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Immunoprecipitation of spliceosomal RNAs by antisera to galectin-1 and galectin-3

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ABSTRACT

We have shown that galectin-1 and galectin-3 are functionally redundant splicing factors. Now we provide evidence that both galectins are directly associated with spliceosomes by analyzing RNAs and proteins of complexes immunoprecipitated by galectin-specific antisera. Both galectin antisera co-precipitated splicing substrate, splicing intermediates and products in active spliceosomes. Protein factors co-precipitated by the galectin antisera included the Sm core polypeptides of snRNPs, hnRNP C1/C2 and Slu7. Early spliceosomal complexes were also immunoprecipitated by these antisera. When splicing reactions were sequentially immunoprecipitated with galectin antisera, we found that galectin-1 containing spliceosomes did not contain galectin-3 and vice versa, providing an explanation for the functional redundancy of nuclear galectins in splicing. The association of galectins with spliceosomes was (i) not due to a direct interaction of galectins with the splicing substrate and (ii) easily disrupted by ionic conditions that had only a minimal effect on snRNP association. Finally, addition of excess amino terminal domain of galectin-3 inhibited incorporation of galectin-1 into splicing complexes, explaining the dominant-negative effect of the amino domain on splicing activity. We conclude that galectins are directly associated with splicing complexes throughout the splicing pathway in a mutually exclusive manner and they bind a common splicing partner through weak protein–protein interactions.

INTRODUCTION

Before transport to the cytoplasm for translation, RNA transcripts in the form of pre-mRNA assemble into a macromolecular structure termed the spliceosome. During subsequent remodeling events of spliceosomes, introns are removed and exons are ligated to form mature mRNA. *In vitro* cell-free assays using simple splicing substrates and nuclear extracts (NE) have established the basic sequence of biochemical events and ordered series of complexes in the pathway (1–4). In this dynamic, multi-step process, over 300 proteins and five ribonucleoprotein particles (snRNPs) orchestrate two *trans*-esterification reactions that result in the formation of mRNA (5–9). We previously identified two polypeptides in NE from HeLa cells (galectin-1 and galectin-3) required for splicing (10,11). Depletion of both galectins from NE, either by lactose–agarose affinity or double antibody adsorption chromatography, abolished splicing activity and halted spliceosome assembly at an early step. Addition of either galectin restored both splicing activity and spliceosome formation. These data suggested that galectins are, indeed, splicing factors and that they are functionally redundant.

Galectins are a family of carbohydrate-binding proteins with specificity for galactose or galactose-containing glycoconjugates that localize both intracellularly and extracellularly (12). Two of the galectins (galectins-1 and -3) are ubiquitous in mammalian tissues and have been documented in the nucleus and cytosol. Galectin-1 (gal-1) is composed of a single carbohydrate recognition domain (CRD) of ∼130 amino acids. Galectin-3 (gal-3) has an N-terminal domain (ND) of ∼130 amino acids containing multiple repeats of the amino acid sequence PGAYPGXXX of unknown function fused to a CRD whose sequence is conserved when compared to the CRD of gal-1 and the other galectins. As we investigated the role of gal-1 and gal-3 in splicing, we noted several key differences. First, recombinant gal-3 is ∼5–10 times more efficient at reconstitution of splicing than gal-1 or the isolated CRD of gal-3 (11). Second, although both galectins co-localize with known splicing factors (i.e. the Sm core polypeptides of snRNPs and SC35) and each other in nuclear

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The authors wish it to be known that, in their opinion, the first two authors should be regarded as joint First Authors

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speckles, there are regions of non-overlap (13). Lastly, the isolated ND of gal-3 inhibits splicing chemistry and spliceosome formation in a dominant negative manner when added to splicing competent NE (14).

Although these data are consistent with galectins being splicing factors, they do not address whether their role in splicing is direct or indirect. In this study, we provide evidence for a direct role of galectins in the splicing pathway by showing that galectin-specific antibodies co-immunoprecipitate pre-mRNA, splicing intermediates and products throughout the entire splicing pathway. We have investigated various characteristics of galectin incorporation into spliceosomes and conclude they interact via weak protein–protein interactions with another spliceosomal component.

MATERIALS AND METHODS

Antibodies

Polyclonal rabbit antiserum against recombinant rat gal-1 was a gift from Doug Cooper (University of California, San Francisco, CA, USA) (14). Additionally, we raised a second polyclonal rabbit antiserum against recombinant human gal-1 expressed as a fusion protein with glutathione *S*-transferase (GST). A human gal-1 cDNA clone was kindly provided by Jun Hirabayashi (15). The gal-1 cDNA insert was isolated after BamH1 digestion and inserted into pGEX-2T. Following expression in DH5α, the fusion protein was purified by glutathione–agarose affinity chromatography and used to immunize rabbits. Several polyclonal rabbit antisera to murine gal-3 were produced as described in Agrwal *et al*. (16). When used in immunoprecipitation experiments (Figures 4 and 6), each galectin antiserum was mono-specific for a galectin (i.e. anti-gal-1 precipitated only gal-1 from splicing reactions and anti-gal-3 precipitated only gal-3). Human autoimmune serum (ENA anti-Sm) was purchased from The Binding Site. Anti-Slu7 antibodies were purchased from Santa Cruz Biotechnology and anti-Ran antibodies from BD Biosciences. A monoclonal antibody against hnRNP C1/C2 (4F4) was provided by Gideon Dreyfuss (University of Pennsylvania). For immunoprecipitation experiments, all antibodies were covalently cross-linked to protein G-Sepharose fast flow 4B beads (Sigma) as previously described (11,14) generally using a 2:1 ratio of antiserum to protein G beads.

