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Distinct Effects on Splicing of Two Monoclonal Antibodies Directed against the Amino-terminal Domain of Galectin-3¹

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Abstract

Previous experiments had established that galectin-3 (Gal3) is a factor involved in cell-free splicing of pre-mRNA. Addition of monoclonal antibody NCL-GAL3, whose epitope maps to the NH₂-terminal 14 amino acids of Gal3, to a splicing competent nuclear extract inhibited the splicing reaction. In contrast, monoclonal antibody anti-Mac-2, whose epitope maps to residues 48–100 containing multiple repeats of a 9-residue motif PGAYPGXXX, had no effect on splicing. Consistent with the notion that this region bearing the PGAYPGXXX repeats is sequestered through interaction with the splicing machinery and is inaccessible to the anti-Mac-2 antibody, a synthetic peptide containing three perfect repeats of the sequence PGAYPGQAP (27-mer) inhibited the splicing reaction, mimicking a dominant-negative mutant. Addition of a peptide corresponding to a scrambled sequence of the same composition (27mer-S) failed to yield the same effect. Finally, GST-hGal3(1–100), a fusion protein containing glutathione S-transferase and a portion of the Gal3 polypeptide including the PGAYPGXXX repeats, also exhibited a dominant negative effect on splicing.

Keywords

lectins; carbohydrate-binding proteins; RNA processing; spliceosome; monoclonal antibodies

Introduction

Galectin-1 (Gal1⁷) and galectin-3 (Gal3) are two members of a family of β -galactoside-binding proteins that contain characteristic amino acid sequences in the carbohydrate recognition

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⁷The abbreviations used are: CRD, carbohydrate recognition domain; Gal1, galectin-1; Gal3, galectin-3; TFII-I, general transcription factor-I for RNA polymerase II; snRNP, small nuclear ribonucleoprotein complex; NE, nuclear extract; ND, the NH₂-terminal domain of galectin-3; GST, glutathione S-transferase; PBS, phosphate-buffered saline; PMSF, phenylmethylsulfonyl fluoride; DTT, dithiothreitol; PCR, polymerase chain reaction; HRP, horseradish peroxidase.

domain (CRD) of their respective polypeptides [1,2]. Using nuclear extracts (NE) derived from HeLa cells, depletion and reconstitution experiments had established that these proteins are two of the many polypeptides required for the splicing of pre-mRNA, assayed in a cell-free system [3–5]. The polypeptide of Gal1 consists of a single domain, the CRD. In contrast, the polypeptide of Gal3 can be delineated into three distinct regions: (a) the first 10–15 residues that contain sites of phosphorylation at Ser 6 and Ser 12 [6]; (b) a domain toward the NH₂-terminal end (ND) containing multiple repeats of a 9-residue motif, PGAYPGXXX; and (c) a COOH-terminal CRD that shows sequence similarity with the corresponding CRDs of other members of the galectin family [1,2].

Gong *et al.* [7] had reported that deletion of the NH₂-terminal 11 amino acids of human Gal3 resulted in loss of immunoblotting by the anti-Mac-2 monoclonal antibody. It was concluded that the antigenic recognition site of anti-Mac-2 is at the amino terminus. In contrast to these results and conclusions, we have mapped the epitope of the anti-Mac-2 antibody to the ND region bearing the PGAYPGXXX motif (residues 48–100) while the epitope of a second monoclonal antibody, designated NCL-GAL3, mapped to the NH₂-terminal 14 amino acids. On the Gal3 polypeptide, if the binding regions of the two monoclonal antibodies were near each other, one might expect that they would have similar effects on a functional assay such as the splicing assay. On the other hand, if the two monoclonal antibodies bound to distinct regions of the Gal3 polypeptide, they could exhibit different effects on the functional assay. Indeed, NCL-GAL3 and anti-Mac-2 did not have the same effect on the cell-free splicing assay, consistent with the notion that their epitopes resided in distinct regions of the Gal3 polypeptide.

On the basis of our results, therefore, the objectives of the present communication include: (a) to report that a previous identification of the epitope of the anti-Mac-2 monoclonal antibody by Gong *et al.* [7] may be in error; (b) to document the distinct epitopes of two monoclonal antibodies directed against Gal3 that have different effects on the splicing assay; and (c) to provide three lines of evidence that implicate the repeating 9-residue motif, PGAYPGXXX, in mediating the interaction of Gal3 with the spliceosome. Although previous studies had documented that the carboxyl-terminal CRD of the Gal3 polypeptide was necessary and sufficient for splicing activity [4], our present results suggest that the Pro- and Gly-rich sequences in the ND are also important in mediating interactions with components of the splicing machinery.

