Vol. 217, No. 1, 1995 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
December 5, 1995 Pages 292-303

GALECTIN-3 IS A NUCLEAR MATRIX PROTEIN WHICH BINDS RNA*

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Received October 19, 1995

SUMMARY: The endogenous galectin-3 is a carbohydrate-binding protein of M_r~30,000 serving in the cytoplasm and on the cell surface as a receptor for ligands containing poly-N-acetyllactosamine sequences. In addition, galectin-3 has been demonstrated to be associated in the nucleus with ribonucleoprotein complexes and to act as a pre-mRNA splicing factor and to be involved in spliceosome assembly. However, little is known about either its nuclear localization or its ligand(s), respectively. We demonstrate directly here that galectin-3 is associated with the RNA protein skeleton of the nucleus,*i.e.*, the nuclear matrix, and binds with single-stranded DNA (ssDNA) and with RNA. The affinity of binding was determined to be 2.3 μM. Lactose, which inhibits galectin-3 binding to glycoconjugates, failed to inhibit either galectin-3-ssDNA or galectin-3-RNA binding. Galectin-3 exhibited the highest affinity to poly(A) ribonucleotide homopolymers. The results presented here shows that galectin-3 may act as a RNA-binding protein in the nuclear matrix in a non-carbohydrate-dependent manner.

* This work was supported by Grant R01-CA46120 from NIH. A.R. is the recipient of the Paul Zuckerman Support Foundation for Cancer Research. K.J.P. is supported by Physician Scientist Award CA60156 and in part by CA57453.

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Abbreviations used are: Bm, maximum binding; BSA, bovine serum albumin; hnRNP, heterogeneous nuclear ribonucleoprotein; IEF, isoelectricfocusing; Kd, dissociation constant; SDS-PAGE, sodium-dodecylsulfate-polyacrylamide gel electrophoresis; snRNP, small nuclear RNA; ssDNA, single-stranded DNA.

0006-291X/95 $12.00

The galectins are a growing family of vertebrate carbohydrate-binding proteins sharing affinity for β-galactosides and conserved sequence homology in the carbohydrate-binding domain (galectin-1 to galectin-8) (1-3). Galectin-3 (a.k.a. Mac-2, CBP-35, mL-34, L-29, hL-31 and εBP) (4-8) is a M_r ~30,000 protein composed of two distinct structural motifs, an amino-terminal half of Gly-X-Y tandem repeats characteristic of collagens with limited homology to hnRNPs (5) and a carboxy-terminal half containing the carbohydrate-binding site (4-9). Although galectin-3 is presumed to be involved in cell growth, differentiation, inflammation, transformation and metastasis via interactions with specific ligands, the mechanism of its diverse actions have not yet been fully elucidated.

Galectin-3 has been localized to the cytoplasm, to the cell surface and to the nucleus. Cell surface galectin-3 has been implicated in cellular recognition and adhesion (10) while nuclear galectin-3 has been associated with pre-mRNA splicing and in spliceosome assembly (12). Small nuclear RNA complexed with proteins (snRNP) hnRNA, the precursors to mRNA, have been shown to be associated with the nuclear matrix (13,14) which is the RNA-protein skeleton of the nucleus involved in DNA and RNA organization and function (15). The nuclear matrix has been shown to be the structural site for RNA binding, processing and splicing (16,17). Thus, we questioned whether galectin-3 is a member of the nuclear matrix proteins, and whether it is capable of DNA and RNA binding. Here, we report that galectin-3 is a structural component of the nuclear matrix exhibiting carbohydrate-independent RNA as well as ssDNA binding properties.

MATERIALS AND METHODS

Nuclear Matrix Preparation. Nuclear matrices were prepared according to the established methodology (15,18). Dunning rat prostate adenocarcinoma MAT-LyLu cells growing in culture were detached using trypsin, which was then neutralized by re-adding media with 10% fetal calf serum. Cells were then spun at 800x g for 10 minutes to produce a pellet and then resuspended on ice with 0.5% Triton X-100 to release the lipids and soluble proteins in a buffered solution containing 2mM vanadyl ribonucleoside, an RNAase inhibitor. Salt extraction with 0.25M ammonium sulfate with vanadyl ribonucleoside was added to release the soluble cytoskeletal elements. DNAase-1 and RNAase-A at 25 degrees centigrade were used to remove the soluble chromatin and RNA. The remaining fraction contains the intermediate filaments and nuclear matrix proteins, and this fraction was disassembled with 8 M urea, and the insoluble components, which consist principally of carbohydrates and extracellular matrix components, were pelleted. The urea was then dialyzed out, and the intermediate filaments then reassemble and were separated out by centrifugation. The soluble nuclear matrix proteins were then ethanol precipitated. All solutions contain freshly made 1 mM phenylmethylsulfonylfluoride (PMSF) to inhibit serine proteases. The protein composition was determined by resuspending the proteins in 0.1 N sodium