Co-immunoprecipitation of ^{32}P-labeled-MINX RNAs

Splicing reactions containing 60% by vol. HeLa NE were assembled with ^{32}P-MINX RNA without or with ATP (1.5 mM) and creatine phosphate (20 mM) and incubated at 30°C for the times indicated as previously described (10,11,14). HeLa cells were obtained from the National Cell Culture Center. Typically 60–100 μl of the splicing reaction mixture was diluted to 0.5 ml with 60% buffer D (D60) and incubated with 30–50 μl antibody cross-linked protein G-Sepharose beads at 4°C for 1–2 h. After washing with D60 containing 0.05% Triton X-100 (three washes, each with 1 ml buffer), the bound material was eluted with 20 μl of SDS–PAGE sample buffer. The eluted sample was divided into two aliquots. RNA was purified from one by incubating at 37°C for 20 min with proteinase K (4 mg/ml final concentration) and diluting to 100 μl with 125 mM Tris (pH 8), 1 mM EDTA, 300 mM sodium acetate. RNA was extracted with 200 μl of phenol–chloroform (50:50 [vol/vol]), followed by 200 μl of chloroform. RNA was precipitated with 300 μl of ethanol at −80°C. The extracted RNA was dissolved in 10 μl of sample buffer (9:1/formamide:bromophenol blue) and subjected to electrophoresis through 13% polyacrylamide (bisacrylamide–acrylamide 1.9:50 [wt/wt])-8.3 M urea gels. The radioactive RNA species were revealed by autoradiography and quantitated by phosphorimage analysis (Molecular Dynamics). The other aliquot was subjected to electrophoresis in 12.5% or 15% SDS–PAGE (bisacrylamide–acrylamide 0.9:30 [wt/wt]) and analyzed by western blotting.

The salt sensitivity of the association of galectins and snRNPs with spliceosomes was determined as follows. Active splicing complexes (incubated for 60 min with ATP at 30°C) were incubated with antibody-coupled beads. Following the initial binding, the beads were washed either with 60, 130 or 250 mM KCl-containing buffer and the bound material was eluted with SDS–PAGE sample buffer. The RNA was extracted and analyzed by denaturing gel electrophoresis as described above.

Native gel electrophoresis

To evaluate binding of the galectins to the splicing substrate, recombinant gal-1 or recombinant gal-3 (expressed in DH5α and purified by lactose–agarose affinity chromatography), or NE was incubated with ^{32}P-MINX under splicing conditions. Following incubation, the reactions were subjected to native gel electrophoresis (4% polyacrylamide gels [bisacrylamide: acrylamide 1:80 wt/wt]) (17) and complex formation revealed by autoradiography. Agarose native gel electrophoresis to identify H or E complexes was performed as described by Das *et al*. (18) followed by autoradiography.

RESULTS

Galectins-1 and -3 are associated with spliceosomes

Previously we showed that depletion of gal-1 and gal-3 from HeLa NE abolished pre-mRNA splicing and arrested spliceosomes before formation of active complexes (i.e. only early complexes formed) (10). Addition of either recombinant gal-1 or gal-3 to the galectin-depleted NE resulted in reconstitution of splicing activity and active spliceosome formation (10,11). We concluded that these two nuclear galectins are redundant splicing factors. However, there was little information to suggest whether their involvement in the splicing pathway was direct or indirect.

To test for a direct association of galectins with spliceosomes, we determined whether radiolabeled splicing substrate in splicing complexes could be precipitated by galectin-specific antisera. As seen in Figure 1 (lane 1), after incubation in HeLa NE with ATP for 60 min, the ^{32}P-MINX splicing substrate is converted to processing intermediates and products. Typically, 20–35% of the RNA detected following 60 min is ligated product. In the absence of ATP, no processing of the MINX substrate occurs (see below). When splicing

reactions that had been incubated for 60 min are immunoprecipitated by galectin-specific antisera, all species of RNA, the pre-mRNA substrate, the intermediates and the mature products, are found in the precipitates (lanes 4 and 5). In contrast, pre-immune serum did not precipitate significant quantities of labeled RNA (lane 2). Human autoimmune serum reactive against the Sm polypeptides of the snRNPs precipitated all species of radiolabeled spliceosomal-associated MINX as expected (lane 3). Thus, gal-1 and gal-3, like Sm proteins, are associated with active spliceosomes.

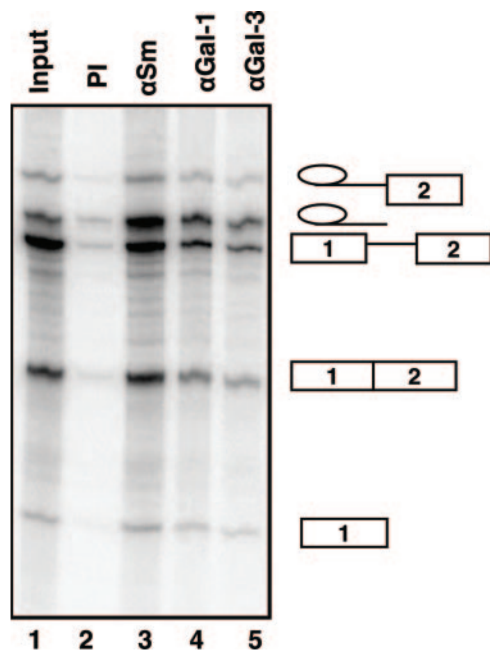

Figure 1. Analysis of spliceosomal RNA species immunoprecipitated by various antisera. Equal aliquots of splicing reactions incubated for 60 min with ^{32}P-MINX and ATP were subjected to antibody adsorption and the bound RNA analyzed by denaturing gel electrophoresis and autoradiography. Lane 1, RNA in the splicing reaction subjected to immunoprecipitation; lane 2, RNA bound to pre-immune serum; lane 3, RNA bound to anti-Sm; lane 4, RNA bound to anti-gal-1 and lane 5, RNA bound to anti-gal-3. The positions of the various RNA species are diagrammed on the right in this and subsequent figures analyzing spliceosomal RNAs.