Materials and Methods

Antibodies and peptides used in functional assays

A rat monoclonal antibody was originally developed against the murine Mac-2 antigen [8], which has been shown to be Gal3 [9]. The hybridoma line producing this monoclonal antibody (M3/38.1.2.8.HL.2) was obtained from the American Type Culture Collection (TIB 166). The hybridoma cells were cultured in serum-free medium (RPMI 1640 containing Nutridoma SP (Boehringer Mannheim)). After pelleting the cells, supernatants from the cultures were pooled, subjected to ammonium sulfate precipitation (45% of saturation), dialyzed exhaustively against phosphate-buffered saline (PBS; 140 mM NaCl, 2.68 mM KCl, 10 mM Na₂HPO₄, 1.47 mM KH₂PO₄, pH 7.4), and stored in aliquots at a concentration of 250 µg/ml. This antibody preparation is hereafter designated as rat monoclonal anti-Mac-2. We also obtained an independent preparation of the anti-Mac-2 antibody from a commercial source (Acris Antibodies, GmbH, Hiddenhausen, Germany).

A murine hybridoma, designated as NCL-GAL3, was derived using recombinant human Gal3 as the immunogen. The NCL-GAL3 antibody used in this study was purchased from Vector Laboratories (VP-G802; hybridoma clone 9C4). Human autoimmune serum reactive with the

Sm epitopes (anti-Sm) of small ribonucleoprotein complexes (snRNPs) was purchased from The Binding Site.

The following peptides were synthesized in the Macromolecular Structure Facility (Michigan State University): (a) 9-mer (PGAYPGQAP) corresponding to residues 42–50 of human Gal3; (b) 18-mer (PGAYPGQAPPGAYPGQAP) corresponding to two iterations of the 9-residue motif; (c) 27-mer (PGAYPGQAPPGAYPGQAPPGAYPGQAP), three iterations; (d) 27mer-S (YPGGGQPPAPQYPGPAAAGYAGPQPA) containing scrambled sequence of the same amino acid composition (<http://bioweb.pasteur.fr/seqanal/interfaces/shuffleseq.html>); (e) 14-mer (MADNFSLHDALSGS) corresponding to residues 1–14 of human Gal3; and (f) mt14-mer (MADNFALHDALSGS), with a S6A substitution.

Assays for pre-mRNA splicing and spliceosome assembly

HeLa S3 cells were grown in suspension culture by the National Cell Culture Center (Minneapolis, MN). Nuclear extract (NE) was prepared in buffer D (20 mM Hepes-KOH, pH 7.9, 20% glycerol, 0.1 M KCl, 0.2 mM EDTA, 0.5 mM phenylmethylsulfonyl fluoride (PMSF), 0.5 mM dithiothreitol (DTT)), as described by Dignam *et al* [10]. NEs were frozen as aliquots in a liquid nitrogen bath and stored at -80°C . Protein concentrations were determined by the Bradford assay [11]. In this study the protein concentration of NE was ~ 6 mg/ml.

NaCl was added to NE in buffer D to 0.5 M and set on ice for 20 minutes. Samples of this NE were dialyzed against 60% buffer D in the presence or absence of the appropriate amounts of antibodies: anti-Mac-2, NCL-GAL3, or anti-Sm. Similarly, recombinant Gal3 [12], GST or GST-hGal3(1–100) was added to the NE at this step to test the effect of the recombinant or GST-fusion proteins on the splicing reaction. Dialysis was carried out for 70 minutes at 4°C in a microdialyzer with a 6–8 kD cutoff dialysis membrane [4]. Splicing reaction mixtures, in a total volume of 12 μl , contained dialyzed NE sample (10 μl), [^{32}P]MINX pre-mRNA [13], 2.5 mM MgCl_2 , 1.5 mM ATP, 20 mM creatine phosphate, 0.5 mM DTT, and 20 U RNasin (Promega). Splicing reactions were incubated at 30°C for 45–60 minutes. The RNAs of the reaction mixture were extracted and analyzed as described [4]. Quantitation of product formation was carried out by exposing the gel to a Storage Phosphor Screen (Amersham Biosciences), scanning on a Storm 860 scanner (Molecular Dynamics), and using the program Image Quant (Molecular Dynamics) to determine the percentage of radioactivity in specific bands in each lane.

The assembly of spliceosomes was monitored by gel mobility shift assay for complex formation [13,14]. Non-denaturing 4% polyacrylamide gels (acrylamide:bisacrylamide 80:1 (w/w)), 50 mM Tris pH 8.8, 50 mM glycine, 10 mM EDTA pH 8.0) were pre-run at 150V for 30 minutes at 4°C . Heparin (1 μl at 10 mg/ml) was added to the splicing reaction, incubated for 15 minutes at 30°C , and set on ice for 5 minutes. Then, 1.3 μl of 10X loading dye (97% glycerol, 1% bromophenol blue, 1% xylene cyanol) was added. Half of each sample was loaded and electrophoresed at 150V for 90 minutes at 4°C . The gel was overlaid on gel blot paper (Schleicher and Schuell), dried, analyzed by autoradiography, and quantitated using the phosphor imaging screen, scanner and quantitation program as described above.

