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It depends on the hinge: a structure-functional analysis of galectin-8, a tandem-repeat type lectin

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Galectin-8, a member of the galectin family of mammalian lectins, is made of two carbohydrate-recognition domains (CRDs), joined by a "hinge" region. Ligation of integrins by galectin-8 induces a distinct cytoskeletal organization, associated with activation of the extracellular-regulated kinase (ERK) and phosphatidylinositol 3-kinase signaling cascades. We show that these properties of galectin-8 are mediated by the concerted action of its two CRDs and involve both protein–sugar and protein–protein interactions. Accordingly, the isolated *N*- or *C*-CRD domains of galectin-8 or galectin-8 mutated at selected residues implicated in sugar binding (E251Q; W85Y, W248Y, W[85,248]Y) exhibited reduced sugar binding, which was accompanied by severe impairment in the capacity of these mutants to promote the adhesive, spreading, and signaling functions of galectin-8. Other mutations that did not impair sugar binding (e.g. E88Q) still impeded the signaling and cell-adherence functions of galectin-8. Deletion of the "hinge" region similarly impaired the biological effects of galectin-8. These results provide evidence that cooperative interactions between the two CRDs and the "hinge" domain are required for the proper functioning of galectin-8.

Key words: cell adhesion/galectins/protein–sugar interactions/signal transduction

Introduction

Galectin-8 is a mammalian lectin made of two carbohydrate-recognition domains (CRDs) joined by a "link peptide" of variable length (Hadari *et al*., 1995, 1997). Like other galectins, galectin-8 is a secreted protein. Upon secretion, it acts as a matrix protein equipotent to fibronectin in promoting cell adhesion by ligation and clustering of a selective subset of cell-surface integrins (Hadari *et al*., 2000; Levy *et al*., 2001). Complex formation between galectin-8 and integrins involves sugar–protein interactions and triggers integrin-mediated signaling cascades such as Tyr phosphorylation of focal adhesion kinase and paxillin, and a robust and sustained activation of the mitogen-activated protein kinase (MAPK) and phosphatidylinositol 3-kinase (PI3K) pathways (Levy *et al*., 2003). In contrast, when present as a soluble ligand, galectin-8 negatively regulates cell adhesion. Such a mechanism allows local signals emitted by secreted galectin-8 to specify territories available for cell adhesion and migration (Hadari *et al*., 2000; Levy *et al*., 2001; Zick *et al*., 2004; Arbel-Goren *et al*., 2005). Owing to its dual effects on the adhesive properties of cells and its association with fibronectin, galectin-8 meets several of the criteria characteristic of matricellular proteins (Bornstein and Sage, 2002). Galectin-8 levels of expression positively correlate with certain human neoplasms, prostate cancer being the best example studied thus far (reviewed in Zick *et al*., 2004). The overexpressed lectin presumably provides these neoplasms with growth and metastatic advantages due to its ability to modulate cell adhesion and cellular growth. Structurally, galectin-8 belongs to the tandem-repeat type galectins. Other members of this family are a 32-kDa galectin from *Caenorhabditis elegans* (CE-galectin) (Hirabayashi *et al*., 1992), as well as galectin-4 (Oda *et al*., 1993), -6 (Gitt *et al*., 1996), -9 (Wada and Kanwar, 1997), -12 (Yang *et al*., 2001), and -14 (Dunphy *et al*., 2002). Galectin-8 shares with them ~30–40% identity, but no homology to other galectins is found in its "link peptide." The *N*- and *C*-terminal domains of galectin-8 share ~35% homology and contain sequence motifs (e.g. HXNPR; WGXEE) that have been conserved among most CRDs of galectins (Wang *et al*., 1991; Drickamer and Taylor, 1993). Although galectin-8 has two CRDs, prototype members of the galectin family (e.g. galectin-1, -2, -7, -10, -11, and -13) function while having a single CRD by forming dimers. This suggests that each CRD of galectin-8 should contain enough structural information to function independently.

In the present study, we undertook to address this question and determine whether the isolated CRDs of galectin-8 can indeed function independently. Furthermore, we wished to assess the importance of the "hinge" region that links the two domains. This was of interest in view of the fact that different isoforms of galectin-8 differ in the length of their "hinge" domain (Bidon *et al*., 2001). Our results indicate that shortening of the "hinge" region severely impairs the biological activity of galectin-8. Furthermore, the isolated CRDs of galectin-8 manifest impaired biological activity, suggesting that tandem-repeat type galectins require cooperative interactions between the two CRDs and a properly oriented "hinge" region for effective function.

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Results

Generation and expression of mutated/truncated forms of galectin-8

Several mutated and truncated forms of galectin-8 were generated as recombinant proteins in bacteria, using the cDNA of rat galectin-8 as a template (Hadari *et al.*, 1995). These proteins served to perform a structure-functional analysis of galectin-8 (Table I). Mutants expressed at relatively low yields as native proteins were generated as glutathione *S*-transferase (GST)-fusion proteins. Mutations were introduced into conserved amino acids implicated in sugar binding of other galectins. These included E88Q and W85Y (at the *N*-CRD) and their corresponding sites E251Q and W248Y at the *C*-CRD. The double-mutant W*2Y (W85Y, W248Y) was generated as well. A unique feature of the *N*-CRD of galectin-8 is the presence of Ile90 instead of a conserved Arg residue within the common WG-E-*R*-PF motif present at the *C*-CRD of galectin-8 and other galectins, which has been implicated in sugar binding (Zick *et al.*, 2004). To assess the potential importance of Ile90, two mutants, I90R and the corresponding R251I, were generated. Truncated forms of galectin-8 were also studied. These included the isolated *N*-CRD (aa 1–158), *C*-CRD (aa 184–316) as well as a mutated form of galectin-8 whose "hinge" domain (S153-L184) was deleted and replaced with a short $(Gly)_6$ peptide. The expression of the above mutants was verified using galectin-8 antibodies (Figure 1A and B). The recombinant wildtype (WT) galectin-8 and its truncated/mutated forms were at least 85% pure following affinity chromatography purification over lactosyl-Sepharose beads, as judged by Coomassie Blue staining (Figure 1C). When resolved by means of gel filtration over a Superdex 75 FPLC (Amersham) column, both the native and the mutant forms of galectin-8 migrated as homogenous proteins having the same molecular mass (not shown). These results suggest that the mutations did not grossly alter the overall structure of the proteins.

Most truncated/mutated forms were biologically active as judged by their ability to agglutinate red blood cells with an agglutination titer of ~0.5 μM (Figure 2A and B). Furthermore, the agglutination resulted from sugar–protein interactions as judged by the ability of thiodigalactoside to inhibit this process (Figure 2C). The only two exceptions were the isolated *N*-CRD, which fails to dimerize and has a single CRD, and the double-mutant W*2Y, which lost most sugar-binding activity (Figure 1D and Table I). GST-galectin-8 and its truncated/mutated forms manifested a lower titer of agglutination (~0.125 μM), consistent with the fact that GST itself tends to form dimers (Maru *et al.*, 1996). Of note, the GST mutants W85Y and W248Y agglutinated red blood cells with a titer that was even lower than

Table I. Lactose-binding of WT and mutated/truncated forms of galectin-8

Construct	Structure	Lactose binding
Galectin-8 (WT)	*N*-CRD Hinge *C*-CRD W85, E88, I90 -------- W248, E251, R253	72% (100%)
NT	*N*-CRD W85, E88, I90	45% (63%)
I90R	*N*-CRD Hinge *C*-CRD R90 ---------- R253	63% (88%)
R253I	*N*-CRD Hinge *C*-CRD I90 ---------- I253	65% (91%)
E88Q	*N*-CRD Hinge *C*-CRD Q88 ---------- E251	70% (98%)
E251Q	*N*-CRD Hinge *C*-CRD E88 ---------- Q251	52% (72%)
GST-galectin-8	GST-*N*-CRD Hinge *C*-CRD W85, E88, I90 ---------- W248, E251, R253	72% (100%)
GST-CT	GST-*C*-CRD W248, E251, R253	48% (67%)
GST-Δ-hinge	GST-*N*-CRD- -GGGGGG- -*C*-CRD W85, E88, I90 W248, E251, R253	54% (75%)
GST-W85Y	GST-*N*-CRD Hinge *C*-CRD Y85 ---------- W248	45% (62%)
GST-W248Y	GST-*N*-CRD Hinge *C*-CRD W85 ---------- Y248	39% (54%)
GST-W*2Y	GST-*N*-CRD Hinge *C*-CRD Y85 ---------- Y248	16% (23%)

Summary of the experiment described in Figure 1. Lactosyl-agarose beads were incubated with 10 or 20 μg of recombinant WT galectin-8 or its mutated/truncated forms for 2 h at 4°C. Next, the beads were intensively washed with PBS. Samples of the proteins that remained bound and samples of the total proteins that were loaded were resolved by 10–15% SDS–PAGE and were stained with Coomassie Blue (Figure 1D). The intensity of the stained bands was quantitated by densitometry, and the percent of galectin-8 that remained bound to the beads out of the total amount of recombinant protein loaded was calculated. The numbers in parentheses refer to the efficiency of binding of each galectin-8 mutant relative to that of WT galectin-8, which was assigned to 100%. Results are an average of duplicate measurements that did not vary by more than 10%.