hydroxide and using the BCA protein assay (Pierce, Rockford, IL), with BSA as a standard. For gel electrophoresis, the ethanol precipitated nuclear matrix proteins were redissolved in a sample buffer consisting of 9 M urea, 65 mM CHAPS, 2.2% ampholytes, and 140 mM dithiothreitol.

Two-Dimensional Electrophoresis and Immunoblot. High resolution two-dimensional gel electrophoresis was carried out utilizing the Investigator 2-D gel system (Millipore Corporation, Bedford, MA). One dimensional isoelectric focusing was carried out for 18,000 volt-hours using 1 mm X 18 cm tube gels after prefocusing. The tube gels were extracted and placed on top of 1 mm 10% sodium dodecyl sulfate-polyacrylamide electrophoresis slab gels and the gels were electrophoresed with 12° C. constant temperature regulation. The gels were then fixed with 50% methanol and 10% acetic acid. The gels were thoroughly rinsed and treated with 5% glutaraldehyde and 5 mM dithiothreitol after buffering with 50 mM phosphate (pH 7.2). After rinsing overnight, the gels were stained with silver stain. (Accurate Chemical Co., Inc., Westbury, NY). Forty micrograms of nuclear matrix protein were loaded for each gel. Protein molecular weight standards were Silver Standards from Diversified Biotechnology (Newton Centre, MA). Isoelectric points were determined using carbamylated standards from Sigma Chemical Company (St. Louis, MO). Immunoblots were performed on two-dimensional gels performed as previously described (Herman et al., 1978), using anti-Mac-2 rat monoclonal antibody (Boehringer Mannheim). Gels were incubated in 0.025 M tris base, 0.04 M glycine and 20% methanol for 5 minutes. The gels were then transferred to Immobilon-P (Millipore Corporation, Bedford, MA) with 0.3 M tris base, 10% methanol and 0.025 M tris base, 10% methanol using the Milliblot graphite transfer apparatus for one hour. The membranes were then removed and processed as follows. The membrane was incubate overnight in tris-buffered saline (TBS) with 0.1% tween and 10% nonfat dry milk at 4° C. The membranes were then washed with TBS/0.1% tween and subsequently incubated with the primary monoclonal anti-galectin-3 antibody at 1:200 dilution in TBS for one hour at room temperature. After additional washes in TBS/0.1% tween, the membranes are incubated with the secondary antibody, horseradish peroxidase labeled anti-rat IgG (H&L) (Pierce Chemical Company, Rockford, IL) diluted 1:10,000 in TBS. Enhanced chemiluminescence (ECL) was performed using the ECL procedure from Amersham International (Arlington Heights, IL).

Human recombinant galectin-3 expression and purification. Recombinant human galectin-3 was expressed in Escherichia Coli and purified as previously described in detail (19).

Gel mobility shift assay. Oligonucleotides (12-20 bases of dA, dC, dG, dT from USB or Pharmacia and 20 random base oligonucleotide synthesized commercially) were labeled with T4 polynucleotide kinase to a specific activity of $2x10^6$ cpm/ µg (20) . *In vitro* RNA transcript of a linearized plasmid from Boehringer Mannheim was prepared according to manufacture's instruction. The DNA template was destroyed by addition of 50U RNase-free DNase. RNA was end-labeled by T4 polynucleotide kinase with a specific activity of $6x10^6$ cpm/mg. ^{32}P end-labeled RNA or oligonucleotides large than ~2000 cpm and purified recombinant galectin-3 in the amount indicated in the text were mixed in 20 µl binding buffer (20mM Tris-HCl pH 7.2, 20mM NaCl, 1mM EDTA, 10% glycerol, and 0.2mM DTT). After incubated at room temperature for 30 min and on ice for another 30 min to allow complex formation, the mixtures were analyzed on 5% non-denatured polyacrylamide gel with 0.5x TBE as running buffer. The gels were then dried and autoradiographed with intensifying screen for more than 16 hours at -70° C.