The effect of peptides on the splicing reaction and on spliceosome assembly was tested by preincubating the peptides with NE in a final volume of 10 μl (containing 60% buffer D, 2.5 mM MgCl_2 , 1.5 mM ATP, 20 mM creatine phosphate, and 0.5 mM DTT) for 20 minutes at 30°C . [^{32}P]MINX and 20 U RNasin were added and splicing was carried out in a total volume of 12 μl at 30°C for 0–45 minutes. Splicing reactions were processed as above for RNA analysis. For complex formation experiments, duplicate samples of the splicing reactions were removed at 0–15 minute time points and snap frozen. Upon thawing, 1 μl of heparin (10 mg/

ml) was added to each sample. The samples were incubated at 30° C for 15 minutes, and on ice for 5 minutes prior to running on the non-denaturing gels.

Construction of fusion proteins containing GST and Gal3

A 5' BamHI restriction site was introduced into the 750 bp human Gal3 cDNA [15] using the 5' primer (ATATATAGGATCCAAATGGCAGACAATTTTTCGCTC) for polymerase chain reaction (PCR). The 3' primer (TAATAAGCGGCCGCACTAGTGATT) includes the 3' NotI restriction endonuclease site. PCR products were purified, digested with BamHI and NotI, ligated into the vector pGEX 5X-2, and transformed into *E. coli* DH5 α cells via electroporation. The harvested plasmid derived from an ampicillin-selected colony was then sequenced using a primer (GGGCTGGCAAGCC-ACGTTTGGTG) complementary to a site just upstream of the multiple cloning region. This confirmed that the human Gal3 insert is present in the correct orientation and reading frame. This plasmid, pGEX-hgal3, expresses the full-length fusion protein, GST-hGal3(1–250) (see Fig. 1, panel A).

Fusion proteins containing GST followed by portions of the human Gal3 sequence (Fig. 1, panel A) were generated by site-directed mutagenesis. Complementary oligonucleotide primer sets with mutations that convert a pair of sense codons into a pair of stop codons at the desired location were designed for each desired mutant: (a) GST-hGal3(1–100) --- 5'-CCAAGTGCCCCCGAGCCTAATAGGCCACTGGCCCCTATGG-3' and 3'-CCATAGGGGCCAGTGGCCTATTAGGCTCCGGGGGCACTTGG-5'; (b) GST-hGal3(1–25) --- 5'-GGATGGCCTGGCGCATGATAGAACCGGTCTGCTGGGGCAGGGGG-3' and 3'-CCCCCTGCCCCAGCAGACCGGTTCTATCATGCGCCAGGCCATCC-5'. (Note that the primers code for a mutation that introduces an AgeI restriction endonuclease site downstream of the stop codons.) (c) GST-hGal3(1–14) --- 5'-GCGTTATCTGGGTCTTGATAAGCTTACCCTCAAGGATGGCCTGGC-3' and 3'-GCCAGGCCATCCTTGAGGGTAAGCTTATCAAGACCCAGATAACGC-3'. (Note that these primers also code for a new HindIII restriction endonuclease site downstream of the stop codons.) Following thermocycling with pGEX-hgal3, the template DNA was cleaved by incubation with the Dam methylation dependent endonuclease DpnI. The reaction mix was then used to transform DH5 α cells via electroporation. Colonies expressing the proper mutation were screened based on new restriction endonuclease sites introduced during the site directed mutagenesis (AgeI and HindIII, respectively), followed by further screening by western blotting of transformed bacterial lysates. The plasmid DNA for each was sequenced to confirm the mutations.

Constructs expressing the fusion proteins GST-hGal3(1–47) and GST-hGal3(46–250) were generated using the XmaI restriction endonuclease sites coded by the DNA base pairs of amino acid residues 48 and 45, respectively. GST-hGal3(1–47) was created by excising a portion of the Gal3 DNA insert in pGEX-hgal3 using the 5' BamHI and internal Gal3 XmaI restriction sites and ligating into pGEX 5X-2 that had been digested with BamHI and XmaI. Similarly, GST-hGal3(46–250) was created by excising a portion of the Gal3 DNA insert in pGEX-hgal3 using the internal Gal3 XmaI and 3' NotI restriction sites and ligating into pGEX 5X-2 that had been digested with XmaI and NotI. These ligation reactions were used to transform DH5 α bacteria by electroporation. Colonies were screened based on the size of the insert excised by double digestion of sites remaining on the pGEX multiple cloning region, BamHI and XhoI for the 1–47 construct and EcoRI and NotI for the 46–250 construct. The plasmid DNA from each was sequenced using the same primer as pGEX-hgal3 to confirm the mutations.