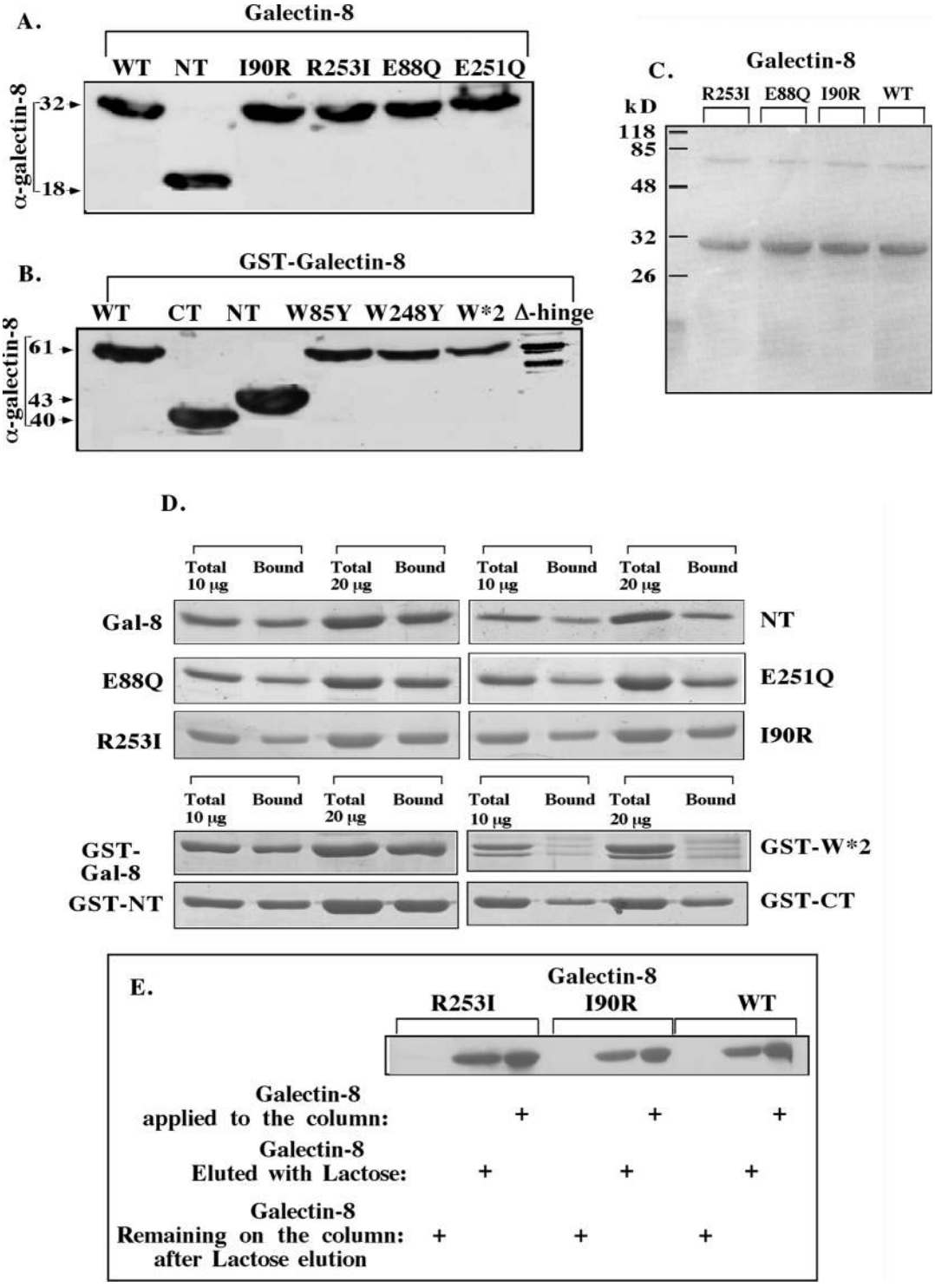

Fig. 1. Expression and lactose-binding capacity of mutated/truncated forms of galectin-8. (**A**) Bacterial expression pET3a plasmids encoding the indicated mutated/deleted forms of galectin-8 were transformed into the pLysS bacterial host. Total bacterial lysate was incubated with lactosyl-Sepharose beads. Bound proteins were eluted with 150 mM lactose. Samples were resolved by 10–15% SDS–PAGE, transferred to nitrocellulose membranes, and were western immunoblotted with galectin-8 polyclonal antibodies. (**B**) pGEX2T plasmids encoding mutated/deleted forms of GST-galectin-8 were transformed in the TOP10 bacterial host. Total bacterial lysates were incubated with glutathione-agarose beads. Bound proteins were eluted with 40 mM glutathione. Samples from each mutant were resolved by 10% SDS–PAGE, transferred to nitrocellulose, and western immunoblotted with galectin-8 polyclonal antibodies. Coomassie Blue stain of some of the samples is shown in (**C**). An aliquot of 10 or 20 μg of recombinant WT galectin-8 or its mutated/truncated forms was incubated with lactosyl-Sepharose beads for 2 h at 4°C. Next, the beads were intensively washed with PBS, and the bound proteins (Bound) were eluted by boiling of the beads in Laemmli's sample buffer (**D**). Some of the samples were eluted instead with 200 mM lactose (**E**). The total amount of the eluted proteins (**D**) and (**E**) and recombinant proteins at equivalent amounts to those originally loaded on the columns (Total) was resolved by 10–15% SDS–PAGE and were stained with Coomassie Blue. Results are of representative experiment carried out twice.

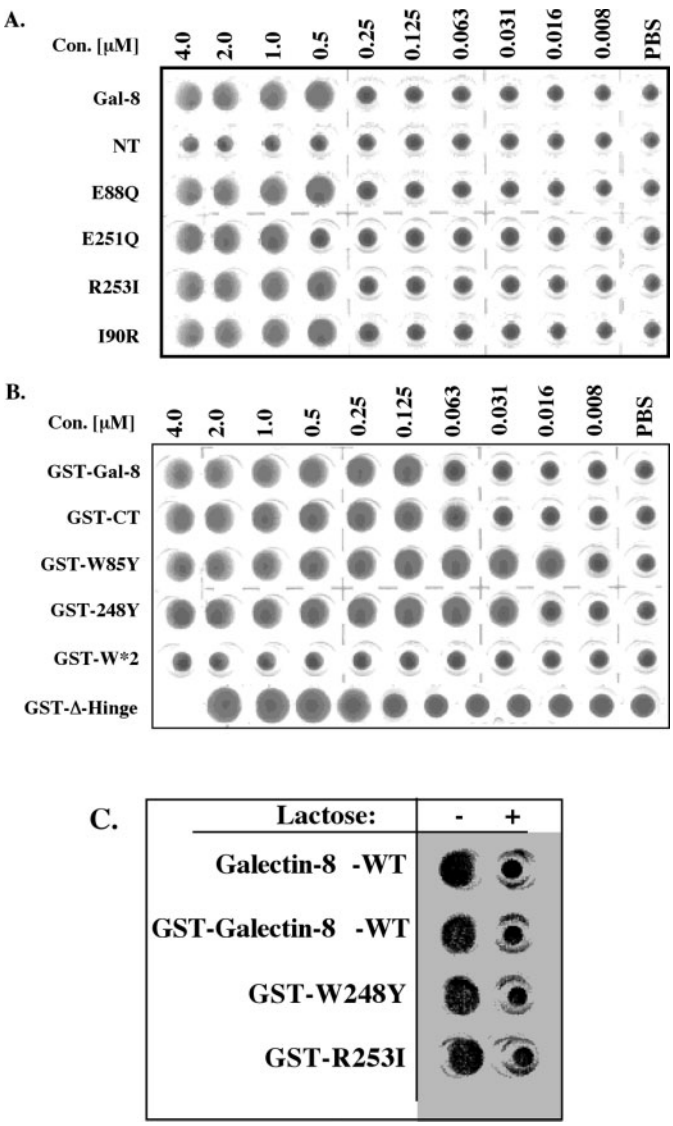

Fig. 2. Agglutination activity of galectin-8 and its mutated/truncated forms. Hemagglutination activity was measured by mixing serial dilutions of galectin-8 in PBS (50 μL per well) with a suspension of packed rabbit erythrocytes in PBS (50 μL per well) in micro-titer U-shape plates (**A**) and (**B**). Some of the samples, as indicated, were also incubated in the presence or absence of thiodigalactoside (20 mM) (**C**). Following 1 h incubation at 22°C, the agglutination activity was determined. Wells that contain spread red dot exhibit agglutination activity, while wells that contain concentrated small red dots lack agglutination ability. Results are of a representative experiment carried out three times.

that of the WT GST-galectin-8 (Figure 2). The reason for this increased agglutination capacity is presently unclear.

Sugar-binding activity of galectin-8 and its mutated/truncated forms

A major feature of galectins is their ability to bind sugars. Therefore, the binding capacity of WT galectin-8 and its mutated/truncated forms to lactosyl-Sepharose beads was examined. Similar results were obtained irrespective of whether the lectins were eluted from the columns with SDS (and boiling) (Figure 1D) or with 0.2 M lactose (Figure 1E). Moreover, no galectin-8 remained on the column following lactose elution (Figure 1E), suggesting that non-specific binding to the column was negligible. Consistent with our previous findings (Hadari *et al.*, 1995), WT galectin-8 was effectively bound to lactosyl-Sepharose beads with >70% of the applied protein being retained by the column (Figure 1D and Table I). Mutation of E88, implicated in sugar binding, had no effect on the ability of the mutant protein to bind to lactosyl-Sepharose. However, single mutations of other residues expected to be involved in sugar binding (W85, W248, and E251) reduced (>30%) the binding capacity of galectin-8 to the immobilized lactosyl residues (Figure 1D and Table I). These results suggest that the mutated amino acids, which are conserved among members of the galectin family, also play a role in the sugar-binding activity of galectin-8. This conclusion was supported by the fact that the double-mutant (W85Y/W248Y) lost almost 80% of its sugar-binding capacity. The reduced sugar-binding capacity of the mutants could be due to decreased affinity, but also due to a part of the protein being selectively modified for interaction with the lactose epitopes present on lactosyl-Sepharose. The involvement of W85, W248, and E251 in sugar binding was further supported by the predicted 3D model of galectin-8 that was based on the known X-ray structures of galectins-1 and -2 (Rini and Lobsanov, 1999). This model suggested that E251 is presumably involved in binding to the glucose moiety of lactose, while W85 and W248 are part of the galactose-binding site.

