galectin-1 (Gal-1) Ab (23) and 1:5000 peroxidase-goat anti-rabbit IgG (Jackson Laboratories). We then visualized Ras proteins and the 14 kDa galectin-1 protein by ECL and quantified the bands by densitometry with Image Master VDS-CL (Amersham Pharmacia BioTech) using TINA 2.0 software (Ray Tests).

**Ras-GTP and phospho-ERK assays**–We co-transfected HEK 293 cells with the combination of 0.15 μg plasmid DNA coding for GFP-H-Ras or for GFP-K-Ras and 5.85 μg empty pcDNA, 2.85 μg plasmid DNA coding for galectin-1 and 3 μg
pcDNA, or 2.85 µg galectin-1 antisense RNA plasmid (galectin-1-AS) and 3 µg pcDNA. Twenty-four h after transfection the cells were serum starved for 24h then stimulated with 100 ng/ml EGF for the times indicated and lysed in 0.5 ml Ras binding domain (RBD) lysis buffer (24) containing 0.1 mM orthovanadate. We used lysates containing 30 µg protein for the determination of total GFP-Ras (Mr about 54 kDa) by Western immunoblotting with pan anti-Ras Ab, and lysates containing 500 µg protein for the determination of GFP-Ras-GTP by the glutathione S-transferase (GST)-RBD pull-down assay followed by Western immunoblotting with pan anti-Ras Ab as detailed previously (21). We determined ERK or phospho-ERK using 30 µg of lysates protein by Western immunoblotting, ECL and densitometry as detailed above. ERK immunoblots were incubated with 1: 2000 rabbit anti-ERK1/2 Ab (Santa-Cruz) then with 1:1000 peroxidase-goat anti rabbit IgG; phospho-ERK immunoblots were incubated with 1:10,000 mouse anti-phospho-ERK Ab (Sigma) then with 1:10,000 peroxidase-goat anti-mouse IgG.

Raf-1 kinase assay—We determined Raf-kinase activity with a Raf-1 immunoprecipitation kinase cascade assay kit (Upstate Biotechnology). HEK 293 cells were co-transfected with plasmids coding for K-Ras, H-Ras, H-Ras (G12V) or its effector loop mutants along with a plasmid coding for galectin-1 or with empty vector as detailed above. All combinations included 1.5 µg of plasmid DNA coding for Raf-1(wt). Twenty-four h after transfection the cells were serum starved for 24h then, either left un-stimulated (activated Ras transfectants), or stimulated with 100 ng/ml EGF for 5 min. The cells were then lysed in 0.5 ml of Raf-1 kinase assay kit lysis buffer. Raf-1 was immunoprecipitated from 300 µl lysate and assayed in the coupled
kinase assay using myelin basic protein (MBP) from Sigma as a substrate and $\gamma^{32}\text{P}$ ATP (3000 Ci/mmol, Dupont, NEN) as a phosphate donor according to the manufacturer’s instructions. The amount of $^{32}\text{P}$-labeled MBP thus formed was estimated in a scintillation counter.

**PI3-K assay**—We determined PI3-K activity using a published procedure (25) in HEK 293 cells that were transfected and treated with EGF as detailed for the Raf-1 kinase assay (omitting the Raf-1 plasmid). We lysed the cells with 0.5 ml of PI3-K lysis buffer (25), immunoprecipitated the enzyme from 500 µl lysate with rabbit anti PI3-K p85 Ab (Upstate Biotechnology) and assayed its activity with 0.5 mg/ml phosphatidylinositol and 125µM ATP and 5 µCi $\gamma^{32}\text{P}$ ATP. We extracted the lipids with chloroform/methanol then separated them by TLC (25). Phospholipid markers were used for the identification of the $^{32}\text{P}$-labeled phosphatidylinositol-3-phosphate (PIP) product (25). The $^{32}\text{P}$-labeled lipid products were visualized by an overnight exposure on an X-ray film and the spots then quantified by TINA analysis.