Protein expression and purification

GST fusion proteins were expressed in 500 ml cultures of *E. coli* BL-21 codon plus (DE3) cells (Stratagene) by induction with 100 μ M isopropyl- β -D-galactopyranoside (IPTG) for 2–

3 hours at 30°C. Cells were pelleted and stored at -70°C. Thawed bacterial pellets were resuspended (one-twentieth of the culture volume) in PBS containing protease inhibitors (4 µg/ml aprotinin, 5 µg/ml leupeptin, 0.2 µg/ml pepstatin A, and 1 mM Pefabloc (Roche)) and sonicated using a microtip probe. Triton X-100 was added to a final concentration of 1% for lysates that would be purified using glutathione beads or to 0.1% for lysates that would be purified using lactose beads. After rocking for 1 hour at 4°C, cell debris was removed by centrifugation at 12,000 x g for 10 minutes at 4°C. The supernatant was aliquotted, snap-frozen, and stored at -70°C.

GST-hGal3(1–250) and GST-hGal3(46–250) were purified by lactose affinity chromatography. All procedures were carried out at 4°C. Frozen stock lysate (5 ml) was thawed and diluted with 90 ml of binding-wash buffer (PBS containing 1 mM DTT and 0.5 mM PMSF) and rotated overnight with 5 ml of lactose-agarose beads (Sigma). The slurry was poured into a poly-prep chromatography column (Bio-Rad) and allowed to flow through. The column was washed with 10 column volumes of binding-wash buffer. The bound protein was eluted with 15 ml of elution buffer (PBS, 0.4 M lactose, 1 mM DTT, 0.5 mM PMSF). In this procedure, the elution buffer was first allowed to flow into the column (approximately 5 ml), the column was stopped for 1 hour and then 15 x 1 ml fractions were collected. Samples from each fraction were electrophoresed on 12.5% SDS-PAGE, silver stained and screened by western blotting with NCL-GAL3, anti-Mac-2, and anti-GST. Fractions were selected for highest quantity and purity, pooled, and concentrated using a Centricon-10 filter unit (Amicon) allowing buffer exchange and removal of lactose. The amount of protein in each concentrated preparation was quantitated using the Bradford assay [11]. Silver staining of the SDS-PAGE gel, compared to known amounts of standards, provided confirmation of this quantitation.

Because GST and the other fusion proteins lacked the CRD of Gal3 (see Fig. 1, panel A), they were purified by glutathione affinity chromatography. All procedures were carried out at 4°C. Frozen stock lysate (5 ml) was thawed, diluted with 45 ml of binding buffer (PBS, 0.1% TritonX-100, 1 mM DTT, 0.5 mM PMSF) along with 1 ml of glutathione beads (Pierce) and rotated for 2 hours. The beads were then pelleted by centrifugation at 2,000 x g for 3 minutes. Supernatant was removed and the beads were washed 3 times with 50 ml binding buffer. After removal of the last wash, the beads were washed with 50 ml of wash buffer (PBS, 1 mM DTT, 0.5 mM PMSF). The beads were then resuspended in 10 ml of wash buffer and loaded into a chromatography column. The column was washed with another 10 ml of wash buffer. Purified proteins were eluted with 10 ml of elution buffer (PBS, 10 mM glutathione, 0.5 mM PMSF), by allowing 1 ml of elution buffer to flow into the column and stopping the column for 15 minutes. Fractions (0.5 ml) were collected and analyzed as above. Selected fractions were pooled, dialyzed, and quantitated.

SDS gel electrophoresis, silver staining, and western blotting

Purified proteins or NE were subjected to SDS-PAGE as described by Laemmli [16] on 12.5% acrylamide gels. Proteins were visualized by silver staining as described by Merrill *et al.* [17]. Some gels were electrophoretically transferred onto Hybond Nitrocellulose paper (Amersham Biosciences) and subjected to immunoblotting. Membranes blotted with rabbit primary antibodies were pre-blocked with unconjugated goat anti-rabbit antibody (Sigma) at 1:2000 dilution. The membranes were then incubated with horseradish peroxidase (HRP)-conjugated secondary antibodies for 1 hour and then washed four times for 15 minutes. The proteins were visualized by use of the Western Lightning Chemiluminescence System (Perkin Elmer Life Sciences).

NCL-GAL3 was used at a dilution of 1:3000. Anti-GST antibodies were affinity purified from serum of rabbits immunized with GST constructs and used at a dilution of 1:6000. Anti-Mac-2 antibody was used at 1:1000 dilution. Goat anti-mouse HRP (Bio-Rad), goat anti-rat HRP

(Roche), and goat anti-rabbit HRP (Bio-Rad), were each used at a dilution of 1:10,000. Peptide inhibition experiments were carried out by incubating the appropriate amounts of antibody and peptide in 0.5 ml PBS at 4°C overnight, followed by appropriate dilution with 1% milk, 10 mM Tris, 0.5 M NaCl, 0.05% Tween 20, pH 7.5 and incubation with membrane as described above.