The mutants I90R and R253I exhibited similar sugar-binding activity as WT, with 88 and 91% of lactose-binding capacity of the WT galectin-8, respectively (Figure 1D and Table I). This suggests that Ile90 does not play a special role in sugar binding. In contrast, a truncated form of galectin-8 which contained only its *N*-terminal (NT) CRD exhibited lower lactose-binding capacity (~60%). Similarly, the isolated *C*-CRD (GST-CT) exhibited 67% lactose-binding capacity of the WT galectin-8, in accordance with the idea that tandem binding of both CRDs is required for optimal interaction of galectin-8 with glycoconjugates. Of interest, truncation of the "hinge" region reduced by 25% of the lactose-binding capacity of galectin-8 (Table I), indicating that impaired orientation of the two CRDs, as in GST-Δ-hinge, affects the sugar-binding capacity of the protein.

Mutated/truncated forms of galectin-8 are impaired in their ability to mediate the adhesive and anti-adhesive functions of galectin-8

We have previously shown that immobilized galectin-8 acts as an extracellular matrix protein that promotes cell adhesion, whereas soluble galectin-8, like soluble fibronectin or collagen, inhibits adhesion of suspended cells (Levy *et al.*, 2001). Therefore, the mutated/truncated forms of galectin-8 were studied for their ability to modulate cell adhesion. As shown in Figure 3A and in Table II, the immobilized E88Q mutant reached only 60% of the maximal adhesive capacity of WT galectin-8, while the immobilized E251Q mutant promoted cell adhesion similar to WT galectin-8. In contrast, soluble E251Q was ineffective in inhibiting cell adhesion to fibronectin-coated plates, while soluble E88Q was only modestly impaired in its ability to inhibit cell adhesion

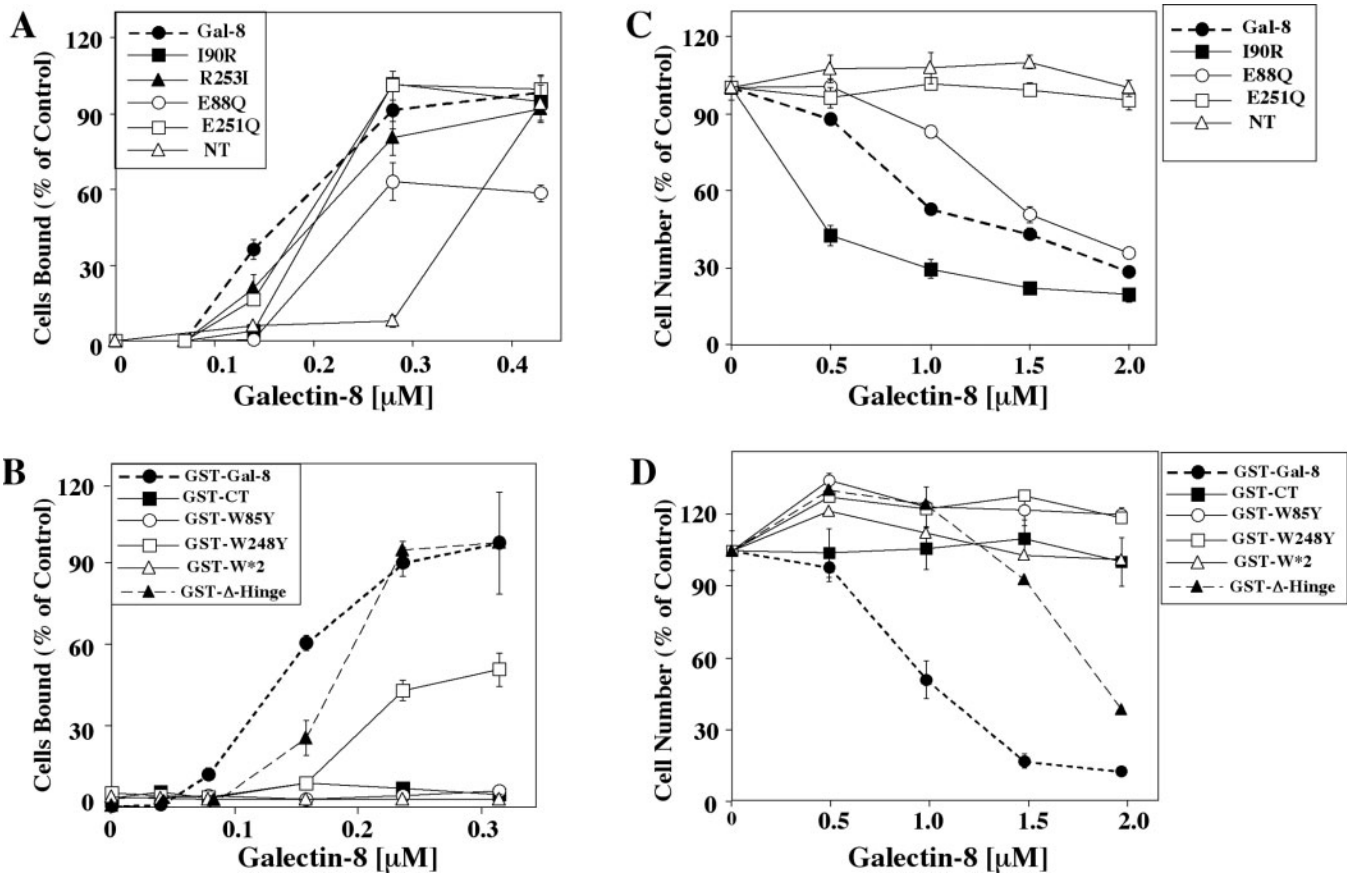

Fig. 3. Modulation of cell adhesion by WT and mutated/truncated forms of galectin-8. (**A**) and (**B**) *Cell adhesion over immobilized galectin-8*: 96-well bacterial plates were precoated with the indicated concentrations of WT galectin-8 or its truncated/mutated forms. CHO-P cells (4×10^6 cells/mL) were resuspended in serum-free medium, and 100 μL aliquots were seeded on the coated 96-well plates. Following 2 h incubation at 37°C, the plates were washed with PBS, stained with Crystal Violet, and the number of adherent cells was determined using ELISA-reader at 540 nm. Values are mean ± SD of tetraplicate measurements of a representative experiment. (**C**) and (**D**) *Inhibition of cellular adhesion by WT and mutated galectin-8*: CHO-P cells were detached from culture plates with 5 mM EDTA and were washed with PBS. The cells were resuspended in serum-free medium at a concentration of a 4×10^6 cells/mL and were incubated in suspension for 1 h at 37°C with the indicated concentrations of WT galectin-8 or its truncated/mutated forms. Next, the cells were washed with PBS, and 100 μL aliquots were seeded on 96-well tissue culture plates that were precoated with fibronectin and were further blocked with 1% BSA. Following 2 h incubation at 37°C, the cells were washed, stained with Crystal Violet, and the number of adherent cells was determined using ELISA-reader at 540 nm. Values are mean ± SD of tetraplicate measurements of a representative experiment.

(Figure 3C and Table II). Of note, the E88Q mutation did not impair the abilities of galectin-8 to bind lactose or to agglutinate RBC, suggesting that modulation of adhesion induced by galectin-8 not only involves protein–sugar interactions, but presumably includes protein–protein interactions as well. The immobilized mutants GST-W85Y and GST-W*2Y were completely inactive in promoting cell adhesion (Figure 3B), while GST-W248Y reached 52% of the maximal adhesive capacity of GST-WT. When added in a soluble form, GST-W85Y, GST-W248Y, and GST-W*2Y were ineffective in promoting the anti-adhesive effects of galectin-8 (Figure 3D). These results suggest that amino acids involved in sugar binding are differently involved in regulating cell adhesion. While amino acids W85 and W248 are more important participants in regulating cell adhesion, amino acids E88 and E251 seem to play a somewhat minor role. Furthermore, it seemed that at least for some of the mutants, there was no direct correlation between loss of sugar-binding activity and adhesive properties of galectin-8. For example, the E88Q mutant that retained maximal sugar-binding activity lost ~40% of its adhesive activity, while E251Q was as effective as WT galectin-8 in promoting cell adhesion, while losing ~30% of its sugar-binding capability. The results further suggest that the inhibitory effects of soluble galectin-8 are more sensitive to mutations than the adhesive properties of the immobilized protein, which promote cell adhesion (Table II). Next, the ability of the mutants I90R and R253I to regulate cell adhesion was examined. These immobilized mutants induced cell adhesion with similar potency as WT galectin-8 (Figure 3A). Similarly, when added as soluble ligands, I90R (Figure 3C) and R253I (not shown) were largely as potent as WT galectin-8 in promoting the anti-adhesive properties of the soluble lectin.