To characterize in greater detail galectin-containing splicing complexes, splicing reactions were subjected to antiserum selection during a time course experiment and RNAs (Figures 2 and 3) and some of the proteins (Figure 4) in the immunoprecipitates were characterized. Each galectin antisera co-immunoprecipitated the splicing substrate throughout the time course of the splicing reaction. At the earliest times sampled, both antisera precipitated MINX pre-mRNA, most probably in the form of H/E complexes (see below). Splicing intermediates and ligated exons were precipitated as they appeared during the kinetic analysis (Figure 2, beginning at 20 min for the anti-gal-1 time course and at 40 min for the anti-gal-3 time course). Less spliceosomal RNAs were precipitated in the gal-3 time course compared to the gal-1 time course in this experiment due to use of a lower quantity of anti-gal-3 serum for precipitation. An internal control for the specificity of RNA precipitation is apparent in these analyses. Degraded RNAs of the gal-1 time course observed at 0, 5 and 10 min (highlighted by arrows to the left of the input lanes in Figure 2) are not detected in the immunoprecipitated complexes at these times. Both galectin antisera appeared to immunoprecipitate the excised lariat RNA species preferentially (compare the ratios of lariat to pre-mRNA species in the precipitates and in the input at the 40 and 60 min time points in Figure 2). Both observations (i.e. no precipitation of degraded RNAs and preferential precipitation of free lariat) argue against non-specific adsorption of radioactive RNA species to beads since the precipitated RNAs do not reflect the same relative amounts of the different RNA species in the sample used for immunoselection (input).

Detecting MINX pre-mRNA in galectin immunoprecipitates at early times in the splicing reaction prompted us to

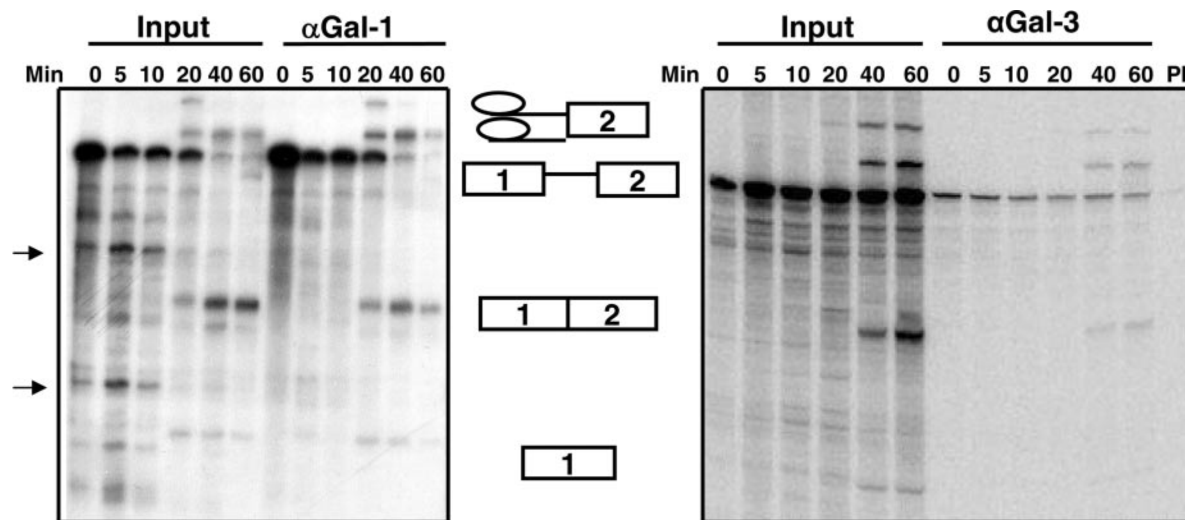

Figure 2. Time course analysis of spliceosomal RNAs precipitated by anti-gal-1 or anti-gal-3 antiserum. ^{32}P-MINX was incubated with ATP in NE at 30°C. At the times indicated, the reactions were subjected to galectin affinity adsorption and the bound fraction separated into two parts. RNA was extracted from one portion and analyzed by denaturing gel electrophoresis. MINX RNA in splicing reactions at 0, 5, 10, 20, 40 and 60 min (input) and MINX RNA precipitated by anti-gal-1 or anti-gal-3 antiserum. Arrows at left indicate degraded RNA species in the input material.

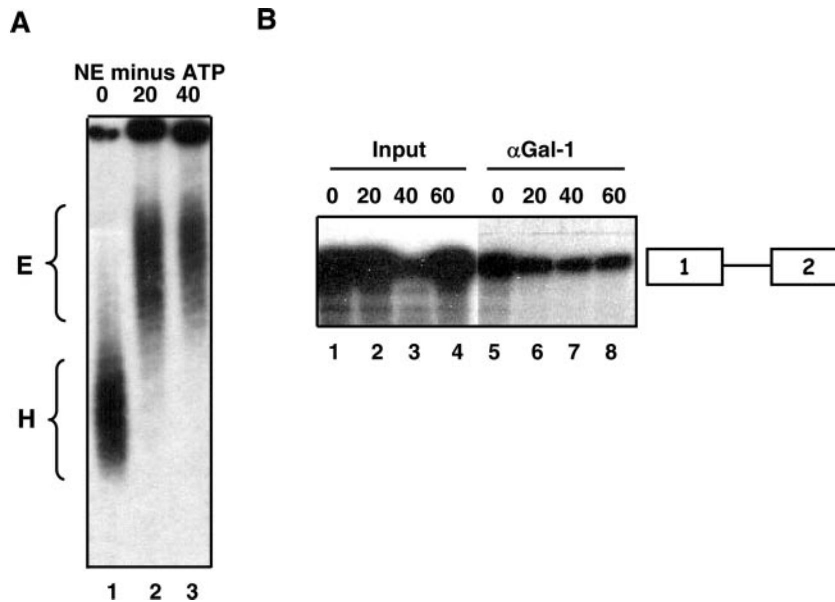

Figure 3. Immunoprecipitation of ^{32}P-labeled RNA assembled into early (H and E) complexes. (**A**) MINX RNA was incubated with NE at 30°C in the absence of ATP for the indicated times. The reaction mixtures were loaded directly onto 1.5% agarose gels. Following electrophoresis, autoradiography was performed to detect RNA-containing complexes. (**B**) Splicing reactions containing MINX RNA in the absence of ATP at 0, 20, 40 and 60 min incubation at 30°C (lanes 1–4) were incubated with anti-gal-1 immobilized and the bound material subjected to analysis by denaturing gel electrophoresis (lanes 5–8).