**RESULTS**

*Co-immunoprecipitation of galectin-1 with active Ras isoforms*—Our previous experiments suggested that the activated forms of H-Ras and of K-Ras4B (referred to as K-Ras), but not of N-Ras, interact with galectin-1. We therefore focused on H-Ras and K-Ras, attempting first to determine whether GDP-bound Ras(wt) interacts with galectin-1. This was done by co-immunoprecipitation assays with H-Ras(wt)
or H-Ras(G12V)/galectin-1 co-transfectants and K-Ras(wt) or K-Ras(G12V)/galectin-1 co-transfectants. The wild type isoforms were GDP bound, and the constitutively active mutant forms were GTP bound. We found that with comparable levels of Ras and galectin-1 expression, galectin-1 co-immunoprecipitated with H-Ras(G12V) or with K-Ras(G12V)—GTP-bound forms—much more efficiently than it did with H-Ras(wt) or with K-Ras (wt) (Fig. 1A). H-Ras (wt) binding of galectin-1 was (mean ± SD, n=3) 32 ± 4% that of H-Ras(G12V) (p<0.05) and K-Ras (wt) binding of galectin-1 was 6 ± 2% that of K-Ras(G12V) (p<0.01). Less galectin-1 co-immunoprecipitated with K-Ras(G12V) than with H-Ras (G12V). Perhaps the affinity of H-Ras-GTP towards galectin-1 is higher than that of K-Ras-GTP. Alternatively, more galectin-1 molecules may associate with H-Ras (G12V) than with K-Ras (G12V). We can not exclude the possibility that the observed differences between the apparent amounts of galectin-1 that were co-immunoprecipitated with K-Ras(12V) and with H-Ras(12V) is a result of a difference in the affinity of the Ras Ab towards H- and K-Ras isoforms. In any case, since the activated forms of Ras seemed to associate with galectin-1, this association could affect the stability of the bound nucleotide and as well as Ras-effector interactions.

**Galectin-1 augments basal and EGF-induced increase in GFP-H-Ras-GTP and GFP-K-Ras-GTP and prolongs the EGF response**—Thus, we looked at the impact of Ras-GTP/galectin-1 interactions on the stability of the bound nucleotide employing Ras(wt) isoforms that alternate between GDP- and GTP-bound states. We expressed GFP-tagged versions of H-Ras and K-Ras to permit a clear distinction
from endogenous Ras and to permit comparison to the previously described galectin-1 influence on localization of these fusion proteins (13). Following transfections in HEK 293 cells and stimulation with EGF, we pulled-down GFP-Ras-GTP with glutathione S-transferase (GST) fused to the Ras binding domain (RBD) of Raf-1 which binds Ras-GTP but not Ras-GDP (21,24). In cells transfected with GFP-H-Ras or GFP-K-Ras we found a typical EGF-stimulated transient increase in Ras-GTP that peaked at 5 min and returned to basal levels after 10-15 min (Fig. 1, B and C). In Ras/galectin-1 co-transfectants we found higher basal and EGF-stimulated Ras-GTP levels and prolongation of the EGF response (Fig. 1, B and C). The apparent levels of GFP-Ras-GTP were about 2-2.5 fold higher in the co-transfectants and remained relatively high even 1 h after stimulation. The higher EGF-induced increase in Ras-GTP was mediated by intracellular galectin-1 since adding galectin (0.5 µM) to the medium failed to increase basal GFP-Ras-GTP levels or the EGF response (Fig. 1D). Moreover, there was no effect of 100 mM lactose, which interferes with galectin-1 binding to glycan ligands, on the response to EGF stimulation in the co-transfectants (not shown).