The capacity of the truncated forms of galectin-8 to affect cell adhesion was also examined. As shown in Figure 3A, immobilized NT-galectin-8 exhibited the same maximal adhesive activity as WT galectin-8, although higher concentrations of the NT-galectin-8 were required. This might stem from the fact that NT-galectin-8, like monomeric

Table II. Activities of WT and mutated/truncated forms of galectin-8

Construct	Lactose binding	Agglutination	Signaling	Cell adhesion	Anti-adhesive activity
Galectin-8 (WT)	(100%)	+	+	+	+
GST-galectin-8	+	+	+	+	+
I90R	+	+	+	+	+
R253I	+	+	+	ND	ND
GST-Δ-hinge	↓	+	↓	↓	↓
NT	↓	None	↓	↓	None
E88Q	+	+	↓	↓	↓
E251Q	↓	+	+	+	None
GST-W248Y	↓	+	↓	↓	None
GST-CT	↓	+	None	None	None
GST-W85Y	↓	+	None	None	None
GST-W*2Y	↓↓↓	–	None	None	None

Summary of the experiment described in Figures 1–8. The activity of WT galcetin-8 was taken as 100%. The ability of the various galectin-8 mutants to bind to lactosyl-Sepharose beads, agglutinate red blood cells, promote or inhibit cell adhesion, and trigger signaling pathways is presented in a qualitative manner, to enable overall comparison of the different mutants.
↓ Reduced activity <50%.
↓↓↓ Reduced activity >50%.
ND, not determined.
None, no activity.

galectins, tends to form dimers when present at high enough concentrations. However, soluble NT-galectin-8 was essentially ineffective in inhibiting cell adhesion to fibronectin-coated plates (Figure 3C). These findings indicate that NT-galectin-8, although capable of sugar binding, is markedly less potent than the full-length protein in modulating cell adhesion. GST-Δ-hinge partially lost its adhesive and anti-adhesive properties, while the truncated form GST-CT was devoid of any adhesive or anti-adhesive activities (Figure 3B and D). These results suggest that the isolated *C*-CRD of galectin-8 is functionally inactive, while the *N*-CRD maintains, at least partially, the properties of the WT protein. They further suggest that improper linking of the two domains, manifested by the Δ-hinge mutant, impairs the biological activity of galectin-8. Hence, proper juxtaposition of the NT and CT domains of galectin-8 might be needed to promote the adhesive and anti-adhesive activities of this lectin, which cannot independently operate with its individual domains. Of note, all mutants bound to tissue culture or bacterial plates with efficiency that was at least equal to that of WT galectin-8 (data not shown). Still, to ensure that failure of certain mutants to support cell adhesion is not due to steric hindrance or denaturation of the immobilized lectins, an alternative approach was used in which GST-galectins were immobilized to glutathione-coated plates. We could demonstrate that GST-CT, like other mutants (GST-W85Y, GST-W248Y, and GST-W*2Y) was immobilized onto glutathione-coated plates with the same efficiency as WT galectin-8 (Figure 4A). Still, unlike WT galectin-8, GST-CT and GST-W248Y (Figure 4B) as well as GST-W85Y and GST-W*2Y (not shown) failed to promote cell adhesion, suggesting that differences in the ability of the mutants to promote cell adhesion could not be attributed to differences in their intrinsic binding properties to the plates.

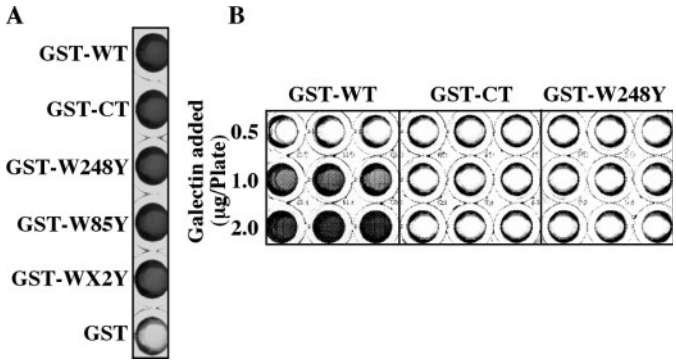

Fig. 4. Cell adhesion onto galectin-8 proteins immobilized on glutathione-coated plates. (**A**) GST-galectin-8 (WT) and GST-galectin-8 mutants: GST-CT, GST-W248Y, GST-W85Y, GST-W*2Y, and GST (control) were added to glutathione-coated 96-well plate at a concentration of 2 μg well. The plate was incubated for 2 h at 22°C and was then washed three times with PBS/0.1% TWEEN-20 and once with PBS. Binding of the different galectin-8 proteins to the plates was determined by ELISA using galectin-8 antibodies and peroxidase-conjugated second antibodies. (**B**) GST-galectin-8, WT, and mutants (GST-CT and GST-W248Y) were added to glutathione-coated 96-well plates at a concentration of 0.5, 1.0, and 2.0 μg/100 μL in triplicates. The plates were incubated for 2 h at 22°C and were then washed three times with PBS/0.1% TWEEN-20 and once with PBS. CHO-P cells 4×10^5/well were seeded on the coated 96-well plates and were incubated for 2 h at 37°C. At the end of incubation, the cells were washed and stained with Crystal Violet.

Impaired adhesive properties of the mutated/truncated forms of galectin-8 translate into impairment in their signaling capacity

We have previously shown that cell adhesion onto immobilized galectin-8 results in activation of the MAPK and PI3K cascades (Levy *et al.*, 2003). We have therefore studied the

ability of the different mutants to activate these signaling pathways. Cells were allowed to adhere onto bacterial plates precoated with mutated/truncated forms of galectin-8; cell extracts were prepared; and activation of extracellular-regulated kinase (ERK)-1,2, protein kinase B (PKB), and p70 S6 kinase (p70S6K) was assayed. Cells adherent to NT-galectin-8 and GST-Δ-hinge exhibited reduced activation of ERK, PKB, and p70S6K (Figures 5 and 6), in accordance with their reduced ability to promote the adhesive and anti-adhesive properties of galectin-8. Signaling mediated by the isolated *C*-CRD could not be assessed because it failed to support cell adhesion (Figure 3).

The signaling capacity of the E88Q mutant was partially impaired, in accordance with its impaired adhesive and anti-adhesive activities. ERK activation was more transient in cells adherent to the E88Q mutant, and the activity returned almost to basal levels already by 30 min (Figure 7A). Similarly, stimulation of PKB and p70S6K was weaker and more transient in cells adherent to the E88Q mutant (not shown). Signaling induced by the GST-W248Y mutant was also impaired. Activation of ERK-1,2 was reduced in cells

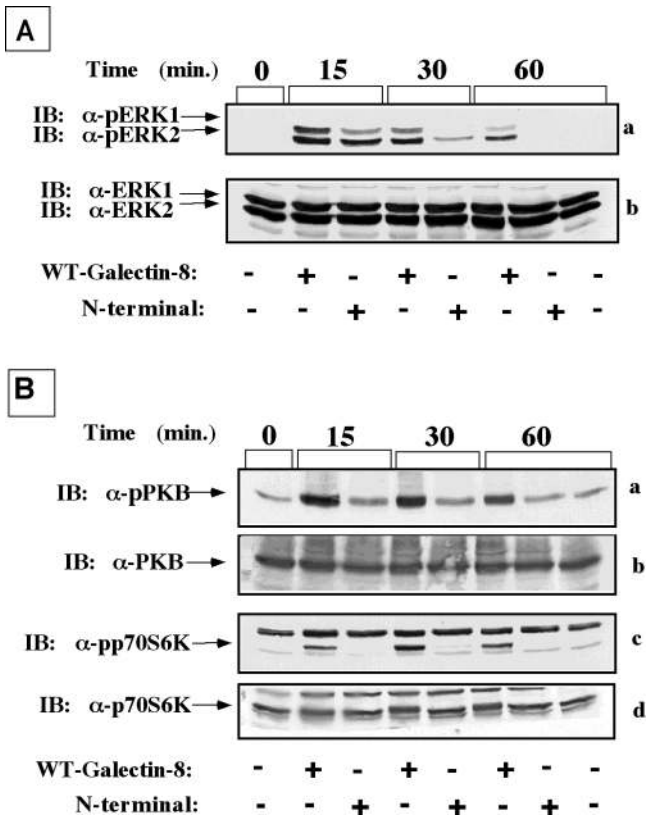

Fig. 5. Effects of galectin-8 and its isolated NT domain (*N*-CRD) on activation of signaling pathways. CHO-P cells were starved, detached with 5 mM EDTA, washed, and were suspended in serum-free medium for 30 min before being seeded for the indicated times on bacterial plates, coated with 0.7 μM of WT or NT galectin-8. Cells were extracted with buffer I and proteins were resolved by SDS–PAGE, transferred to nitrocellulose membranes, and western immunoblotted with anti-phospho and total Erk-1,2 (**A**—a, b), anti-phospho and total PKB (**B**—a, b), or anti-phospho and total p70S6k (**B**—c, d) antibodies. Results are of a representative of two independent experiments.

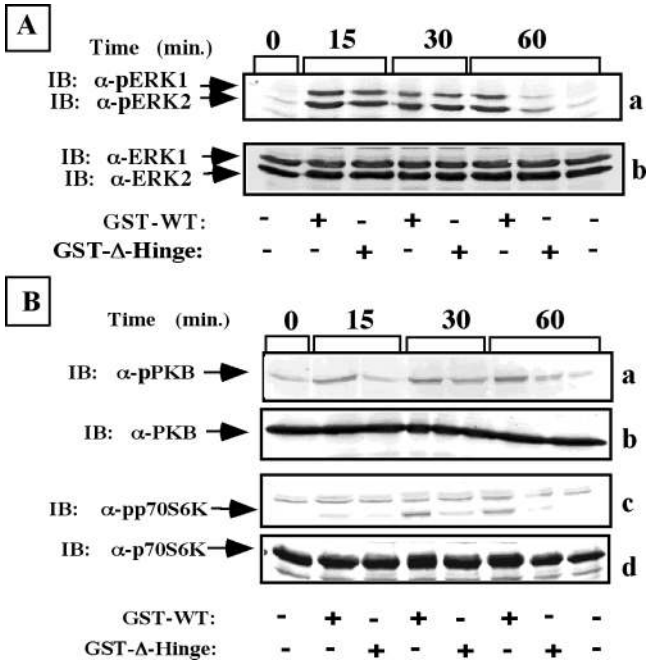

Fig. 6. Effects of galectin-8-Δ-hinge on activation of signaling pathways. Bacterial plates were coated with 0.7 μM GST-WT galectin-8 or GST-galectin-8-Δ-hinge. Suspended CHO-P cells were added to the plates, and the experiment was carried out as described in the legend to Figure 5. Results are of a representative of two independent experiments.