investigate the association of galectins with complexes characterized as H or E splicing complexes. H complexes form upon addition of a substrate to a splicing extract even when incubated on ice. Following incubation at elevated temperatures, H complexes are converted to E (early) complexes that are the immediate precursors of active spliceosomes. Neither H nor E complex assembly requires ATP (18). To test for galectin association with these two complexes, MINX pre-mRNA was incubated without ATP in NE at 30°C for 0 min to assemble H complexes or 20 and 40 min to chase H complexes into E complexes. Figure 3A shows MINX pre-mRNA formed H complexes upon addition to NE (lane 1). Nearly all of the MINX RNA was chased into E complexes following incubation for 20 and 40 min at 30°C (lanes 2 and 3). In a time course experiment, anti-gal-1 antiserum immunoprecipitated splicing competent pre-mRNA assembled in H complexes at 0 time (Figure 3B, lane 5) and E complexes after 20 through 60 min of incubation at 30°C (Figure 3B, lanes 6–8). Thus, complexes formed in the absence of ATP with mobilities characteristic of H and E pre-splicing complexes contain gal-1. Similar results for gal-3 were obtained (data not shown).

To confirm further that the immunoprecipitated complexes represented spliceosomes, antisera specific for several splicing factors were used to probe the precipitated fractions (Figure 4). Antiserum specific for gal-1 co-precipitated the Sm B/B′ core polypeptides of snRNPs, hnRNP C1/C2 and a factor required for in the second *trans*-esterification reaction Slu7. In contrast, gal-3 was not detected in the anti-gal-1 precipitate. We have also tested for and failed to find other nuclear proteins such as Ran co-immunoprecipitated by anti-gal-1 (data not shown). As expected, gal-1 antiserum precipitated gal-1. Similar results were obtained when the immunoprecipitates of anti-gal-3 antiserum were analyzed by western analysis (data not shown).

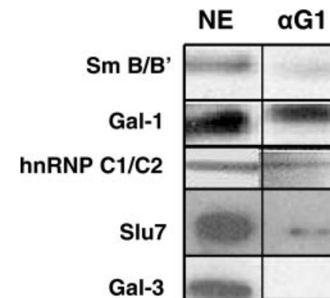

Figure 4. Analysis of proteins co-immunoprecipitated by gal-1 antiserum. HeLa NE (NE) and bound fractions from anti-gal-1 immunoprecipitates (αG1) were blotted for Sm B/B′, hnRNP C1/C2, Slu7, gal-1 and gal-3.

Galectins should be detected in spliceosomes isolated by precipitation with antiserum directed against another splicing factor. A reciprocal co-immunoprecipitation experiment was performed using anti-Sm antisera (Figure 5). As expected, spliceosomes selected by the Sm antiserum contained the splicing substrate, intermediates and products. Immunoprecipitation with human IgG revealed only background levels of splicing RNAs. Most importantly, gal-1 was detected in the Sm selected complexes, but not detected in the material precipitated by the control human IgG. These data strongly support our contention that galectins are splicing factors associated with the splicing machinery.

Galectin-1 and galectin-3 reside on distinct splicing complexes

Spliceosomes selected by galectin-specific antisera are a heterogeneous mixture of complexes (e.g. spliceosomes containing pre-mRNA substrate would be expected to contain different RNA and protein components than spliceosomes

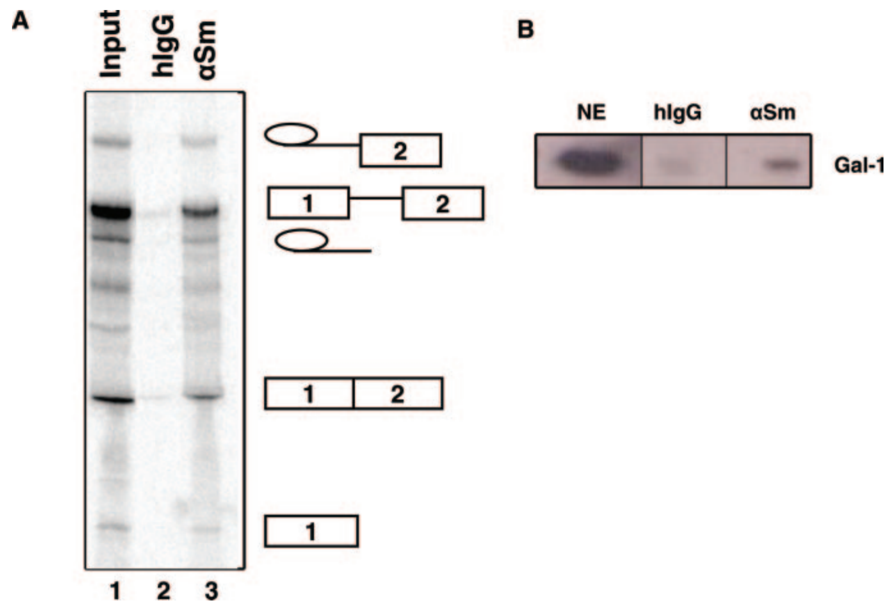

Figure 5. Immunoprecipitation of spliceosomes by anti-Sm antiserum. Splicing reactions were incubated with ATP for 60 min. and then were immunoprecipitated with human anti-Sm antiserum or human IgG (control). Bound fractions were eluted and RNA analyzed by gel electrophoresis and autoradiography (**A**) and gal-1 detected by western blotting (**B**).