The above results, in keeping with the preferential co-immunoprecipitation of galectin-1 with Ras-GTP over Ras-GDP, suggest that galectin-1 binds preferentially to Ras-GTP, stabilizing it in a conformation that may also render Ras less sensitive to Ras-GTPase activating proteins (GAPs) (26). Accordingly, galectin-1 might have been expected to raise basal Ras-GTP levels by stabilizing the small percentage of Ras-GTP that exists under normal conditions; this was indeed the case (Fig. 1, B and C). In the presence of EGF, the level of Ras-GTP is higher, and more is available to
bind to galectin. Thus, the fold increase in Ras-GTP in response to EGF was similar
in the absence and in the presence of galectin-1--the values recorded in GFP-Ras
transfectants and in GFP-Ras/galectin-1 co-transfectants were 2.1 and 2.5
respectively. This is consistent with the idea that when Ras-GTP molecules are
formed, they “search” for galectin-1 partners. To build up in the membrane, they
have to find such partners, and the more galectin there is, the more Ras-GTP can
accumulate. We gained additional support for the above assertion by knocking down
endogenous galectin-1 in HEK 293. These cells express galectin-1 (13,23) and
galectin-1 antisense RNA (galectin-1-AS) specifically abolished galectin-1
expression; antisense RNA for the related galectin-3 protein has no effect on
galactin-1 levels or on GFP-H-Ras(G12V) in HEK 293 cells (13). We co-
transfected cells with galectin-1-AS and GFP-Ras, then stimulated the co-
transfectants with EGF, and found that both basal and EGF-induced increases in
Ras-GTP were strongly inhibited (Fig 1, B and C). EGF still induced a 2-fold
increase in GFP-Ras-GTP as in cells singly transfected with GFP-Ras,
demonstrating a shift in Ras-GTP/Ras-GDP equilibrium in the opposite direction
from the one induced by galectin-1 overexpression. These experiments establish a
functional link between galectin-1 and active Ras suggesting that this linkage may
have an impact on Ras effectors.

Galectin-1 enhances basal and EGF-stimulated increases in Raf-1 activity
and in active ERK--We next examined the impact of Ras/galectin-1 interactions on
Raf-1, a canonical Ras effector, using cells transfected with galectin-1 or Ras(wt)
alone, or Ras(wt) and galectin-1 together. The cells were stimulated for 5 min with
EGF, a time point at which peak Ras-GTP levels after EGF stimulation were observed (Fig 1, B and C). We then measured Raf-1 activity in the cell lysates by immunoprecipitating Raf-1 and assaying its activity in a kinase cascade Raf kinase assay. We found that while basal activities in galectin-1, H-Ras or K-Ras transfectants were elevated compared with pcDNA controls, EGF induced an increase in Raf-1 activity, which was stronger in the galectin-1/Ras(wt) co-transfectants compared with galectin-1 or Ras(wt) transfectants (Fig. 2A). In line with the observed equilibrium shift in basal and in EGF-stimulated increases in Ras-GTP (Fig1, B and C), the fold increase in Raf-1 activity was, however, not altered by galectin-1; the recorded values were respectively, 2.2 ± 0.3, 1.85 ± 0.2, 1.7 ± 0.1, 1.6 ± 0.1, 1.7 ± 0.09 and 1.7 ± 0.2 (mean ±SD, n=4), in pcDNA controls, galectin-1, H-Ras(wt), H-Ras(wt)/galectin-1, K-Ras(wt) and K-Ras(wt)/galectin-1 transfectants. Thus, the galectin-1 enhancement of the EGF-stimulated increase in Ras-GTP (Fig. 1, B and C) promoted elevated Raf-1 activity (Fig. 2A).

We then used GFP-H-Ras or GFP-K-Ras transfectants and GFP-Ras/galectin-1 co-transfectants to examine whether the enhancement of EGF-induced Raf-1 activity propagated to ERK. We found that galectin-1 enhanced the EGF-stimulated increase in the active phospho-ERK protein (Fig. 2, B and C). Similar to its effects on the EGF-induced increase in GFP-Ras-GTP (Fig. 1, B and C), galectin-1 promoted a stronger and more prolonged activation of ERK and galectin-1-AS inhibited the EGF response (Fig. 2, B and C). We conclude that galectin-1-Ras interactions enhance Ras’ activation of the Raf/MEK/ERK pathway. This prompted the question whether such an effect of galectin-1 is confined to this pathway or might be operative in a different pathway.
*Galectin-1 inhibits EGF-induced signal to PI3-K*—We therefore next examined the effects of galectin-1 expression on a second well-established Ras effector, PI3-K type I, the catalytic subunit of which interacts directly with active Ras-GTP (4,5). We studied H-Ras or K-Ras transfected cells or cells co-transfected with galectin-1 and each of these two Ras isoforms. The cells were stimulated for 5 min with EGF and PI3-K was then immunoprecipitated from the cell extracts by anti PI3-K p85 Ab. The precipitates were subjected to a PI3-K assay. We found, as expected, that EGF stimulated PI3-K activity inducing, respectively, 1.6 ± 0.1 and 1.8 ± 0.3 (means ± SD, n=3) fold increases in PI3-K activity in H-Ras and in K-Ras transfectants (Fig. 3). However, co-transfection with galectin-1 strongly inhibited the EGF-mediated stimulation of PI3-K activity (Fig. 3). The degrees of inhibition of EGF stimulation by galectin-1 were respectively 84±12% and 63± 8% (means ± SD, n=3, p< 0.05) in the H-Ras and K-Ras cells. Thus, under the very same conditions where galectin-1 enhanced basal and EGF-stimulated increases in Ras-GTP and activation of the Raf-1/MEK/ERK cascade, it inhibited PI3-K activation by the growth factor. These results suggest that the interactions of galectin-1 with H-Ras-GTP or with K-Ras-GTP provide selectivity to the Ras signal. Apparently the signal of galectin-1/Ras-GTP mimics that of the Ras(T35S) effector loop mutant that activates Raf-1 but not PI3-K (9,10).