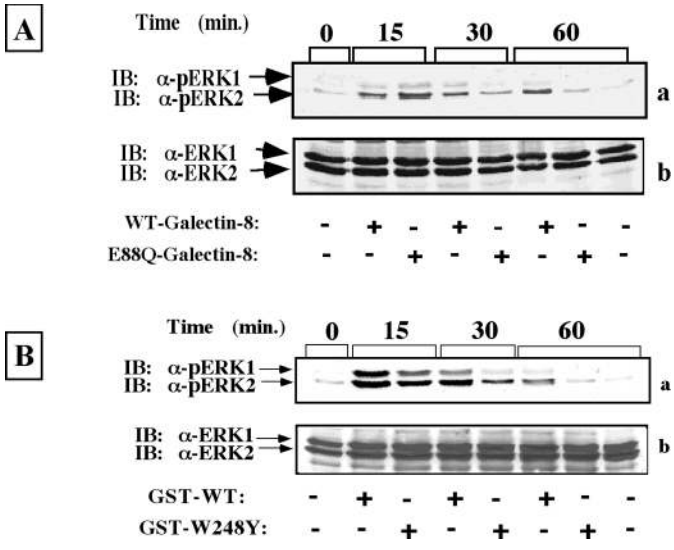

Fig. 7. Effects of galectin-8 mutants E88Q and W248Y on ERK activity. CHO-P cells were starved, detached with 5 mM EDTA, washed, and were suspended in serum-free medium for 30 min before being seeded for the indicated times on bacterial plates, coated with 0.7 μM of WT, E88Q (**A**), or W248Y (**B**) mutants of galectin-8. Cells were extracted with buffer I, and proteins were resolved by SDS–PAGE, transferred to nitrocellulose membranes, and western immunoblotted with anti-phospho or total Erk-1,2. Results are of a representative of two independent experiments.

adherent to GST-W248Y (Figure 7B); similarly, activation of PKB and p70S6K was largely reduced (not shown). These results indicate that the impaired adhesive capacity

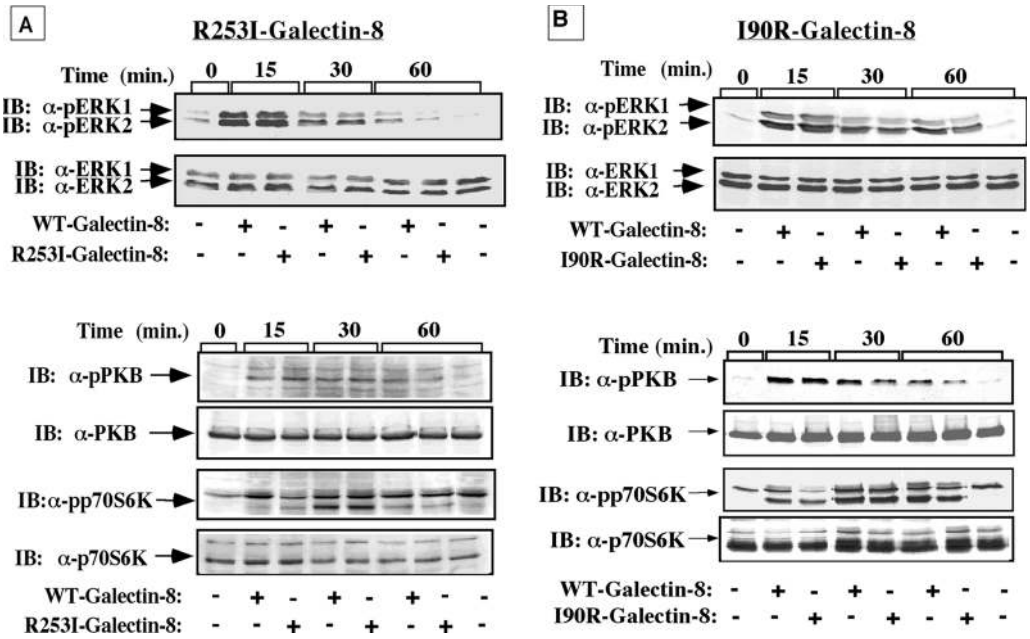

Fig. 8. Activation of the MAPK and PI3K signaling pathways in CHO-P cells adherent onto the I90R and R253I mutants of galectin-8. CHO-P cells were starved, detached with 5 mM EDTA, washed, and were suspended in serum-free medium for 30 min before being seeded for the indicated times on bacterial plates, coated with 0.7 μM of WT, R253I (**A**), or I90R (**B**) mutants of galectin-8. At the end of incubation, cells were extracted with buffer I, and proteins were resolved by SDS–PAGE, transferred to nitrocellulose membranes, and western immunoblotted with anti-phospho and total Erk-1,2, anti-phospho and total PKB, or anti-phospho and total p70S6K antibodies, as indicated. Results are of a representative of two independent experiments.

of the galectin-8 mutants translates to impairment in their ability to signal to downstream effectors. Accordingly, the mutants R253I, I90R (Figure 8), and E251Q (not shown), the adhesive properties of which were largely preserved, activated the ERK and PI3K pathways to the same extent as WT galectin-8. The signaling of the W85Y and the W*2 mutants could not be recorded because these mutants failed to support cell adhesion.

Cytoskeletal organization of cells adherent to mutated/truncated forms of galectin-8

Cell adhesion and spreading over galectin-8 leads to a distinctive cytoskeletal organization and formation of F-actin microspikes (Levy *et al.*, 2003). Therefore, the ability of cells to spread and form microspikes over mutated/truncated forms of galectin-8 was studied. Chinese hamster ovary (CHO)-P cells adherent for 10 min onto the E88Q, E251Q, I90R, and R253I mutants of galectin-8 exhibited the same cytoskeletal organization as cells adherent onto WT galectin-8, characterized by formation of sheet-like projections, termed lamellipodia (Figure 9). After 2 h, microspikes were observed as in cells adherent onto WT galectin-8 (Figure 9). In contrast, cells adherent onto the GST-W248Y and the GST-Δ-hinge mutants were still round after 10 min of adhesion and did not properly spread even after 2 h (Figures 9 and 10). The impaired ability of cells to spread over these two mutants was also reflected by the attenuated rate of cell adherence to these mutants (Figure 11). This contrasted with other mutants (e.g. R253I) that supported cells adhesion at the same rate as WT galectin-8 (Figure 11). These effects were not restricted to CHO-P cells but appeared to be a more general phenomenon. As shown in Figure 12, spreading of human endothelial (HE) cells was practically abolished, and no microspikes were formed when the cells adhered onto GST-W248Y galectin-8. Similarly, cell spreading over the GST-Δ-hinge mutant of galectin-8 was associated with the formation of elongated lamellipodium protrusions that differed from the smooth network of radial microspikes observed when cells spread over WT galectin-8.

Truncation of the *C*-CRD of galectin-8, exemplified by NT-galectin-8, not only inhibited cell adhesion (Figure 3) and signaling (Figure 5) but also affected cell spreading. After 10 min, most of the cells failed to spread over NT-galectin-8 (Figure 9), while after 120 min, the cells exhibited elongated filopodia (Figure 10) that were not observed when the cells spread over WT galectin-8. These results suggest that the *C*-terminal half of galectin-8 may be involved in regulating microspike length and density. They further suggest that impaired sugar binding and/or improper juxtaposition of the two CRDs of galectin-8 (as in the GST-Δ-hinge galectin-8) mainly affects its ability to promote cell spreading, while having less severe impact on cell adhesion.

Discussion

In the present study, we performed structure-functional analysis of galectin-8 to determine the elements necessary for its function as a modulator of cell adhesion. Because galectin-8 is a tandem-repeat type galectin having two CRDs, the main question we aimed to address was whether each of the two CRDs of galectin-8 is functionally independent. For that