containing ligated exon product). While the stoichiometry of most of the proteins in spliceosomes has not been determined, it is assumed that under standard splicing conditions using a single splicing substrate each distinct processing pathway intermediate will contain the same complement of proteins. Thus, it is reasonable to predict that spliceosomes containing gal-1 would also contain gal-3. However, in our previous depletion–reconstitution studies, we showed that either gal-1 or gal-3 could restore splicing activity to a galectin-depleted NE. Obviously, under these reconstitution conditions, spliceosomes containing a galectin would contain only that particular member of the galectin family.

To distinguish between these two formal possibilities (spliceosomes precipitated by one galectin antibody contain one or both nuclear galectins), we performed sequential immunoprecipitations as outlined in Figure 6A. Standard splicing reactions were incubated for 60 min. and divided into two equal portions. One aliquot was immunoprecipitated with anti-gal-1 and the other with anti-gal-3. The unbound fractions were then subjected to a second immunoprecipitation using the other galectin antiserum. Radiolabeled RNA in the bound fractions from each immunoprecipitation was analyzed (Figure 6B). Roughly the same quantity of spliceosomes was precipitated by the anti-gal-1 antiserum in the two sequential selections (compare lanes 3 and 10). Similar results were obtained following the two anti-gal-3 immunoprecipitations (compares lanes 4 and 8). In order to interpret these results, the efficiency of each galectin antiserum to quantitatively immunoprecipitate its cognate antigen was determined. We analyzed the bound and unbound fractions from the first immunoprecipitation for gal-1 and gal-3 (Figure 6C). The bound fraction from the first anti-gal-1 precipitate showed only gal-1 (Figure 6C, lane 3) with no detectable gal-3. Further, the unbound fraction of this precipitation showed nearly quantitative depletion of gal-1 (lane 6; the amount of gal-1 in this fraction represents <10% of the total gal-1 in the reaction used for immunoprecipitation). Similar results were obtained with gal-3. Analysis of the bound fraction of the first anti-gal-3 precipitation showed only gal-3 (lane 4) and nearly all of gal-3 was removed by this immunoprecipitation (lane 5; <15% of the total gal-3 in the reaction remained in the unbound fraction of the first precipitation). We interpret these data to indicate that gal-1 and gal-3 were quantitatively removed during the initial immunoselection and that the two galectins reside on different splicing complexes. Finally, spliceosomal RNAs could be immunoprecipitated by anti-Sm serum from the material remaining after the two sequential galectin adsorptions (data not shown), indicating that some spliceosomal complexes contained neither gal-1 nor gal-3.

As an additional evaluation of the nearly quantitative and specific removal of each galectin by its respective antiserum, this experiment was repeated with the following modification. The unbound fraction of the first immunoprecipitation was incubated with the same antiserum as that used in the first antibody selection (i.e. unbound material of anti-gal-1 precipitation was rebound to anti-gal-1 coated beads). Less than 5% of the initially precipitated RNA was bound to the antiserum in the second round of precipitation (data not shown). This low level of binding to the same antiserum matched the low levels of galectin found in the unbound material after the first precipitation.

It is important to note that these results provide another control for the specificity of the spliceosomes precipitated by the anti-galectin antisera. The fact that each galectin antiserum precipitates only its respective antigen indicates that these antibodies do not precipitate nuclear proteins/complexes non-specifically and further suggests that galectins do not bind to splicing complexes non-specifically.