*Signaling selectivity conferred on Ras by galectin-1*— Our results raised the possibility that galectin-1 induces a conformational change in Ras-GTP that permits interaction with Raf-1 but not with PI3-K. We examined this possibility using H-Ras(G12V/T35S) which preferentially binds and activates Raf-1 and H-
Ras(G12V/Y40C) which preferentially binds and activates PI3-K (5,9-12). The use of constitutively active Ras mutants enabled us to examine the effect of galectin-1 on the selectivity of Ras signaling without the possible interference of its effect on Ras-GTP stabilization. We first tested this assertion in H-Ras(G12V) and in H-Ras(G12V)/galectin-1 co-transfectants. Compared with vector controls, H-Ras(G12V) induced a 4.7 ± 0.9 fold increase in Raf-1 activity and a 12.2 ± 1.6 fold increase (mean ±SD, n=4) in PI3-K activity (Fig 4, A and B). Raf-1 activity in galectin-1/H-Ras(G12V) co-transfectants (Fig. 4A) was no higher than it was in H-Ras(G12V) transfectants, but PI3-K activity (Fig. 4B) was strongly inhibited (72 ± 12%, mean ±SD, n=4), p<0.01. These results demonstrate that the effects of galectin-1 on H-Ras-GTP stabilization and on signal selectivity are separable.

We could then use H-Ras(G12V) effector loop mutants to explore the nature of the galectin-1 induced shift in the Ras signal. We found, as expected, that H-Ras(G12V/T35S) induced a 4.2 ± 0.3 (mean ±SD, n=4) fold increase in Raf-1 activity but did not increase PI3-K activity (Fig. 4A). Raf-1 activity was not enhanced in galectin-1/H-Ras(G12V/T35S) co-transfectants (Fig. 4A). In H-Ras(G12V/Y40C) transfectants we detected a small increase in Raf-1 activity and a strong 9.0 ± 0.9 fold increase (mean ±SD, n=4) in PI3-K activity (Fig. 4, A and B), confirming the known selectivity of this mutant. Raf-1 activity in the galectin-1/H-Ras(G12V/Y40C) co-transfectants was as low as in the H-Ras(G12V/Y40C) transfectants (Fig. 4A), but the PI3-K activity was as high as in the Ras(G12V/Y40C) transfectants (Fig 4B). Thus, the ability of galectin-1 to inhibit PI3-K activation in galectin-1/H-Ras(G12V) co-transfectants was not apparent at all in galectin-1/H-
Ras (G12V/Y40C) co-transfectants, demonstrating that galectin-1 does not alter the signal in a mutant that is already locked in a conformation that favors interaction with a distinct Ras effector. This is also consistent with the observation that galectin-1 neither enhanced nor inhibited Raf-1 activity in the galectin-1/H-Ras(G12V/T35S) co-transfectants (Fig. 4A). Accordingly, the conformation conferred on H-Ras(G12V) by the T35S mutation or by interactions with galectin-1, but not by the Y40C mutation, should permit association with galectin-1. This was indeed observed in co-immunoprecipitation assays in galectin-1/H-Ras(G12V/T35S) and galectin-1/H-Ras(G12V/Y40C) co-transfectants (Fig. 4C). We found that galectin-1 co-immunoprecipitated with H-Ras(G12V/T35S) while it exhibited a weak interaction with H-Ras(G12V/Y40C). Compared with H-Ras(G12V) the amounts of galectin-1 co-immunoprecipitated with H-Ras(G12V/T35S) and with H-Ras (G12V/Y40C) were respectively 56 ± 9% and 7%± 3% (means ±SD, n=3, p<0.01).