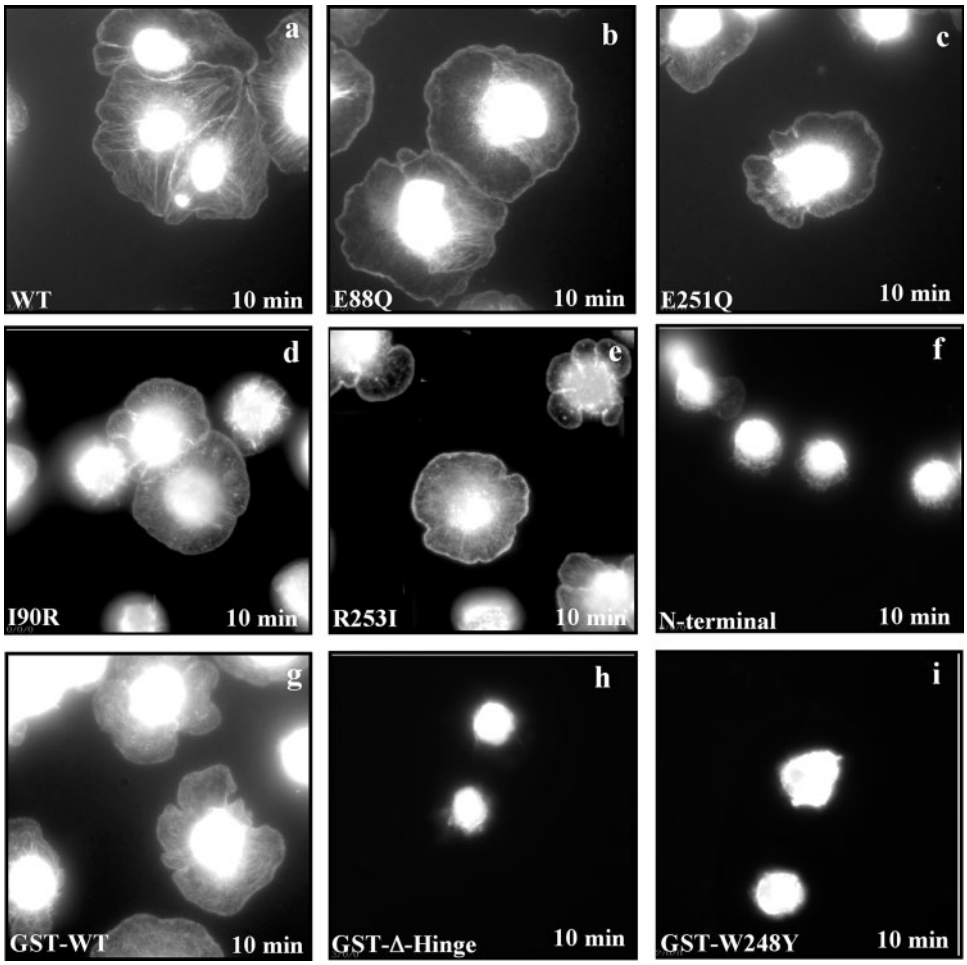

Fig. 9. Short-term spreading of CHO-P cells on galectin-8 and its mutated/truncated forms. Cover glasses were precoated for 2 h at 22°C with galectin-8 or its mutated/truncated forms (0.7 μM): WT (**A**), E88Q (**B**), E251Q (**C**), I90R (**D**), R253I (**E**); *N*-CRD (**F**), GST-WT (**G**), GST-Δ-hinge (**H**), or GST-W248Y (**I**). CHO-P cells were starved for 16 h, detached with 5 mM EDTA from their plates, washed, and were suspended in serum-free medium for 30 min. Then, the cells were seeded on the indicated coated slides. Following 10 min of incubation, cells were fixed and stained with TRITC-phalloidin for actin staining.

purpose, we deleted regions and mutated amino acids that were implicated in sugar binding of galectins (Barondes *et al*., 1994). Then, we explored the ability of the mutated/truncated forms of galectin-8 to bind sugars, to induce intracellular signaling cascades, and to modulate cell adhesion. The results of the above analysis led us to conclude that the two CRDs of galectin-8 are not functionally independent. A proper orientation of the two CRDs, determined by the length of the linker peptide, is required for the proper functioning of this lectin. The results further suggest that while sugar-binding activity is a key feature required for the proper functioning of galectin-8, other structural elements, not involved in sugar binding, affect the signaling capacity and adhesive properties of this lectin.

Several lines of evidence support these conclusions. First, we could show that mutations that have only a minor impact on the sugar-binding capacity of galectin-8 have profound effects on its ability to promote transmembrane signaling exemplified by the activation of the PI3K and MAPK cascades (Table II). The best example is the E88Q mutant, whose sugar-binding activity is essentially identical to that of the WT galectin-8, while its ability to stimulate the PI3K pathway is severely impaired. In contrast, its counterpart mutant E251Q lost almost 30% of its sugar-binding capacity without appreciable effects on its signaling capacity. The dichotomy between sugar binding and signaling activity suggests that structural elements not directly involved in sugar binding might also mediate the signaling capacity of galectin-8. These elements, most likely, are involved in protein–protein interactions between galectin-8 and its cell-surface receptors. Still, the importance of sugar binding for the proper functioning of galectin-8 should not be dismissed. This was evident by the fact that mutations that severely impaired sugar binding of galectin-8 such as the double-mutant W85/248Y resulted in complete loss of its signaling and adhesive properties. Even the single W248Y mutation severely impaired the signaling and adhesive capabilities of galectin-8. These results also support our model, which implies involvement of ERK, PKB, and p70S6K signaling pathways in adhesion and spreading of cells on galectin-8 (Levy *et al*., 2003).

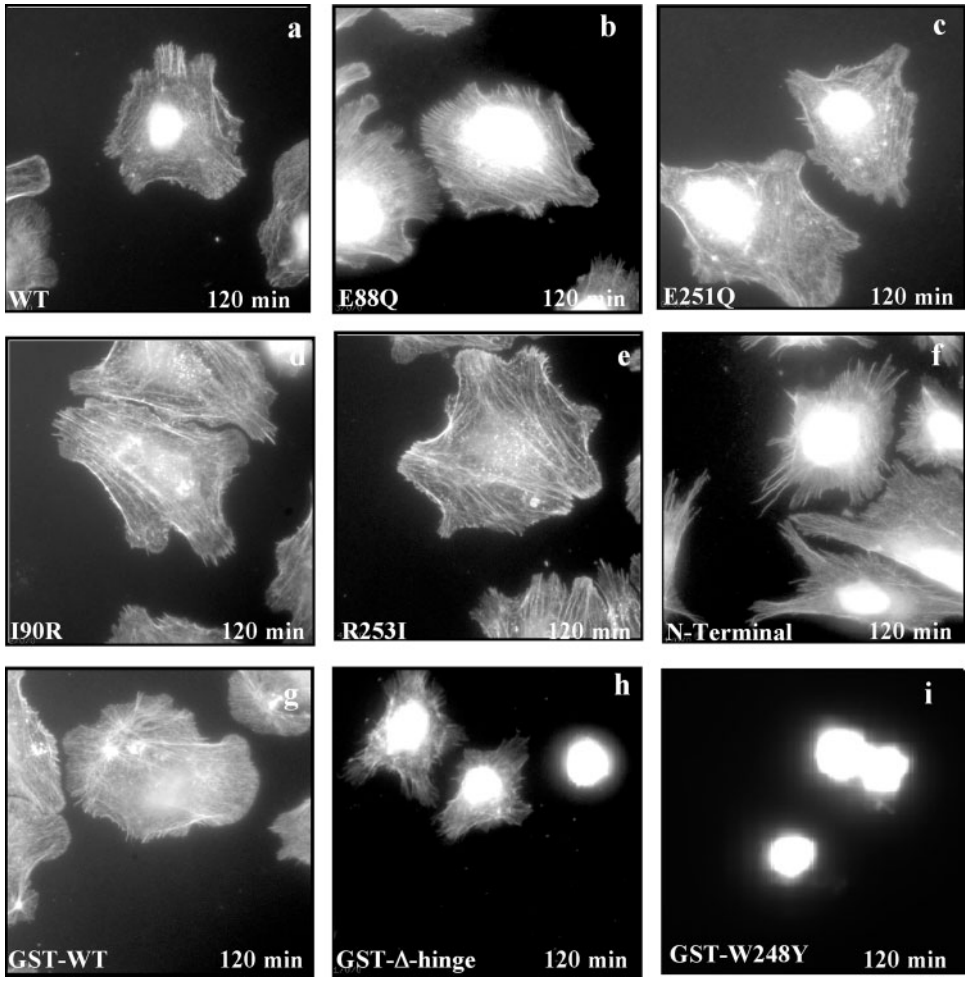

Fig. 10. Long-term spreading of CHO-P cells on galectin-8 and its mutated/truncated forms. Cover glasses were precoated for 2 h at 22°C with galectin-8 or its mutated/truncated forms (0.7 μM): WT (**A**), E88Q (**B**), E251Q (**C**), I90R (**D**), R253I (**E**), *N*-CRD (**F**), GST-WT (**G**), GST-Δ-hinge (**H**), or GST-W248Y (**I**). CHO-P cells were starved for 16 h, detached with 5 mM EDTA from their plates, washed, and were suspended in serum-free medium for 30 min. Then, the cells were seeded on the indicated coated slides. Following 120 min of incubation, cells were fixed and stained with TRITC-phalloidin for actin staining.

In several cases (e.g. E251Q), the impairment in the ability of the soluble lectins to inhibit adhesion to fibronectin-coated plates was more prominent than the impaired adhesive functions of the same immobilized lectin. This suggests that fibronectin might interact with a wider spectrum of integrins than galectin-8; therefore, galectin-8 mutants that have lost binding to a given integrin subtype might fail to inhibit cell adhesion to fibronectin, while still maintaining partial binding to other integrin subtypes which enable them to support cell adhesion when immobilized.

Some of the mutants such as NT or the GST-Δ-hinge had impaired signaling, which translated into impaired adhesion, anti-adhesion, and/or spreading capacity of cells on the mutants (Table II). Impaired cell adhesion and impaired signaling correlated best, indicating that when galectin-8 mutants lose binding even to a subset of integrins, it translates into impaired signaling ability. Still, alterations in adhesive properties and signaling characteristics did not always translate into changes in cytoskeletal organization (cf. E88Q). This suggests that effective compensatory mechanisms enable cells adherent to these mutants to maintain proper cytoskeletal organization even when major signaling cascades such as the PI3K and MAPK pathways are only partially functional. Furthermore, only limited aspects of the putative signaling repertoire of galectin-8 were studied here (e.g. activation of PKB, S6K1, and MAPK). Therefore, it is plausible that defects in other signaling pathways translate into impaired adhesion, spreading, or both.