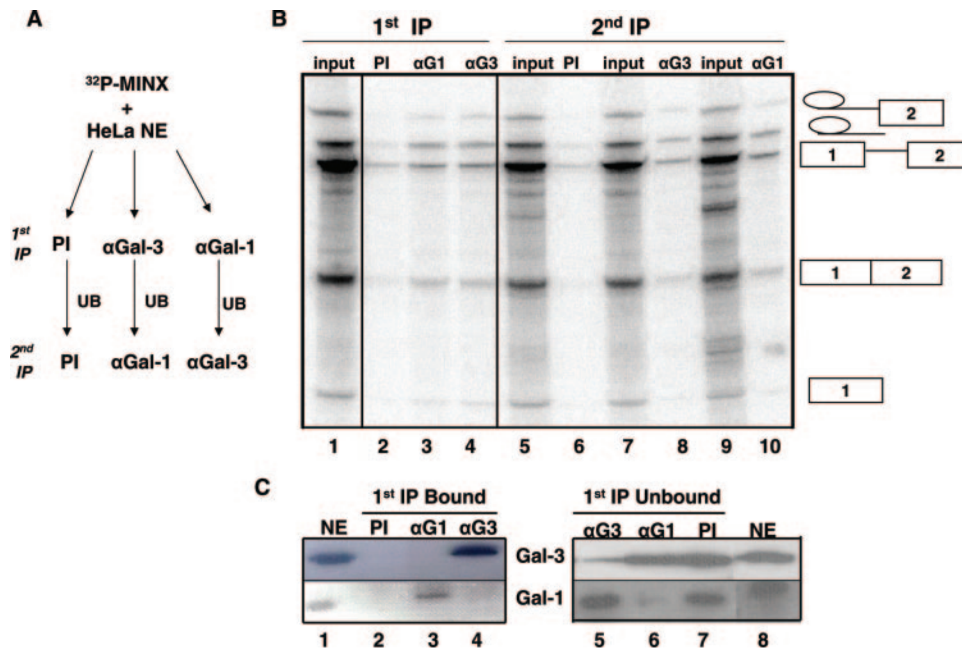

Figure 6. Sequential immunoprecipitation of splicing complexes by gal-1 and gal-3 antisera. (**A**) protocol for sequential immunoprecipitation. Splicing reactions were incubated for 60 min with ^{32}P-MINX and divided into equal aliquots. The aliquots were incubated with the indicated antiserum as described in Materials and Methods. Part of the unbound fraction was then incubated with the other galectin antiserum. After washing, the bound material from the first and second immunoprecipitations was eluted and analyzed for MINX RNA (**B**). Lanes 1, 5, 7 and 9 represent aliquots of the bound fraction used for immunoprecipitation (input). Lanes 2–4, represent the MINX RNA bound to the indicated antiserum in the first immunoprecipitate and lanes 6, 8 and 10 represent MINX RNA bound to the indicated antiserum in the second immunoprecipitate. (**C**) western blotting analysis of gal-1 and gal-3 in the bound (lanes 2–4) and unbound (lanes 5–7) fractions of the first immunoprecipitation.

Galectin-1 and galectin-3 do not bind MINX RNA directly

The findings described above could be explained by a direct interaction of the galectins with the MINX pre-mRNA. If this interaction was with a unique site in the pre-mRNA, then only one galectin would bind per pre-mRNA and spliceosomes containing this pre-mRNA would have either gal-1 or gal-3 associated. To test for direct galectin–MINX interactions, we used an electrophoretic mobility shift assay (Figure 7). ^{32}P-MINX RNA migrated in native polyacrylamide gels as shown in lane 1 of Figure 7A and B. Incubation of MINX with recombinant human gal-1 (0.5 or 2.5 μg, Figure 7A, lanes 2 and 3, respectively) or recombinant gal-3 (0.8 or 4 μg, Figure 7B, lanes 2 and 3, respectively) did not alter the mobility of MINX when compared to the mobility of the substrate alone. Various incubation conditions (i.e. changing temperature, time of incubation and incubation with or without ATP) yielded the same results. As a positive control for altered mobility, MINX was incubated with NE for 15 min on ice. As expected, MINX was assembled into an H complex that had a slower mobility in the native gel (lane 4 of Figure 7A and B). We conclude that the association of galectins with spliceosomes occurs through interaction with another splicing component rather than through direct binding to the splicing substrate.

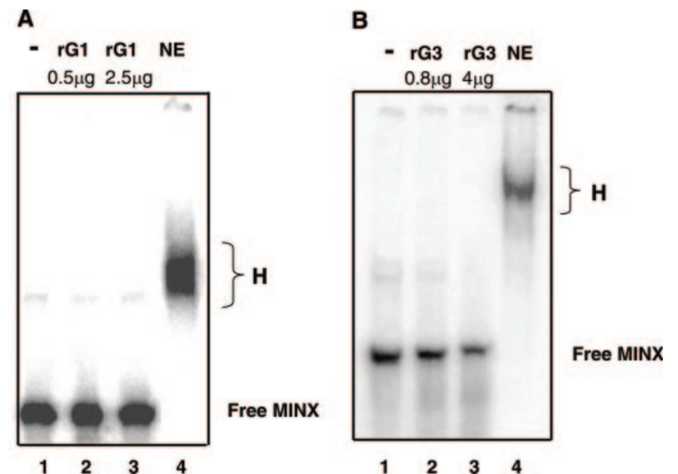

Figure 7. Native gel analysis of splicing substrate incubated with recombinant gal-1, recombinant gal-3 or NE. (**A**) ^{32}P-MINX was incubated on ice for 15 min with 0.5 (lane 2), 2.5 (lane 3) μg recombinant human gal-1, HeLa NE ~25 μg protein (lane 4), or nothing (lane 1). (**B**) ^{32}P-MINX was incubated on ice for 15 min 0.8 (lane 2), 4.0 (lane 3) μg recombinant gal-3, HeLa NE ~25 μg protein (lane 4) or nothing (lane 1). Following incubation, heparin was added and the samples analyzed on 4% polyacrylamide native gels followed by autoradiography. The migration of free MINX RNA and MINX RNA in H complexes (indicated between the two panels) was detected by autoradiography.

ND of gal-3 blocks gal-1 association with spliceosomes

Splicing activity in a complete HeLa NE is inhibited in a dose dependent manner by the addition of the ND of gal-3. At the highest concentration of ND, neither splicing activity nor spliceosome complex formation could be detected even though gal-1 was available (14). Our finding that each galectin resides on separate splicing complexes suggests a mechanism for this dominant negative effect of the ND. Splicing

requires binding of galectins to another splicing factor. Likely this partner is shared by the galectins in a mutually exclusive manner. We suggest that excess ND binds to this partner and blocks the interaction of this partner with both nuclear galectins. To test this prediction, we quantitated spliceosomes immunoprecipitated by anti-gal-1 antiserum in reactions inhibited by the ND of gal-3 (added as a GST fusion protein). The results are shown in Figure 8. As previously reported (14), the GST-ND polypeptide inhibited product formation nearly 100% (lane 5) compared to reactions incubated with (lane 3) or without (lane 1) GST. The addition of GST to the reaction did not inhibit the precipitation of spliceosomes by anti-gal-1 (compare lane 4 to lane 2). In contrast, the addition of GST-ND nearly completely inhibited the association of gal-1 with the splicing machinery (compare lane 6 to lanes 2 and 4). Thus, the ND of gal-3 exerts its dominant negative effect by regulating the incorporation of gal-1 (and gal-3) into splicing complexes.