DISCUSSION

We have demonstrated that galectin-1 exhibits preferential interactions with H- or K-Ras-GTP over Ras-GDP, shifts the basal and the EGF-stimulated Ras-GTP/Ras-GDP steady state towards Ras-GTP and prolongs the EGF-induced response. Taken together these data suggest a role for galectin-1 in determining the strength and duration of the Ras signal; both of these parameters have long been thought to be important determinants in the outcome of Ras activation by growth factors (27). The findings presented here also demonstrate that galectin-1 chaperons active H- or K-Ras, stabilizing them in the GTP bound state. Galectin-1/Ras-GTP complexes are capable of signaling since they strongly activate Raf-1. This indicates
that galectin-1 does not compete with Raf-1 for the Ras effector domain with which Raf-1, other Ras effectors and Ras-GAP interact (3,7,8). Therefore, galectin-1 may have an allosteric effect on Ras-GTP reducing the effect of Ras-GAP on GTP hydrolysis by Ras. Alternatively, the galectin-1-induced increase in Ras-GTP may be explained by a direct interference of galectin-1 with the binding of Ras-GAP to Ras-GTP. If this were the case galectin-1 and Ras-GAP would share a common site in Ras that does not overlap the Ras effector domain. The detailed nature of such mechanisms are an important subject for further investigation.

Our results also demonstrate that Ras signaling selectivity may be achieved not only through a mutation as in the Ras(T35S) effector loop mutant (9,10), but also under physiologically relevant conditions through the interactions of wild-type active H-Ras or K-Ras with natural constituents of the normal cell, i.e. galectin-1. The use of wild-type Ras activated by EGF together with the constitutively active H-Ras(G12V) and its effector domain mutants, provided a useful approach to explore the specific effects of the galectin-1 on Ras signaling selectivity. Our results show that H-Ras (G12V/T35S), which preferentially activates Raf-1 over PI3-K (9,10), binds galectin-1 (Fig. 4C). On the other hand, H-Ras(G12V/Y40C), which preferentially activates PI3-K over Raf-1(5,9-12), does not bind galectin-1 (Fig. 4C). Together with the demonstrated selectivity of wild type H-Ras-GTP, K-Ras-GTP or H-Ras(G12V) towards Raf-1 (Figs 3 and 4), this strongly suggests that galectin-1 induces a conformational change in Ras that mimics the conformation conferred on Ras by the T35S mutation. This conformation appears to permit binding of Ras to Raf-1, but not to PI3-K. To the best of our knowledge this is the first demonstration
of signaling selectivity conferred on Ras through its interaction with an escort protein. This precedence raises the possibility that additional Ras binding proteins yet to be identified may divert Ras signals towards effectors other than Raf-1. The expression levels of galectin-1 and of other galectins are known to vary significantly between normal and cancer cells, with impact on their growth and invasion-related properties (16,17,23,28-30). These levels can therefore serve as important determinants in the outcome of Ras activation either by oncogenic mutation or due to growth factor receptor stimulation. We note that previous studies have demonstrated preferential activation of Raf-1 by K-Ras and of PI3-K by H-Ras (31,32) which was not apparent in our experiments and in other studies (33). While these differences may arise from different experimental conditions they may well be associated with different levels of expression of Ras escort proteins that could divert Ras signals as shown here for galectin-1.