Results

Epitope mapping for the Mac-2 and NCL-GAL3 monoclonal antibodies

The cDNA for human Gal3 was cloned into the pGEX 5X-2 vector and full-length Gal3 was expressed as a fusion protein with glutathione S-transferase (GST), designated as GST-hGal3 (1–250). In addition, site-directed mutagenesis was carried out to introduce translation termination codons at various positions so that the expressed fusion protein was truncated at specific residues of the Gal3 polypeptide chain (see Fig. 1A). Finally, a construct was also engineered to express the GST fusion protein in which the NH₂-terminal 45 amino acids of Gal3 are missing (GST-hGal3 (46–250)). The GST-fusion proteins were purified by glutathione-affinity or lactose-affinity chromatography and subjected to SDS-PAGE analysis. Silver staining provided documentation on the purity of each of the fusion protein preparations (Fig. 1B). Each of the fusion proteins was also detectable by immunoblotting with polyclonal rabbit anti-GST (Fig. 1C). Both of these techniques also ascertained that the molecular weight of the predominant polypeptide was in agreement with the expected size of the fusion protein (Fig. 1A).

When the various GST-fusion proteins were subjected to immunoblotting with NCL-GAL3 (a murine hybridoma generated against human Gal3), a positive reaction was observed with each except GST-hGal3 (46–250) (Fig. 1D). GST itself also failed to react with NCL-GAL3 (Fig. 1D, lane 6). These results suggest that the epitope of NCL-GAL3 lies in the first 14 residues of the galectin-3 polypeptide. This notion is consistent with the observation that a Gal3 S6A mutant (serine to alanine mutation at residue 6) resulted in the loss of reactivity with the NCL-GAL3 monoclonal antibody (data not shown).

Anti-Mac-2 is a rat monoclonal antibody originally derived through immunization with mouse macrophages expressing Gal3 [8]. When the various GST-fusion proteins were subjected to immunoblotting with anti-Mac-2, the antibody reacted with GST-hGal3 (1–250), GST-hGal3 (1–100), and GST-hGal3 (46–250) (Fig. 1E, lanes 1, 2, and 7). In contrast, the fusion proteins missing residues 48–100 of the Gal3 polypeptide all failed to react (Fig. 1E, lanes 3–5). These results suggest that the epitope of anti-Mac-2 lies between residues 48 and 100. This conclusion differs from that of Gong *et al.* [7], who assigned the epitope of the anti-Mac-2 antibody to the first 11 residues of Gal3.

Specific peptide inhibition of immunoblotting by monoclonal antibodies

The region of the Gal3 polypeptide between residues 48 and 100 contains multiple repeats of a 9-residue motif with a consensus sequence PGAYPGXXX [1,2]. For example, residues 41–67 of the murine Gal3 sequence contain three perfect tandem repeats of PGAYPGQAP. On this basis, three peptides were synthesized containing three iterations (27-mer), two iterations (18-mer), and a single iteration (9-mer) of this sequence and were tested for their ability to block immunoblotting of recombinant GST-hGal3(1–250) (*M_r* ~55 kD) by the anti-Mac-2 antibody.

Over a concentration range of 2.5–250 nM (about 1.3- to 130-fold excess over antibody), the 18-mer peptide inhibited the reaction between anti-Mac-2 and Gal3 in a concentration dependent fashion (Fig. 2A, compare lanes 2–4 versus lane 1). Similar results were obtained

with the 27-mer (data not shown). In contrast, the 9-mer showed no effect at the corresponding molar concentrations (Fig. 2A, lanes 5–7). Neither the 9-mer nor the 18-mer affected the immunoblotting by the NCL-GAL3 monoclonal antibody (Fig. 2B). These results suggest that the epitope of the anti-Mac-2 antibody: (a) requires two iterations of the PGAYPGQAP sequence; or (b) overlaps the end of one repeat with the beginning of the second one (e.g., ⁴⁶PGQAPP⁵⁴GAY⁵⁴). In light of our observation that GST-hGal3 (46–250), which contains such a sequence, reacted positively with anti-Mac-2 (Fig. 1E, lane 7), we favor the latter hypothesis.