Southwestern blot analysis. Four µg recombinant galectin-3 was separated on 12.5% SDS-PAGE and electrophoretically transferred to nitrocellulose filters

(BioTrace NT, Gelman Sciences). The filters were renatured for 1 hour at 4°C in the solution same as gel motility shift binding buffer plus 0.1% NP-40, and blocked for 1 hour at 4°C in renaturing solution with 5% non-fat milk. Then the filters were probed in the same blocking buffer with ^{32}P-labeled ssDNA (~10^5 cpm) for 1 hour at 24°C. The filters were washed 3 times with renaturing buffer and exposed overnight at -70°C.

Filter binding assay. The assay was performed as previously described (21) with the following modification: Fifty fmol ^{32}P-labeled 20 base random sequence oligonucleotides were mixed with increasing amount of purified galectin-3 in 50 µl gel motility shift binding buffer for 30 min at room temperature and another 30 min on ice. The mixtures were then filtered through a pre-soaked nitrocellulose filter (BioTrace NT, Gelman Sciences), and washed three times with 50 µl binding buffer. The amount of radioactivity on the filter was determined by liquid scintillation counting. Each assay includes a control for DNA retention without any added protein. This value was never larger than 3 % input DNA. For each set of measurements, the background was subtracted. The dissociation constant Kd and maximum binding Bm were determined by plotting the fraction of bound oligonucleotides (maximum binding of no sugar as 100%) as a function of galectin-3 concentration using GraphPad Prizm software package.

RESULTS

Galectin-3 in nuclear matrix. Nuclear matrices of Dunning rat prostate adenocarcinoma MAT-LyLu cells were extracted from semi-confluent cultures and resolved by IEF followed by SDS-PAGE as previously described (15,18). The silver stain of the MAT-LyLu nuclear matrix separated by the 2D-gel electrophoresis revealed the typical pattern multiple spots (Fig. 1A) and confirming the overall pattern previously reported (22), including a protein of 29-34 Kilodalton with a pI of 6.1. To determine whether this protein spot is the endogenous galectin-3 present in the nuclear matrix, a non-stained duplicate gel was electrotransferred onto immobilon membrane and

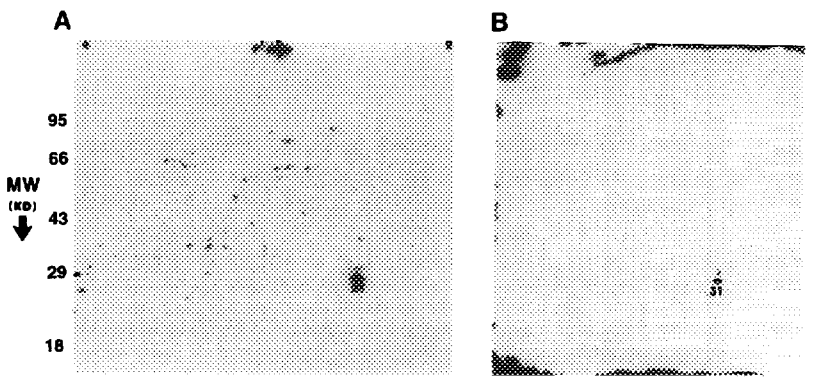


Figure 1. Two-dimensional gel electrophoresis of Dunning rat prostate adenocarcinoma MAL-LyLu nuclear matrix. A. Silver stain B. Immunodetection of galectin-3 by the rat anti-Mac-2 monoclonal antibody.

processed for immunodetection of galectin-3 utilizing the anti-mouse Mac-2 monoclonal antibody. Fig. 1B clearly shows that the monoclonal antibody recognizes the ~30 Kilodalton spot as galectin-3. Thus, it can be concluded that the nuclear matrix contains the galectin-3.

Galectin-3 interacting with single-stranded DNA. Single-stranded DNA was chosen initially for studying possible nucleic acids binding to galectin-3 due to its overall equivalence to RNA in binding properties of some hnRNPs (23-29). Protein-oligonucleotides interactions were detected *in vitro* by three different methods: 1) Southwestern blot analysis, 2) gel mobility shift assay and 3) filter binding assay.

For this study we have used the human recombinant galectin-3 which was purified by affinity chromatography and has been shown to retain the structural and functional properties of the endogenous galectin-3 (19).