H-Ras and K-Ras share with each other a high degree of sequence homology, as well as biochemical functions (3,7,8). They differ from each other significantly at the hyper-variable C-terminal domain but share a common carboxy-terminal farnesyl cysteine methyl ester (34-36). H-Ras has two palmitoyl moities while K-Ras is not palmitoylated but contains a stretch of six lysine residues. This, and additional sequences in the C-terminal domain appear to dictate several important differences among Ras isoforms in (i) the routes of trafficking (37,38); (ii) membrane microdomain localization (39,40); (iii) activation of the Ras effectors Raf-1 and PI3-K (31,32); and (iv) interactions with Ras-GEFs (41). Our present findings show, however, that galectin-1 can bind both active H-Ras and active K-Ras and confers
on both similar properties with respect to GTP stabilization and signal selectivity. Thus, galectin-1 should bind to a site common to H-Ras and K-Ras; at the same time this site may not be the Ras effector domain. This and the previous observation that N-myristoylated unfarnesylated H-Ras(G12V) failed to interact with galectin-1 (13), point to the possibility that galectin-1 binds the farnesyl cysteine carboxymethylester of Ras. It would then be analogous to the interaction of the geranylgeranyl cysteine carboxymethylester of Rho GTPases with Rho-GDIs (42). Interestingly, the carboxy terminal region of the Rho GTPase Cdc42 binds to the immunoglobulin-like domain of Rho-GDI (43). This domain folds into a $\beta$ sandwich that allows the insertion of the geranylgeranyl moiety into a hydrophobic pocket residing between the two layers of the $\beta$ sheets (43). Galectin-1 structure also consists of a sandwich of two $\beta$ sheet layers, which in some manner are reminiscence of the Rho-GDI immunoglobulin-like domain. (Reviewed in (44)). This raises the possibility that a putative hydrophobic pocket in galectin-1 may associate with the farnesyl moiety of activated H- or K-Ras.

In spite of the similarities in the interactions of galectin-1 with H- or K-Ras, the present study and previous results point to several significant differences between the interactions of galectin-1 with these Ras isoforms. First, we have shown that less galectin-1 co-immunoprecipitated with K-Ras (G12V) than with H-Ras (G12V). This is consistent with earlier observations using a cross-linking procedure (13) and may suggest that the affinity of H-Ras-GTP towards galectin-1 is higher than that of K-Ras-GTP. Alternatively, more galectin-1 molecules may associate with H-Ras(G12V) than with K-Ras(G12V). Second, we have previously shown that
downregulation of galectin-1 in HEK-293 cells resulted in robust mislocalization of GFP-H-Ras (G12V) but not of GFP-K-Ras (G12V) (13). Third, we found that targeting galectin-1 for downregulation by galectin-1 AS-RNA resulted in a far stronger inhibition of the EGF-induced effects on wild type H-Ras than on wild type K-Ras. This was apparent in both the EGF-induced increases in GTP-bound Ras (Figs 1B and C) and in phospho-ERK (Figs 2B and C). The two latter differences may be related to each other because downregulation of galectin-1 does not affect membrane localization of wild type GFP-H-Ras or of GFP-K-Ras whether the latter is GDP or GTP bound. On the other hand, it does affect the plasma membrane localization GFP-H-Ras-GTP (13). Accordingly, once GFP-H-Ras is GTP loaded due to EGF stimulation it may be dislodged in part from the cell membrane in the absence of galectin-1, resulting in a stronger inhibition of the EGF response. This does not occur with GFP-K-Ras. The differences between the impacts of galectin-1 on activated H-Ras and on activated K-Ras are likely associated with the above-mentioned hyper-variable C-terminal domain of Ras proteins. Consistent with the possible involvement of the hyper-variable region in galectin-1 and H- or K-Ras interactions is the observation that N-Ras, which like other Ras isoforms is farnesylated (35), does not interact with galectin-1 (13).

In conclusion, our results provide evidence for a novel mechanism controlling the function of Ras. We have shown that galectin-1 augments and prolongs Ras activation at the same time that it shifts the interaction of the activated molecule away from PI3-K and towards Raf-1. These, and the documented preferential activation of Raf-1 by K-Ras and of PI3-K by H-Ras (31,32), suggest that the strength (27) and
nature of Ras signals in any given cell are likely to be defined by the Ras isoforms expressed and the partners, including galectin-1, which they find. In fact, galectin-1 levels, which are known to vary among normal and cancer cells (16,17,23,45), may define the functional outcome of activating mutations of Ras or upstream receptors. The observed selectivity of Ras-galectin-1 towards Raf-1 and not towards PI3-K may be further exploited in the design of new Ras inhibitors as anti cancer drugs. Until recently, galectin-1 was viewed as a *bona fide* lectin. Our study reveals a new function of this protein as a regulator of Ras signaling, an activity also serving as potentially promising therapeutic target.