Similarly, a peptide corresponding to the sequence of the first 14 residues of human Gal3, MADNFS¹LHDALSGS¹⁴, was also synthesized. This peptide, designated 14-mer, was tested for the ability to inhibit blotting of endogenous Gal3 (*M_r* ~30 kD) present in NE of HeLa cells by the NCL-GAL3 antibody. Over a concentration range of 0.07–7 nM (3–300-fold excess over antibody), the 14-mer peptide inhibited the immunoblotting by NCL-GAL3 (Fig. 2C, compare lanes 2–4 versus lane 1). In contrast, the 14-mer peptide containing a serine to alanine substitution at position 6 (mt 14-mer) failed to inhibit immunoblotting by NCL-GAL3 over an identical concentration range (Fig. 2C, lanes 5–7). These findings support the conclusion that the NCL-GAL3 epitope lies within the first 14 amino acids of Gal3 and also indicate that serine 6 constitutes an important determinant in the epitope. The 14-mer peptide did not inhibit blotting by the anti-Mac-2 antibody (Fig. 2D, lanes 2–4): (a) indicating that it does not inhibit antibody-antigen interactions non-specifically, and (b) lending additional support to the conclusion that the anti-Mac-2 epitope lies outside of the first 14 amino acids of Gal3.

Different effects of two monoclonal antibodies against Gal3 on pre-mRNA splicing

The effects of these two monoclonal antibodies were tested on splicing competent NE derived from HeLa cells. In this system, human autoimmune serum reactive against the Sm epitopes of snRNPs (anti-Sm) served as the positive control. Anti-Sm inhibited the conversion of the pre-mRNA substrate into the mRNA product (Fig. 3, lane 8 in both panels A and B). Tested under the same conditions, the NCL-GAL3 monoclonal antibody also inhibited splicing in a dose-dependent fashion (Fig. 3A). Partial inhibition was observed at a concentration as low as ~9 µg/ml (~60 nM) (Fig. 3A, lane 2) and complete inhibition was achieved at a concentration of ~20 µg/ml (~140 nM) (Fig. 3A, lane 5). In contrast, the anti-Mac-2 monoclonal did not inhibit splicing over the same concentration range of 9 – 23 µg/ml (Fig. 3B, lanes 2–7). Both products of the splicing reaction, ligated exons and free intron lariat, were observed at all concentrations tested.

NCL-GAL3 recognizes a single polypeptide, corresponding to human Gal3, in total extracts [18] as well as in NE (Fig. 2C, lane 1) of HeLa cells. In previous studies, we had shown that addition of recombinant Gal3 alone to a splicing competent NE had no effect on the splicing reaction [19]. On the other hand, the inhibitory effect of NCL-GAL3 on splicing can be overcome by prior incubation with recombinant Gal3 (data not shown). These results suggest that the effect of NCL-GAL3 on splicing was due to specific recognition of its antigen.

At various early time points, aliquots of the splicing reaction mixture were analyzed by non-denaturing gel electrophoresis to assess progress in spliceosome assembly. In the absence of antibody addition, the radiolabeled pre-mRNA initially found in the region labeled as the H-complex (*t*=0) is converted to the A and B active spliceosomal complexes within five minutes (Fig. 3D). Further incubation results in the formation of more active complexes at the expense of the H-complex. Essentially identical results were observed in splicing reactions carried out in the presence of anti-Mac-2 (Fig. 3D). It should be noted that the native gel system used in the present study does not resolve H- and E-complexes [20] so our use of the term H-complex is meant only to indicate a region of the gel rather than a distinction between the two early complexes of the spliceosome assembly pathway.

At a concentration of ~24 µg/ml, the NCL-GAL3 antibody inhibited the splicing reaction (Fig. 3A, lane 7). This is paralleled by an almost complete arrest of the progression of the H-complex to the higher order A- and B-complexes (Fig. 3C). At a concentration of ~10 µg/ml, NCL-GAL3 only partially inhibited spliceosome assembly (Fig. 3C) and the splicing reaction (Fig. 3A, lane 3).

Effect of addition of PGAYPGQAP peptides on the in vitro splicing reaction

The failure of anti-Mac-2 to inhibit splicing might be rationalized in terms of its epitope being buried in protein-protein interactions with components of the spliceosome and those Gal3 molecules assembled into splicing complexes are inaccessible to the monoclonal antibody. Thus, we wanted to test whether peptides bearing its epitope, the PGAYPGQAP repeating motif, can perturb the splicing reaction. Control NE exhibited good splicing activity, converting ~35% of the pre-mRNA substrate into the mature RNA product (Fig. 4A, lane 4). Addition of the 27-mer synthetic peptide containing the PGAYPGQAP motif inhibited the splicing reaction. At concentrations of 300 µM and 600 µM, product formation was reduced to ~15% and <5%, respectively (Fig. 4A, lanes 2 and 3). There were also lower levels of the intermediates of the splicing reaction (free exon 1 and lariat-exon 2) and higher levels of the starting substrate.