1) Southwestern blot analysis. Galectin-3 and bovine serum albumin (BSA, negative control) were separated on SDS-PAGE, and were either stained with Commassie-Blue (Fig. 2A) or electrotransferred into nitrocellulose membrane (Fig. 2B).

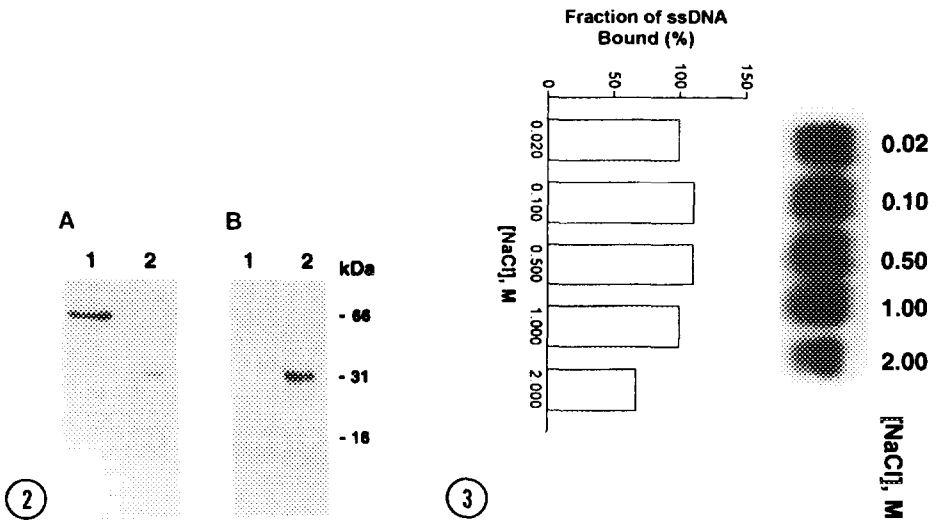


Figure 2. Southwestern analysis of purified galectin-3 binding activity with ssDNA. (A) Coomassie brilliant blue-stained gel. Four µg bovine albumin (lane 1) and galectin-3 (lane 2) were separated by 12.5% SDS-PAGE and stained with Coomassie brillant blue. (B) Southwestern analysis. A gel identical to that of panel A was transferred onto a nitrocellulose filter which was hybridized with ^{32}P-labeled 20 base random sequence ssDNA. The migration of protein molecular weight markers is indicated on the right.

Figure 3. Gel mobility shift assay analysis of galectin-3 binding to ssDNA in the presence of different concentration of NaCl. 16 µM galectin-3 were mixed with 2000cpm ^{32}P-labeled 20 base random sequence oligonucleotides and in the presence of 20 mM, 100mM, 500mM, 1M and 2 M NaCl,respectively. Autoradiograms at several exposures were quantified on a densitometer. Value in the presence of 20 mM NaCl is arbitrarily designated as 100.

After quenching, the filter was probed by ^{32}P-labeled 20 base random sequence oligonucleotides. The 31 kDa band corresponding to galectin-3 was visualized after autoradiography (Fig. 2B, lane 2), while the band corresponding to BSA was not visualized (Fig. 2B, lane 1). These results revealed that the ssDNA binds specifically to galectin-3 as it fails to bind to BSA or any other proteins present in the quench solution.

2) Gel mobility shift assay. Gel mobility shift assay was employed for further studies on galectin-3 binding to random sequence and to homo oligonucleotides (12-18 bases dA, dC, dG and dT). Consistent with above results, the galectin-3 bound to random sequence ssDNA (Fig. 3). Addition of NaCl to the binding solution as high as 1 M concentration did not significantly affect the galectin-3 DNA-binding (Fig. 3), at 2 M NaCl the binding was reduced by 40% (Fig. 3). This relative salt resistance of galectin-3-DNA binding is not surprising and resembles several hnRNPs that bind specific RNA homopolymers in a salt-resistant manner (30-31). In order to further examine possible preference for galectin-3 binding to specific ssDNA sequences, competition assays were performed in fixed amount of ^{32}P-labeled random sequence DNA with 10-fold excess amount of unlabeled homo oligonucleotides (12-18 bases oligo dA, dC, dG and dT). Although galectin-3 could bind to all of four homo oligonucleotides (Fig 4A), the competition assay revealed that galectin-3 exhibited the highest affinity toward poly(A) than other the sequences (Fig 4B). Thus, galectin-3 can function as a ssDNA binding protein *in vitro* and exhibited differential affinity among ssDNA.