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FOOTNOTES

1 The abbreviations use are: PI3-K, phosphoinositide 3-kinase; Ral-GDS, Ral-guanine nucleotide dissociation stimulator; EGF, epidermal growth factor; ERK, extracellular signal-regulated kinase; GFP, green fluorescent protein; IP, immunoprecipitation; IB, immunoblotting; AS, antisense; RBD, Ras binding domain; GST, glutathione S-transferase; MBP, myelin basic protein; GAP, GTPase activating protein;
FIGURE LEGENDS

FIG. 1. Galectin-1 interacts with active H-Ras or K-Ras and increases basal and EGF-stimulated levels of GFP-H-Ras-GTP and GFP-K-Ras-GTP. Cos-7 cells were co-transfected with plasmid coding for galectin-1 along with plasmid coding for each H-Ras (12V), H-Ras (wt), K-Ras (12V) or K-Ras (wt) and lysed 48 h later. Lysates were subjected to immunoprecipitation (IP) with pan anti-Ras Ab followed by SDS-PAGE and immunoblotting (IB) with either anti-Ras Ab or anti-galectin-1 (Gal-1) Ab, visualization by ECL and densitometry. Results of a representative experiment (A) are shown. Similar results were obtained in two additional experiments demonstrating that galectin-1 preferentially co-immunoprecipitated with active H-Ras or K-Ras. The relatively lower Mr of wild H-Ras reflects the fact that of the vectors used here only the wild type H-Ras has no tag. In a second set of experiments HEK 293 cells were co-transfected with GFP-Ras/pcDNA, GFP-Ras/galectin-1 or GFP-Ras/galectin-1 antisense (B and C). Twenty-four hours after transfection the cells were serum-starved overnight then stimulated with EGF for the periods indicated and lysed. Lysates were subjected to SDS-PAGE followed by immunoblotting with pan anti-Ras Ab for the determination of total GFP-Ras or subjected to the GST-RBD pull-down assay followed by SDS-PAGE and immunoblotting with pan anti-Ras Ab for the determination of GFP-Ras-GTP. The experiments were repeated three times for the set of GFP-H-Ras (B) and the set of GFP-K-Ras co-transfectants (C). The immunoblots, visualized by ECL, were then
subjected to densitometry. The upper panels show immunoblots of a representative experiment of each set. The lower panels show the results of densitometry (means ±SD) in arbitrary units (AU). Differences between each mean in the Ras transfectants and in the co-transfectants were statistically significant (p<0.01). In an additional set of experiments cells were co-transfected with GFP-H-Ras and galectin-1 as detailed above and serum-starved overnight. The cells were then treated with 0.5 µM extracellular galectin-1 for 1h or left untreated and subsequently incubated for 5 min in the absence or in the presence of EGF and lysed. Lysates were subjected to the GST-RBD assay for the determination of Ras-GTP as detailed above. Results of a representative experiment (D) performed in triplicates show that extracellular galectin-1 did not enhance the EGF-induced increase in Ras-GTP.