In contrast, addition of the 9-mer (Fig. 4A, lanes 9–12) and the 18-mer (Fig. 4A, lanes 5–8) synthetic peptides did not yield the same result. Moreover, the 27mer-S peptide, with the same amino acid composition but with a scrambled sequence, also showed no effect on the splicing reaction (Fig. 4B, lanes 2–4). These results suggest that multiple repeats (more than two) of the PGAYPGXXX motif are necessary to perturb the endogenous Gal3 interaction with components of the splicing reaction.

Consistent with this notion, GST-hGal3 (1–100), which contains seven repeats of the PGAYPG motif (some repeats are imperfect such as PGVYPGPPSG), inhibited the splicing reaction (Fig. 5A, lanes 2–4 and panel B). On the other hand, GST alone did not inhibit splicing over the same concentration range (Fig. 5A, lanes 5–7 and panel B). Similarly, addition of the carboxyl-terminal CRD (also approximately 100 residues but lacking the PGAYPGXXX motif) to a complete NE did not perturb the splicing reaction [19]. Comparison of the dose-responses indicated that the concentration required to achieve full inhibition was observed at a much lower concentration of GST-hGal3 (1–100) (~100 µM) than the 27-mer synthetic peptide (~600 µM). Together, the results provide confirmation of the dominant negative effect and suggest that the PGAYPGXXX motif contributes to the interaction of the Gal3 polypeptide with the splicing machinery.

Effect of the 27-mer on the kinetics of spliceosomal assembly and product formation

When the splicing reaction was carried out with control NE, the products of the first cleavage reaction, free exon 1 and lariat-exon 2, were observed after 10 minutes (Fig. 6A). Product bands (ligated exon 1-exon 2 and intron) appeared within 15 minutes and increased monotonically as a function of time (Fig. 6A and 6B). Together, these bands accounted for ~35% of the total radioactivity of a splicing reaction at 30 minutes (Fig. 6B). In the presence of the 27-mer peptide inhibitor (600 µM), however, neither intermediates nor products could be observed at 15 minutes (Fig. 6A). The intermediates appeared to accumulate at 20–30 minutes, but product formation remained less than 3% over the time course (Fig. 6 B).

In the same manner, we monitored the kinetics with which early complexes (H- and E-complexes) are chased into higher order spliceosomal A-, B-, and C-complexes (Fig. 7A). Both in the absence and presence of the 27-mer peptide inhibitor, the H-complex disappeared with the same kinetics (Fig. 7, panels A and B). However, while ~50% of the radioactive pre-mRNA

in the reaction without the inhibitor has progressed into B-complexes within the first 5 minutes (Fig. 7D), ~50% of the pre-mRNA in the reaction containing the peptide inhibitor is still in the A-complex at the same time point (Fig. 7C). Therefore, the peptide appears to slow the progression into the B-complex, resulting in an accumulation of the A-complex at 5 minutes and a persistently higher amount of the A-complex compared to controls (Fig. 7C). Thus, a steady increase in the active C-complex in the reaction without the inhibitor precedes the first signs of an active C-complex in the reaction containing the peptide (Fig. 7E). This is consistent with the observation that, in the presence of the peptide, there is not a hint of the products of the first cleavage reaction until 20 minutes have elapsed (Fig. 6A).

Discussion

The key findings of the present study include: (a) the epitope of the NCL-GAL3 monoclonal antibody resides in the first 14 residues of the Gal3 polypeptide, a region containing sites of phosphorylation at Ser 6 and Ser 12 [6]; (b) the epitope of the anti-Mac-2 monoclonal antibody maps to residue 48–67 of the Gal3 polypeptide, corresponding to one or two repeats of the motif PGAYPGQAP; (c) addition of NCL-GAL3 to a splicing competent NE inhibits the *in vitro* splicing reaction whereas parallel addition of anti-Mac-2 fails to yield the same effect; and (d) addition of a 27-mer synthetic peptide, bearing three iterations of PGAYPGQAP, to NE inhibits the splicing reaction. Together, the results indicate that, in addition to the CRD, sequences in the ND of Gal3 also interact with the splicing machinery.

Inhibition of *in vitro* splicing has been demonstrated with other antibodies and synthetic peptides to implicate the involvement of specific proteins in spliceosome assembly and the splicing reaction. For example, Yuryev *et al.* [21] monitored the effect on splicing by a monoclonal antibody directed against the carboxyl-terminal domain of the large subunit of RNA polymerase II. At an antibody concentration of ~33 μ M, there was a concomitant loss of the products, as well as intermediates, of the splicing reaction. In the same study, Yuryev *et al.* [21] also used a peptide consisting of eight consensus repeats in the carboxyl-terminal domain of RNA polymerase II large subunit to inhibit splicing. Four peptides that inhibit Ca^{2+} -dependent calmodulin kinase II were shown to block spliceosome assembly and pre-mRNA splicing *in vitro* [22]. One of the peptides (designated GS) was derived from the sequence of glycogen synthase and competitively inhibited the kinase from binding its substrate. This GS peptide inhibited splicing at a concentration of ~300 μ M. More interestingly, Parker and Steitz [22] observed splicing products after prolonged incubation. This delay (hours) in appearance of splicing products is consistent with the observation of stalled spliceosome assembly at the B-complex stage. These results are similar to our own observations with the 27-mer peptide, in which there is a delay in the appearance of intermediates and products, as well as in slowing the rates of A- to B-complex progression.