3) Filter binding assay. Filter binding assay was used to quantitatively estimate the binding of galectin-3 to ssDNA. As depicted in Fig. 5, galectin-3 bound to 20 base random sequence oligonucleotides in a dose-dependent manner, and the dissociation constant Kd is 2.3 μM.

Effect of saccharides on galectin-3 binding to ssDNA. Galectin-3 is composed of two structurally and functionally distinct domains which are independently folded (4-9). Therefore, it was of interest to determine which domain might be involved in galectin-3 binding to ssDNA. The effect of lactose, which is the competitive inhibitor of galectin-3 carbohydrate-binding domain interacting with glycoconjugates, on galectin-3 binding to ssDNA was thus examined, since it may unveil whether this domain is involved in galectin-ssDNA binding (Fig.5). Assuming the maximum binding (Bm) is 100% in the absence of any sugar, addition of 100 mM lactose did not affect the binding to single-stranded oligonucleotides (Kd=1.5 μM, Bm=98%). The same amount

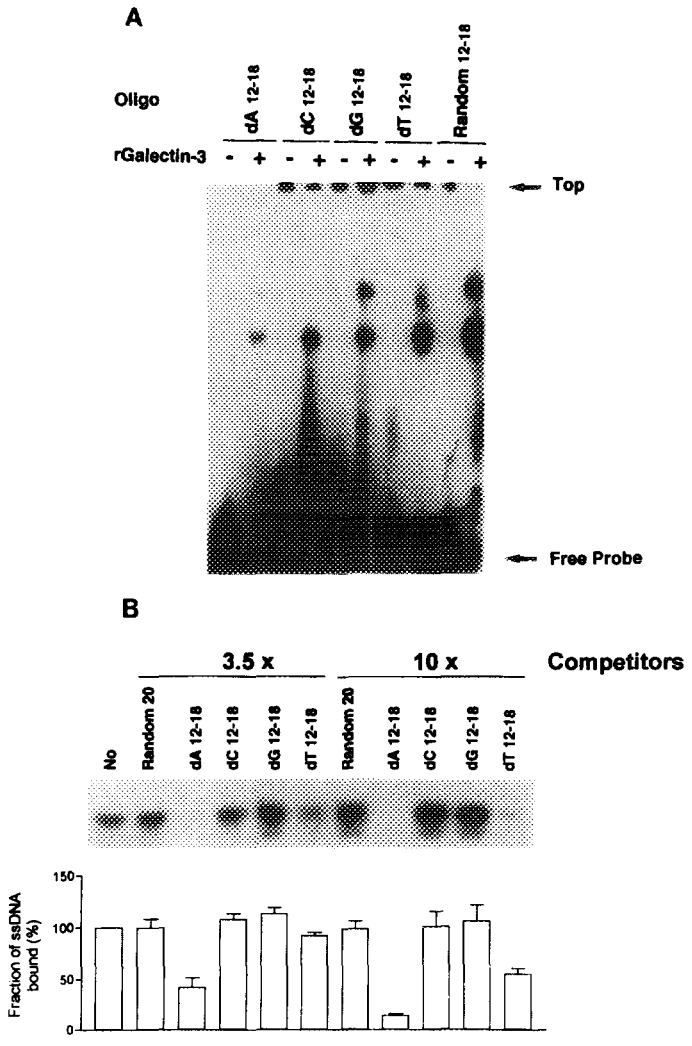
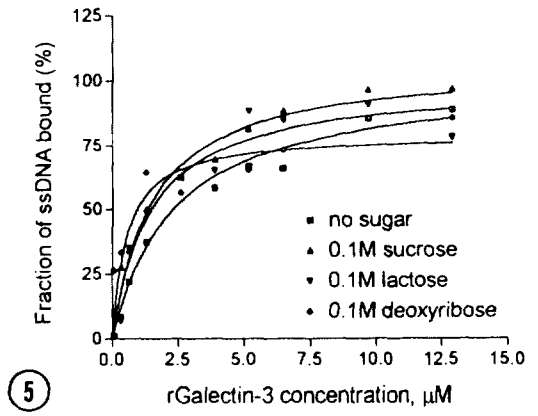