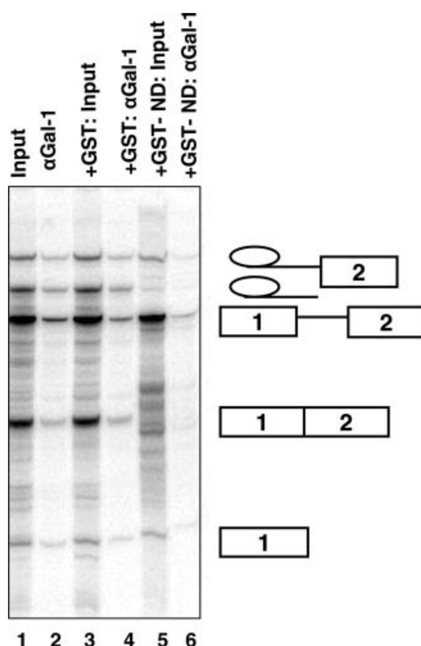

Figure 8. Analysis of spliceosomes immunoprecipitated by anti-gal-1 antiserum in reactions incubated with GST or GST-ND of gal-3. Equivalent splicing reactions were incubated with no addition (lane 1, input), GST (lane 3, +GST: input, 100 μM) or GST-ND (lane 5, +GST-ND: input, 100 μM) for 60 min. Anti-gal-1 beads were added to each reaction and RNA in the bound fractions was analyzed by gel electrophoresis and autoradiography (lanes 2, 4 and 6).

Galectin association with spliceosomes is salt labile

The strength of the association of galectins with spliceosomes was evaluated in relation to the stable association of the snRNPs with splicing complexes. Splicing complexes formed after a 60 min. splicing reaction (Figure 9, lane 1) were incubated with each galectin antiserum or pre-immune serum in 60 mM KCl buffer (the buffer used for optimal splicing efficiency). Aliquots of the antibody-bound spliceosomes were then washed with 60 mM (lanes 2–5), 130 mM (lanes 6–9) or 250 mM (lanes 10–13) KCl buffers. The bound fractions were eluted and analyzed for radiolabeled RNA. Salt concentrations of 130 or 250 mM released most of the splicing substrate from the galectin-selected columns (>90% of the spliceosomes were released as determined by quantitation from phosphorimage analysis) whereas 130 mM KCl had no effect on spliceosomes selected by anti-Sm antiserum. Even when the salt was increased to 250 mM KCl, ~20% of the snRNPs remained stably associated with spliceosomes. The loss of spliceosomal RNAs from the antibody columns was due to dissociation of the complexes from each galectin and not due to release of the galectins from their respective antibody. At 130 mM KCl, virtually no gal-1 or gal-3 was released from the immobilized antibodies compared to the galectins bound at 60 mM KCl. At 250 mM KCl, more than 70% of the galectins remained bound to the antibodies (data not shown). We conclude that the association of galectins with the splicing machinery is sensitive to perturbation of ionic strength.

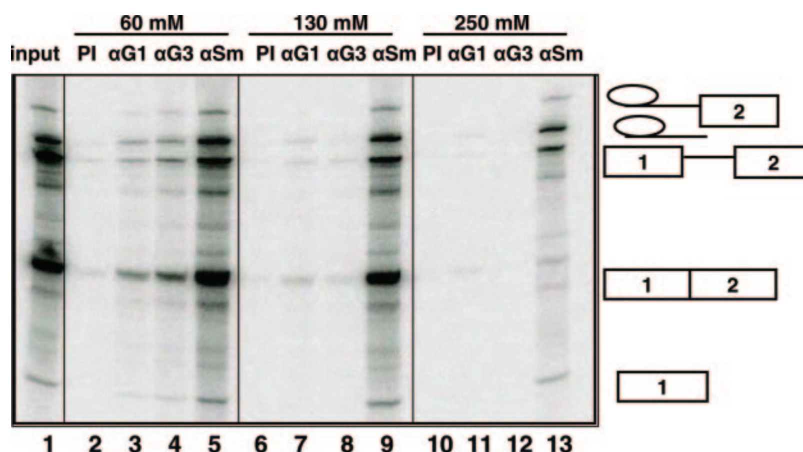

Figure 9. Analysis of spliceosomes precipitated by various antisera at increasing salt concentrations. HeLa NE was incubated with ^{32}P-MINX for 60 min in the presence of ATP. Immobilized pre-immune serum (lanes 2, 6 and 10); anti-gal-1 (lanes 3, 7 and 11); anti-gal-3 (lanes 4, 8 and 12) or anti-Sm (lanes 5, 9 and 13) was incubated with the reactions in 60 mM KCl-buffer for 60 min. Beads were then washed extensively with 60 mM KCl-buffer (lanes 2–5); 130 mM KCl-buffer (lanes 6–9); or 250 mM KCl-buffer (lanes 10–13). The bound fractions were eluted and RNA analyzed by denaturing gel electrophoresis and quantitated by phosphorimage analysis (Molecular Dynamics) following autoradiography. Lane 1 indicates the RNA subjected to immunoprecipitation.

DISCUSSION

We previously have shown by depletion–reconstitution experiments that galectins-1 and -3 are required splicing factors (10,11). Further, the splicing activity of the galectins appears to be redundant in that either galectin can reconstitute splicing in a galectin-depleted NE. Now we document a direct association of these galectins with spliceosomal complexes. The key findings are (i) mono-specific galectin antisera immunoprecipitate splicing substrate RNAs in H, E and active spliceosomes along with Sm proteins of snRNPs, hnRNP C1/C2 and Slu7 (all known splicing factors), (ii) spliceosomal complexes contain either gal-1, gal-3 or no galectin (iii) neither galectin interacts directly with the pre-mRNA substrate, (iv) the amino terminus of gal-3 exerts a dominant negative effect on splicing by regulating the entry of gal-1 (and presumably gal-3) into splicing complexes and (v) the association of galectins with spliceosomes is salt-sensitive compared to the stable association of the snRNPs.