FIG. 2. **Galectin-1 increases EGF-stimulated Ras activation of Raf-1 and ERK.** A, Galectin-1 enhances Raf-1 activation. Lectin-1, H-Ras(wt) or K-Ras(wt) and H-Ras(wt)/galectin-1 or K-Ras (wt)/galectin-1 co-transfectants and pcDNA transfectants were stimulated with EGF (5 min) then lysed as detailed in Fig. 1B and C. Immunoblot analysis verified that levels of Ras isoforms or of Raf-1 expression under the different co-transfection conditions were similar. Lysates were subjected to immunoprecipitation with anti-Raf-1 Ab followed by a kinase cascade Raf-1 kinase assay. The amounts of 32P (DPM) incorporated into MBP were then determined. The mean values (±SD) recorded in 4 separate experiments are shown. Differences between each mean in the Ras and in the Ras/galectin-1 co-transfectants were statistically significant (p<0.05). B and C, Galectin-1 enhancement of EGF-stimulated GFP-Ras activation of ERK. GFP-Ras, GFP-Ras/galectin-1 or GFP-
Ras/galectin-1 antisense co-transfectants were stimulated with EGF for the indicated periods of time. ERK and active phospho-ERK were then determined by immunoblotting with anti ERK and anti phospho-ERK Abs followed by ECL and densitometry. Upper panels show representative immunoblots. Lower panels show the results of the analysis (means ±SD, n=3) in arbitrary units (AU). Differences between each mean in the Ras transfectants and in the co-transfectants were statistically significant (p<0.01 and p<0.05, respectively in GFP-H-Ras and GFP-K-Ras transfectants).

FIG. 3. **EGF-stimulated H-Ras and K-Ras activation of PI3-K is inhibited by galectin-1.** H-Ras(wt) or K-Ras(wt) and H-Ras(wt)/galectin-1 or K-Ras(wt)/galectin-1 co-transfectants, and pcDNA transfectants were stimulated with EGF (5 min) then lysed as detailed in Fig. 1B and C. Immunoblot analysis verified that Ras isoform expression patterns under the different co-transfection conditions were similar. Lysates were subjected to immunoprecipitation with anti PI3-K p85 Ab and PI3-K activity was then determined. The $^{32}$P-labeled lipid products separated by TLC were visualized by an overnight exposure to an X-ray film. The phosphatidylinositol-3-phosphate (PIP) spots were then subjected to quantitative densitometry. The results of a representative experiment are shown where Gal-1 (-) denotes empty-vector transfections. Similar results were obtained in 2 additional experiments.

FIG. 4. **Signaling selectivity conferred on Ras by galectin-1.** Raf-1 and PI3-K activities were determined 48 h after transfection in H-Ras(G12V), H-
Ras(G12V/Y40C) and H-Ras(G12V/T35S) transfectants, or in the H-Ras(G12V)
mutants/galectin-1 co-transfectants and in pcDNA transfectants. Other experimental
details were as in Figs 2 and 3. A, Raf-1 activity in the cell lysates. Mean values
(±SD) of Raf-1 activity (\(^{32}\)P incorporated into MBP) from quadruplicate experiments
are shown. B, PI3-K activity in the cell lysates. The PIP spots of a representative
experiment as detected on an X-ray film are shown. Similar results were obtained in 3
additional experiments. C, Galectin-1 co-immunoprecipitates with H-Ras(G12V) or
with H-Ras(G12V/T35S) but not H-Ras(G12V/Y40C). The experiments were
performed as detailed in Fig 1A. Proteins were co-immunoprecipitated (IP) with
anti-Ras Ab from lysates of galectin-1/ H-Ras (G12V), galectin-1/ H-Ras
(G12V/T35S), or galectin-1/ H-Ras (G12V/Y40C) co-transfectants and
immunoblotted (IB) with either anti-Ras Ab or anti galectin-1 (Gal-1) Ab.
Immunoblots of a representative experiment are shown. Similar results were obtained
in two additional experiments.
FIG. 1
FIG. 2
FIG. 3

Gal-1 transfection

- - - - - + + +

EGF

- + - - + + +
**FIG. 4**

A

Graph showing Raf-1 activity (DPM x 10^-4) with different transfection conditions:

- **Gal-1 transfection**
  - pcDNA
  - H-Ras(12V)
  - H-Ras(12V40C)
  - H-Ras(12V35S)

B

Western blot analysis of PIP with the following conditions:

- **Gal-1 transfection**
  - pcDNA3
  - H-Ras(12V)
  - H-Ras(12V40C)
  - H-Ras(12V35S)

C

Immunoprecipitation (IP) and immunoblotting (IB) with anti-Ras and anti-gal-1 antibodies.
Galectin-1 augments ras activation and diverts ras signals to Raf-1 at the expense of phosphoinositide 3-kinase
Galit Elad-Sfadia, Ronit Haklai, Eyal Ballan, Hans-Joachim Gabius and Yoel Kloog

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