Figure 4. Sequence preference binding of galectin-3. (A) Binding of galectin-3 to four different homooligonucleotides. 2000cpm of ^{32}P-labeled oligonucleotides were incubated individually with or without 16 μM galectin-3 and run on 5% nondenatured PAGE as described in Materials and Methods. (B) Competition assay between 20 base random oligonucleotides and dA, dC, dG, dT homo oligonucleotides. 16 μM galectin-3 were mixed with 0.1 μM ^{32}P-labeled 20 base random oligonucleotides in the presence of 3.5- and 10-fold excess of unlabeled 12-18 base homo oligonucleotides dA, dC, dG and dT or random sequence oligonucleotide as described in Materials and Methods. Autoradiograms at several exposures were quantified on a densitometer. Values represent the mean and range of three determinations and the value in the absence of any unlabeled oligonucleotides is arbitrarily designated as 100.

of control disaccharide, i.e., sucrose also failed to interfere in binding (Kd=1.4 μM and Bm=106%). We also tested the effects of sugars that are involved in DNA structure. The 100mM deoxy-ribose also did not have any inhibition effect on galectin-3-ssDNA



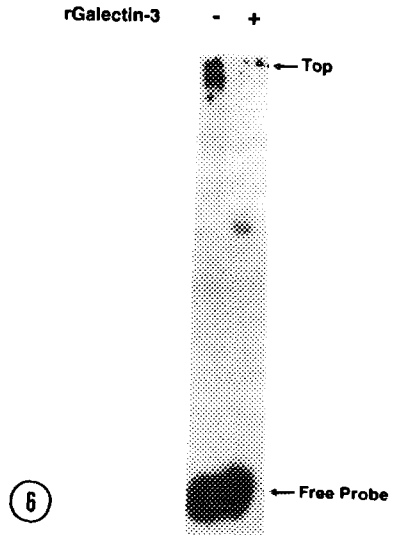


Figure 5. Binding of galectin-3 ssDNA in the presence of four different sugars. Increasing amount of galectin-3 were incubated with 50 fmol ^{32}P-labeled random sequence ssDNA and processed as described in Materials and Methods. The binding affinity of galectin-3 was measured by a filter binding assay. Each point represents the average of three independent binding reactions. The legend to the data points is shown in the lower right corner of the plot.

Figure 6. Gel mobility shift assay analysis of galectin-3 binding to RNA. 16 μM galectin-3 was incubated with ~2000cpm ^{32}P-labeled *in vitro*-transcribed RNA and processed as described in Materials and Methods. Lane -, no galectin-3; lane +, with galectin-3.

interaction (Fig. 5). Accordingly, it is unlikely that the carbohydrate-binding domain of galectin-3 is involved in binding to ssDNA.

RNA-binding activity of galectin-3. In order to examine the binding of galectin-3 to RNA more directly, gel mobility shift assay was carried out to analyze the possible interaction of galectin-3 with a 760 base *in vitro* transcription mRNA end-labeled by T4 polynucleotide kinase. The result demonstrated that galectin-3 binds directly to RNA (Fig. 6, lane +) and lactose (100 mM) did not alter this binding (data not shown).

DISCUSSION

The results presented here establish that (*i*) galectin-3 is a nuclear matrix protein; (*ii*) that galectin-3 is capable of RNA and ssDNA bindings; (*iii*) that galectin-3 exhibits the highest affinity to poly(A) ribonucleotide A homopolymer and (*iv*) that binding to nucleic acids is sugar independent. In addition the affinity of galectin-3 to ssDNA was established. The ssDNA has been chosen here for studying in detail the

interaction with galectin-3 because of convenience and the overall equivalence between RNA and ssDNA in protein- recognition and binding (23-27).