The presence of Ile instead of an Arg at position 90 of galectin-8 is a hallmark of this lectin. Overall, mutation of this residue to Arg, the corresponding amino acid at the *C*-CRD of galectin-8, did not result in any major functional alterations. The only difference was that the soluble I90R mutant inhibited cell adhesion with a greater potency than WT galectin-8, suggesting that Ile90 negatively regulates the anti-adhesive properties of soluble galectin-8. Mutation of R73 of galectin-1 that corresponds to I90 and R253 of galectin-8 abolished its sugar-binding activity (Hirabayashi and Kasai, 1991). Because the mutants I90R and R253I

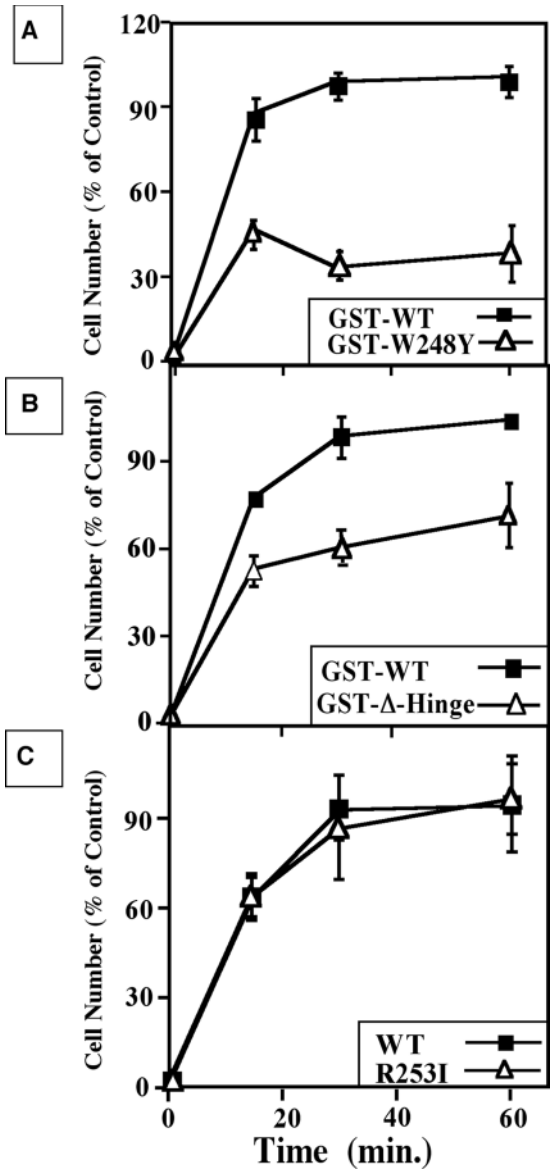

Fig. 11. Rate of adhesion of CHO-P cells on galectin-8 or its truncated/mutated forms. ELISA plates were coated for 2 h at 22°C with 0.7 μM of WT galectin-8, GST-WT galectin-8, or its truncated/mutated forms GST-W248Y (**A**); GST-Δ-hinge (**B**); or R253I (**C**). CHO-P cells were starved for 16 h, detached with 5 mM EDTA from their plates, washed, and were suspended in serum-free medium for 30 min. Then, the cells were incubated on the coated plates for the indicated times. The plates were washed and stained with Crystal Violet. The number of adherent cells was determined using ELISA-reader at 540 nm. Values are mean ± SD of tetraplicate measurements of a representative experiment.

showed almost the same lactose-binding capacity as WT galectin-8, we conclude that Ile90 has the same effects on sugar binding as Arg253.

A major thrust of this study was aimed at elucidating the functional autonomy of the two CRDs of galectin-8. Our results clearly indicate that the isolated domains of galectin-8 are functionally impaired. This translates into impaired sugar binding, adhesive and anti-adhesive effects, reduced signaling capacity, and altered cytoskeletal organization. The impairment is better manifested by the isolated *C*-CRD, which is practically devoid of adhesive and anti-adhesive capabilities. This might reflect the fact that cell-surface integrins of CHO-P cells lack the specific sugar epitopes required for binding to GST-CT. The isolated *N*-CRD maintains impaired adhesive functions but no anti-adhesive capacities. The impaired adhesive and signaling properties of the *N*-CRD are reflected by the reduced rate of cellular spreading on this truncated lectin and by the unique cytoskeletal organization of cells having elongated filopodia that were not observed when cells spread over WT galectin-8. These findings clearly indicate that the presence of both CRDs is necessary for the proper functioning of galectin-8. They further suggest that the function of this tandem-repeat type galectin is not a simple additive effect of its isolated domains (Table II).

An important outcome of this study is the realization that the proper functioning of galectin-8 clearly depends not only upon the presence of its two CRDs but also upon their proper orientation, determined by the length of the linker or "hinge" region. This was an unexpected result in view of the fact that the model structure of galectin-8, which was based upon the crystal structure of galectin-1 dimers, clearly indicated that the interactions of the two CRDs occur along interphases that do not involve the linker peptide (Zick *et al.*, 2004). One could therefore predict that replacement of the native "hinge" region with a Gly_6 peptide will not alter the juxtaposition of the two CRDs. This apparently was not the case as the Δ-hinge mutant was severely impaired in its capacity to promote the adhesive and signaling capabilities of galectin-8. This was clearly evident by the reduced activation of MAPK and PI3K pathways in cells adherent to the Δ-hinge mutant, as well as by the impaired ability of the cells to organize their cytoskeleton in a manner characteristic of cells adherent onto WT galectin-8. These results differ from findings related to galectin-9, where it was demonstrated that shortening of the "hinge" domain had little impact on the functioning of this lectin (Sato *et al.*, 2002). Hence, galectin-8 and galectin-9 presumably evolved differently with regard to their capacity to coordinate the functions of their isolated domains.

In conclusion, our results are consistent with the hypothesis that galectin-8 must be viewed as a single functional entity whose two CRDs must be properly oriented and act in concert to elucidate the adhesive and signaling functions of this lectin. This contrasts with the more prevailing view of an "antibody model" in which each CRD of a tandem-repeat type galectin can function independently. Because galectin-8 can exist in several isoforms, which vary in the length of their "hinge" domain, further studies are required to determine how such variations affect the overall function of this lectin.

Materials and Methods

Materials

The galectin-8 used was a bacterially expressed recombinant protein, encoded by the cDNA of rat galectin-8 (Hadari *et al.*, 1995). Isopropyl-β-D-thiogalactopyranoside

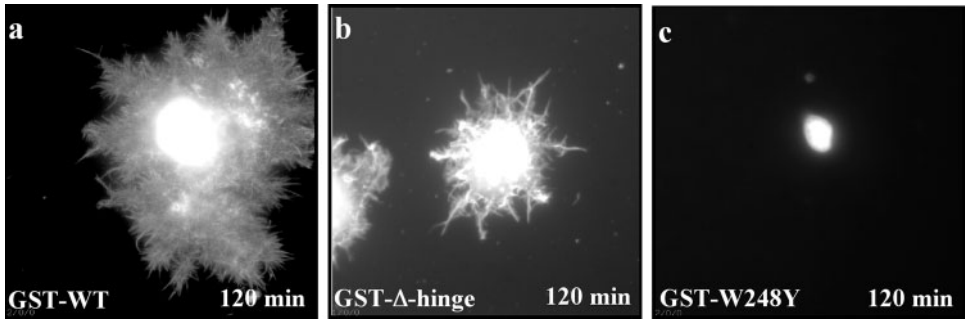

Fig. 12. Long-term spreading of HE cells on galectin-8 or its mutated/truncated forms. Cover glasses were precoated for 2 h at 22°C with galectin-8 or its mutated/truncated forms (0.7 μM): GST-WT (**A**), GST-Δ-hinge (**B**), or GST-W248Y (**C**). HE cells were starved for 16 h, detached with 5 mM EDTA from their plates, washed, and were suspended in serum-free medium for 30 min. Then, the cells were seeded on the indicated coated slides. Following 120 min of incubation, cells were fixed and stained with TRITC-phalloidin for actin staining.

was purchased from MBI Fermentas (Amherst, NY). Glutathione-coated HS 96-well plates, glutathione-agarose beads, Crystal Violet, and tetramethylrhodamine isothiocyanate (TRITC)-labeled phalloidin were purchased from Sigma Chemicals (St. Louis, MO). Restriction enzymes were from New England BioLabs (Beverly, MA). Agarose and Optimem were from GIBCO BRL (Grand Island, NY). Glutathione was purchased from Merck (Darmstadt, Germany).

Cell cultures

Naïve CHO-P cells were grown in F12 medium containing 10% fetal calf serum (FCS). HE ECV304 cells (Takahashi *et al*., 1990) were grown in DMEM-F12 medium containing 10% FCS.

Antibodies

Polyclonal anti-phospho-PKB (Ser^{473}), anti-p70S6K, and anti phospho-p70S6K (Thr^{389}) antibodies were from New England BioLabs. Polyclonal anti-ERK1,2, anti-PKB, and monoclonal anti phospho-ERK1,2 (Thr^{183}, Tyr^{185}) antibodies were kindly provided by R. Seger (Weizmann Institute, Rehovot, Israel). Affinity-purified polyclonal antibody (1.1) against galectin-8 was generated as described (Hadari *et al*., 1995).

Generation of mutated/truncated forms of galectin-8

Site-directed mutagenesis was performed using a QuikChange kit (Stratagene, Cedar Creek, TX) according to the manufacturer's instructions. pET-3a-galectin-8 (Hadari *et al*., 1995; Paz *et al*., 1996) served as a template.

NT. The NT fragment of galectin-8 (aa 1–158), in which Glu159 was replaced by a stop codon, was generated using a set of overlapping primers: 5′-GCTCGGATTTACAG AGTATGTAA*AGATCT*ACTCTGGGACTG-3′ and 5′-CAGTCCCAGAGT*AGATCT*TTACATACTCTGTAAA TCCGAGC-3′ (an additional restriction site for Bgl-II is italicized).

I90R. Mutation of Ile 90 to Arg was introduced using a set of overlapping primers: 5′-GGGGCTGGG AGGAG*CGTACG*CACGACATGCCTTTCAG-3′ and 5′-CTGAAAGGCATGTCGTG*CGTACG*CTCCAGCCC-3′ (an additional restriction site for BsiWI is italicized).

R253I. Mutation of Arg 253 to Ile was introduced using a set of overlapping primers: 5′-GGATGCCTGGGGAGA AGAGGAG*ATTAAT*ATTACCTGCTTCCC-3′ and 5′-GGGAAGCAGGTAAT*ATTAAT*CTCCTCTTCTCCCC A GGCATCC-3′ (an additional restriction site for AseI is italicized).

E88Q. Mutation of Glu 88 to Gln was introduced using a set of overlapping primers: 5′-CTGACAAATGAGAAA TGGGGCTGGCAGGAGATCAC*TCATGA*CATG-3′ and 5′-CATG*TCATGA*GAGATCTCCTGCCAGCCCCATTT CTCATTTGTCAG-3′ (an additional restriction site for BspHI is italicized).