The data presented are significant in evaluating the role of galectins as splicing factors. First, these data support our hypothesis that nuclear galectins are indeed splicing factors that can be incorporated into the canonical model for pre-mRNA splicing (1). The fact that both galectin antibodies co-immunoprecipitate pre-mRNA associated with early (i.e. H and E) complexes illustrates the initial entry of galectins during spliceosome assembly. While a precise molecular function of galectins during spliceosome assembly is not known, it appears to be in the recruitment or supplying of snRNPs to pre-mRNA based on two observations: (i) depletion of galectins from splicing extracts inhibits transition of early (e.g. H and/or E) complexes to active spliceosomes (1) and (ii) galectins are associated with gemin4 in SMN complexes (14) which are implicated in recycling snRNPs to pre-mRNA in the early commitment complex (19).

Since the anti-galectin antibodies precipitated not only the pre-mRNA substrate but also the intermediates and products of the splicing reaction generated on the spliceosome, our results suggest that gal-1 and gal-3 are associated with the assembled machinery throughout the reaction cycle. It is clear that these galectin-containing complexes are spliceosomes as the hnRNP C1/C2 polypeptides [known splicing factors (5–9,20)], the snRNP-specific Sm proteins and Slu7 (2,5–9) co-precipitate with the substrate intermediates and products. Results of three controls strengthen our interpretation of the data. First, the nuclear shuttling protein Ran, which has not been found associated with spliceosomes, is not co-precipitated by the galectin antisera. Second, gal-1-containing spliceosomes are not precipitated by antibodies specific for gal-3 and vice versa. These results indicate (i) the galectin antisera are mono-specific and (ii) galectins do not adhere non-specifically to splicing complexes. Third, spliceosomes isolated by precipitation with antibodies directed against the Sm polypeptides of snRNPs co-precipitate galectins.

An obvious question of our contention that galectins are splicing factors is that they have not been identified in any of the proteomic analyses of spliceosomes (5–9). Several reasons can be offered to explain this discrepancy. First, most of the spliceosome isolation procedures use 150–250 mM salt during binding or washing to select stable complexes. The rationale has been to correctly identify components with a stable association in the spliceosome, rather than catalogue all associations. As we have shown (Figure 9), ionic conditions >60 mM release the galectins from spliceosomal complexes. We contend the buffer conditions we use for immunoprecipitation (which are optimal for *in vitro* splicing activity) allow a more complete cataloguing of spliceosomal proteins. Could the transient/loose association of galectins hint to a regulatory role? Second, the galectins may be in low abundance in spliceosomes. Our observation that not all spliceosomes contain galectins speaks to this point. Third, it is possible that galectins only assist in initiating spliceosome assembly (i.e. only associate with a complex containing the pre-mRNA substrate) and are not stable components of active splicing complexes. Only a thorough and careful evaluation of these early complexes would reveal this association. Finally, the stringency set for the identification of peptides and subsequent database searches results in missing members of a complex. For example, of the four massive proteomic analyses published, none have detected the U6-associated polypeptide LSm5 and there are several instances where one of the four studies detected a core spliceosomal component and the other three did not.

Other significant aspects of our findings include providing an explanation for functional redundancy of the galectins and hinting at the nature of the spliceosome-associated binding partner for the galectins. Reconstitution of a galectin-depleted NE can be achieved by either gal-1 or gal-3 (10,11). The sequential immunoprecipitation data provide experimental proof for the exclusive incorporation of only a single galectin into a splicing complex in a complete (i.e. non-depleted) splicing extract. Thus, functional redundancy means spliceosomes contain only one galectin. We show that the ND of gal-3 regulates the entry of gal-1 into spliceosomes. In aggregate, these data suggest the galectins share a common binding partner. This partner is probably a polypeptide splicing factor that weakly interacts with the galectins. In a splicing extract, gal-3 interacts with this partner via its two domains (the ND which contains the PGAYPGXXX repeats of unknown function and the C-terminus which is the CRD) whereas gal-1 only binds via its single CRD. The observation that gal-3 is 8–10 times more efficient in reconstituting splicing activity in galectin-depleted extracts compared to gal-1 supports this contention (11). Addition of excess ND binds to this common partner and blocks binding of gal-1 or gal-3. Abrogation of gal-1 and gal-3 binding results in inhibition of splicing. It remains to be determined whether the galectin-binding partner is assembled into splicing complexes when bound to the isolated ND.

Crucial to providing a mechanistic interpretation of these data regarding the association of galectins with spliceosomal complexes is the identification of the splicing partner for the galectins. While we have identified gemin4 as a galectin binding protein (14), neither gemin4 nor other members of the SMN complex (19) have been identified as spliceosomal components in proteomic analyses (5–9). Is this due to the fact that gemin4 and interacting proteins are not spliceosomal proteins or, as with the galectins, that they are weakly associated with spliceosome complexes and released under the conditions used for spliceosome isolation? From a different

perspective, gal-1 has been shown to be a component of the nuclear matrix (21) that has been proposed to serve as a scaffold for the splicing process. The nuclear matrix partner with which gal-1 interacts is unknown. Also, proteomic analysis of interchromatin granule clusters (IGC) has identified a galectin as a member of this nuclear organelle. Over 80% of the proteins identified as IGC components play a role in RNA biogenesis with >50% having a splicing function (22). Both of these findings lend additional, albeit indirect, support to our assertion that the galectins are splicing factors.

Galectin-containing spliceosomes have been precipitated with several different galectin-specific antisera using three conditions to assemble various splicing complexes. The association of galectins with spliceosomes is through a salt-sensitive protein–protein interaction rather than a galectin-splicing substrate interaction. The commonly shared binding partner/splicing factor explains the mutually exclusive incorporation of gal-1 or gal-3 into splicing complexes and the dominant negative inhibition of splicing demonstrated by the ND of gal-3.

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