Previously, it has been shown that galectin-3 expression is associated with transformation (32), differentiation (33), inflammation (8) and that stimulation to proliferate of quiescent 3T3 fibroblasts as well as HIV-1 infection of T-lymphoblastic cells are associated with an increased nuclear localization of the lectin (34-35), suggesting that galectin-3 expression is associated with some aspects of cell growth. Its exact function, *in vivo,* however, remains largely unknown. Galectin-3 has a unique structure, and is composed of two distinct halves: a globular carboxy-terminal domain encompassing the carbohydrate recognition site and an amino-terminal domain containing a collagen-like sequence characterized by Gly-X-Y tandem repeats. The function of the amino-terminal half of the molecule is not yet known although it was recently shown to contain a unique cleavage site for matrix metalloproteinases-2 and -9 (36). Based on partial sequence homology of this amino-terminal domain, nuclear localization and fractionation analysis it was suggested that galectin-3 is a component of hnRNP(5). While this work was in progress it was reported that galectin-3 is a factor in pre-mRNA splicing, and that splicing is carbohydrate dependent, however the nuclear component that interacts with galectin-3 has not yet been identified (11). Thus, we questioned whether galectin-3 may be a structural component of the nuclear matrix and whether it exhibits an RNA binding ability.

In addition to the central role in DNA organization and nuclear structure, the nuclear matrix plays an important role in RNA organization and function (15). Small nuclear RNA complexed with proteins as well as hnRNA, which are the precursors to messenger RNA, have been localized to the nuclear matrix (13,14). Ciejek, et al., have demonstrated that all precursors of RNA were found to be exclusively associated with chick oviduct nuclear matrix supporting the notion that the nuclear matrix may be the structural site for RNA processing. In a series of experiments using HeLa nuclei, it was demonstrated that RNA is synthesized at the nuclear cage (i.e. nuclear matrix) (37), and that RNA is attached in a specific manner to the nuclear matrix. Using HeLa cells infected with adenovirus type 2, further provided evidence that adenoviral-specific nuclear matrix RNA contains precursors, intermediates, and products of RNA processing (38). The nuclear matrix also has been shown to play a role in RNA splicing *in vitro* (38,40) and that actively transcribed genes are associated with the nuclear matrix (41-45). How RNA, its precursors and intermediates interact with the

nuclear matrix is largely undefined. Our results suggest that galectin-3 may play an as yet undetermined role in nuclear matrix-RNA interactions.

The results presented here show that galectin-3 binds RNA, and with 20 base random sequence oligonucleotide as substrates we were able to determine the dissociation constant of this interaction, 2.3 μM. This is in the range of some other RNA binding proteins like the hnRNP A1 splicing factor (5 μM to 3 nM) (24). The competition of binding studies have revealed that galectin-3 exhibits a sequence preference toward poly(A) ribonucleotide homopolymers, suggesting that the molecule might have a sequence-specific RNA-binding domain like hnRNPs, which remains to be determined.

We report here that galectin-3-RNA binding is carbohydrate independent. This is in contrast to the report showing that competitive carbohydrates specifically inhibit the galectin-3 mediated pre-mRNA splicing (11). It is possible that galectin-3 has two functional domains. The amino-terminal may act as an RNA binding domain and with binding independent of carbohydrate structure. Alternatively, binding of the RNA to the carbohydrate binding domain may be utilized for direct RNA splicing, or for complex formation with either a splicing factor or a general inhibitor of splicing, as been suggested (11). This would suggest that galectin-3 has two distinct functions *e.g.*, RNA -binding and -splicing, which are carbohydrate independent and dependent, respectively. The concept that carbohydrate-binding proteins may exhibit distinct functions independent of their sugar binding specificities is not new. Macrophage asialoglycoprotein-binding protein I has a carbohydrate-binding site and a distinct cell recognition signal, RGD (46). Elastin/laminin receptor (47) and human lymphocyte low-affinity Fc receptor (48) have distinct sites for protein-protein and protein-carbohydrate interaction. Selectins contain an amino terminal of lectin-like domain followed by and epidermal growth factor-like motif and consensus repeats related to the complement-regulating proteins (49). The mannose-binding protein, lung surfactant protein A and conglutinin have similar structural similarity to galectin-3. They contain a collagenous domain of repeating Gly-X-Y sequence and a carboxy-terminal containing the carbohydrate recognition site, and were found to be recognized by the complement subcomponent C1q receptor through their collagenous domain (50). More recently, it was found that galectin-3 can interact in a carbohydrate independent fashion via protein-protein recognition with other carbohydrate-binding proteins in the nucleus (12) and metalloproteinases-2 and -9 (36). The results reported here should facilitate the unveiling of the physiological role(s) of galectin-3 and to the understanding of the structure-function relationship of the various domains of the molecule.

ACKNOWLEDGMENTS

We gratefully acknowledge Mr. Victor Hogan and Dr. Robert Getzenberg for their invaluable assistance, and Ms. Vivian Powell for typing and editing.

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