E251Q. Mutation of Glu 251 to Gln was introduced using a set of overlapping primers: 5′-CAGGATGCCTGGG GAGAACAGGAGAGA*AATATT*ACCTGC-3′ and 5′-GCAGGT*AATATT*TCTCTCCTGTTCTCCCCAGGCAT CCTG-3′ (an additional restriction site for SspI is italicized).

All PCR products were digested at their respective additionally introduced restriction sites to confirm the mutations. Next, all mutations were confirmed by DNA sequencing.

Generation of mutated/truncated forms of GST-galectin-8

Site-directed mutagenesis was performed using a QuikChange kit (Stratagene) according to the manufacturer's instructions. pGEX-2T encoding GST-galectin-8 (Hadari *et al*., 1995; Paz *et al*., 1996) served as a template.

GST-Δ-hinge. The hinge region of galectin-8 (Ser 153-Leu184) was replaced by six glycines (*GGGGGCGGAGG GGGCGGG*; $(Gly)_6$) and was generated using pET-3a-galectin-8 as a template. First, constructs encoding NT-$(Gly)_6$ and $(Gly)_6$-CT were generated using the following primers—for NT-$(Gly)_6$: 5′-ATGTTGTCCTTAAGCAA TCTACAA-3′ and 5′-AAATGG*′CCC′GCC′CCC′TCC′ GCC′CCC′*GAATCTGAACCCGATGGA-3′; for $(Gly)_6$-CT: 5′-AGATTC*′GGG′GGC′GGA′GGG′GGC′GGG′*CCATTTG AAGCAAGGTTG-3′ and 5′-CTACCAGCTCCTTACAT

CCAG-3′. Next, the constructs were annealed in their overlapping $(Gly)_6$ sequence. To generate GST-fusion proteins, BamHI and EcoRI restriction sites were introduced by PCR using the above annealed product as a template and a set of primers: 5′- GGG GGG GGATCC CATATG TTG TCC TTA AGC AAT CTA CAA-3′ and 5′- GGG GGG *GAA TTC* GGATCC CTA CAA GCT CCT TAC ATC GAC-3′. The PCR products were cloned into pGEM-T plasmid, digested with BamHI and EcoRI, and ligated into pGEX-2T plasmid.

GST-CT. The *C*-terminal fragment of galectin-8 (aa 184–316), in which Leu182 and Ser183 were replaced by a restriction site for BamHI, was generated using the following set of overlapping primers: 5′-GTCTGGCAAGCTC CAT*GGATCC*CTGCCATTTGAAGCAAGGTTG-3′ and 5′-CAACCTTGCTTCAAATGGCAG*GGATCC*ATGGA GCTTGCCAGAC-3′. The PCR product was digested with BamHI and was ligated into pGEX-2T.

GST-W85Y. Mutation of Trp85 to Tyr was introduced using the following set of overlapping primers: 5′-CAAATG AGAAATACGGCTGGGAGGAGATCAC*TCA TGA*C ATGCC-3′ and 5′-GGCATG*TCATGA*GTGATCTCCTC CCAGCCGTATTTCTCATTTG-3′ (an additional restriction site for BspHI is italicized).

GST-W248Y. Mutation of Trp 248 to Tyr was introduced using the following set of overlapping primers: 5′-CTC CTTTCTTCAGG*ATGCAT*ACGGAGAAGAGGAGAG AAACATTAC-3′ and 5′-GTAATGTTTCTCTCCTCTT CTCCGT*ATGCAT*CCTGAAGAAAGGAG-3′ (an additional restriction site for NsiI is italicized).

*GST-W*2 (W85Y + W248Y).* Mutation of Trp 248 to Tyr was introduced as above (GST-W248Y), using pGEX-2T-W85Y-galectin-8 as a template. All mutations were confirmed by restriction site analysis and DNA sequencing.

Expression and purification of mutated/truncated forms of galectin-8

To express tag-free mutated/truncated forms of galectin-8, pET-3a constructs were introduced into the pLysS bacterial host. Alternatively, GST-galectin-8 and its mutated/truncated forms, in the pGEX-2T constructs, were introduced into TOP10 bacterial host. The tag-free mutated/truncated forms of galectin-8 were produced and were affinity-purified over a column of lactosyl-Sepharose as we described (Hadari *et al*., 1995). GST-galectin-8 and its mutated/truncated forms were purified over glutathione-agarose beads and were eluted with 40 mM of reduced glutathione in phosphate-buffered saline (PBS), pH = 7.2.

Binding assay of galectin-8 to lactosyl-Sepharose beads

Galectin-8 and its mutated/truncated forms (10–20 μg) were incubated with 30 μL of packed lactosyl-Sepharose beads for 2 h at 4°C. Next, the beads were washed twice with 1 mL of 1% Triton X-100 in PBS and once with 1 mL PBS. The bound proteins were dislodged from the column with Lamellae's sample buffer, boiled for 5 min, resolved by means of 10–15% SDS–PAGE, and were stained with Coomassie Blue. Alternatively, the bound proteins were eluted with 0.2 M lactose. The amount of the proteins eluted from the column was calculated by densitometry of the intensity of the eluted bands and the intensity of the samples of the total protein amounts (10 or 20 mg) that were loaded on the columns. The ratio of eluted to total protein loaded yielded the binding efficiency of each galectin-8 isoform. Non-relevant proteins (BSA control) failed to interact with the column.

Agglutination activity of galectin-8

Hemagglutination activity was measured by mixing serial dilutions of galectin-8 in PBS (50 μL per well) with a suspension of packed rabbit erythrocytes in PBS (50 μL per well) in micro-titer U-shape plates. Following 1 h incubation at 22°C, the agglutination activity was determined as we described (Hadari *et al*., 2000).

Cell-adhesion assay

Bacterial or tissue culture plates were precoated for 2 h at 22°C with galectin-8 or its deleted/truncated forms in PBS, pH 7.2. CHO-P cells, grown in F12 medium containing 10% FCS, were detached from the plates with 5 mM EDTA, washed with PBS, resuspended in serum-free medium, and re-seeded on the coated plates. At the indicated times, cells were washed, and the adherent cells were stained with 0.2% Crystal Violet in a solution containing 20% methanol in PBS for 10 min at 22°C. Excess dye was removed by washes with water, and cells were solubilized in 1% SDS for 1 h at 22°C. The amount of adherent cells was quantified by measuring the absorbance at 540 nm in an ELISA plate reader-TECAN (Spectra, Austria). Specific binding was defined as the difference between the absorbance of cells bound to ligand-coated wells and the absorbance of cells bound to BSA-coated wells. All assays were performed in tetraplicates.

Anti-adhesion assay

96-well bacterial or tissue culture plates were precoated at 37°C with 100 μL of 0.01 μM fibronectin. Unbound ligand was washed after 1 h, and the plates were further blocked with 1% BSA in PBS for 1 h at 37°C. CHO-P cells were detached from tissue culture plates with 5 mM EDTA, washed once with PBS, and resuspended in serum-free medium (4×10^6 cells/mL) in the absence or presence of soluble galectin-8 or its truncated/mutated forms. Following 1 h incubation at 37°C, the cells were washed with PBS and were seeded (100 μL per well) on the fibronectin-coated wells at 37°C. After 2 h, the cells were washed three times with PBS, and the number of adherent cells was quantified following staining with Crystal Violet as described above.

Preparation of cell extracts and immunoblotting

Cell extracts were prepared in buffer I (25 mM Tris/HCl, 25 mM NaCl, 0.5 mM EGTA, 2 mM sodium orthovanadate, 10 mM NaF, 10 mM sodium pyrophosphate, 80 mM β-glycerophosphate, 1% Triton X-100, 0.5% deoxycholate, 0.05% SDS, 5 μg/mL leupeptin, 10 μg/mL trypsin inhibitor, and 1 mM phenylmethylsulfonyl fluoride, pH 7.5). Insoluble

material was removed by 15 min centrifugation (20,000 × *g*) at 4°C. Supernatants were mixed with 5× concentrated Laemmli's sample buffer, boiled for 5 min, and were resolved on 10% SDS–PAGE under reducing conditions. Proteins were transferred to nitrocellulose membranes and western immunoblotted with the indicated antibodies.

Immunofluorescence microscopy

Glass coverslips, placed in 12-well plates, were precoated at 22°C with 1 mL solutions of galectin-8 or its truncated/mutated forms (0.7 μM in PBS). After 2 h, the ligands were removed by suction. CHO-P or HE cells were starved for 16 h in serum-free medium at 37°C. The cells were detached from the tissue culture plates with 5 mM EDTA and were suspended for 30 min at 37°C in serum-free medium (2–4 × 10^5 cells/mL). Then, the cells were allowed to adhere to the precoated slides for 10–120 min. At the end of the incubation, cells were washed three times with PBS, fixed, and permeabilized for 5 min with paraformaldehyde (3%) containing 0.5% Triton-X 100 and were further fixed for 25 min with paraformaldehyde (3%). Cells were washed (×3) with PBS and were incubated with TRITC-phalloidin for actin staining. The coverslips were washed, mounted onto glass microscope slides, and were examined on Zeiss Axioskop microscope.

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Conflict of interest statement

None declared.

Abbreviations

CHO-P, Chinese hamster ovary; CRDs, carbohydrate-recognition domains; ERK, extracellular-regulated kinase; FCS, fetal calf serum; GST, glutathione *S*-transferase; HE, human endothelial; MAPK, mitogen-activated protein kinase; NT, *N*-terminal; p70S6K, p70 S6 kinase; PBS, phosphate-buffered saline; PI3K, phosphatidylinositol 3-kinase; PKB, protein kinase B; TRITC, tetramethylrhodamine isothiocyanate; WT, wildtype.

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