Several lines of evidence have now been accumulated to indicate that the interaction of Gal3 with components of the splicing machinery is mediated, at least in part, by the ND of the polypeptide. First, although a CRD (Gal1 alone or the COOH-terminal domain of Gal3) is sufficient to restore splicing activity to a galectin-depleted NE, the minimum concentrations required for reconstitution are four to eight times higher than that of the intact (full-length) Gal3 polypeptide [4]. It was hypothesized that the Gal3 ND, containing the proline- and glycine-rich repeats, plays a role in protein-protein interactions providing the basis for enhanced interactions with the splicing machinery. Second, that such protein-protein interactions occur between the ND and the spliceosome was suggested by the inhibition of splicing observed when the activity of NE is assayed in the presence of exogenously added ND. This could be demonstrated using the corresponding ND sequences of either human Gal3 (GST-hGal3(1–100)) or synthetic peptides bearing the predominant structural motif of the ND, the PGAYPGXXX repeats. Finally, we have recently documented that antibodies directed

against either Gal1 or Gal3 can immunoprecipitate RNAs from a splicing reaction mixture containing ^{32}P -labeled pre-mRNA [5]. The addition of GST-Gal3ND inhibited the precipitation of radioactive spliceosomal RNA by anti-Gal1, suggesting that it blocked the incorporation of Gal1 into the splicing machinery. Thus, it appears that the ND exerts its dominant negative effect by competing for the spliceosomal component with which Gal3 (and Gal1) is associated. Alternatively, the ND can interact with Gal3 (or Gal1), resulting in a conformational change that precludes the Gal3 molecule from association with the spliceosome. ND interactions with itself or with the CRD have been implicated by electron microscopic imaging, by nuclear magnetic resonance, by cross-linking, and by analysis of positive cooperativity in the binding of Gal3 to multivalent ligands [23–25].

The differential effects of the monoclonal antibodies NCL-GAL3 and anti-Mac-2 on the splicing reaction are interpreted in this context. The epitope of anti-Mac-2 resides in the PGAYPGXXX repeats of the ND. Therefore, its failure to inhibit the cell-free splicing reaction is consistent with the notion that this region is buried in interactions with the splicing machinery and is inaccessible to the antibody. On the other hand, it appears that the NH_2 -terminus of the Gal3 polypeptide, even when associated with the spliceosome, remains accessible to the NCL-GAL3 antibody, which inhibits the splicing reaction.

A key consideration in the interpretation of the antibody effects is the assignment of the epitope location. Gong *et al.* [7] reported that deletion of the NH_2 -terminal 11 amino acids of human Gal3 resulted in loss of immunoblotting by the anti-Mac-2 antibody. It was concluded that the antigenic recognition site of anti-Mac-2 is at the amino terminus. This is clearly inconsistent with the results of our mapping studies, which showed that the anti-Mac-2 antibody immunoblotted GST fusion proteins containing residues 46–100 of the Gal3 polypeptide but failed to immunoblot GST-hGal3 (1–14), GST-hGal3 (1–26), GST-hGal3 (1–47), all of which contained the NH_2 -terminal 11 residues. Moreover, the 18-mer and 27-mer peptides bearing at least two iterations of the PGAYPGQAP motif between residues 46–100 inhibited the immunoblotting of the anti-Mac-2 antibody without any effect on the immunoblotting of the NCL-GAL3 antibody. In contrast, the 14-mer peptide containing the NH_2 -terminus of human Gal3 failed to inhibit the immunoblotting observed with anti-Mac-2. Finally, the NCL-GAL3 antibody, which does map to the NH_2 -terminal 14 residues of Gal3, exhibited a drastically different effect on the *in vitro* splicing reaction than anti-Mac-2.

The original source of our anti-Mac-2 antibody was clone M3/38.1.2.8.HL.2 from ATCC TIB 166, identical to that reported in Gong *et al.* [7]. Because of this apparent discrepancy with the results and conclusions of Gong *et al.* [7], we obtained an independent preparation of the anti-Mac-2 antibody from a commercial source (Acris Antibodies GmbH, Hiddenhausen, Germany). Using our GST fusion protein reagents, we found that the epitope of this preparation of the anti-Mac-2 antibody also mapped to residues 48–100, rather than the NH_2 -terminal 11 residues of the Gal3 polypeptide. On this basis, we do not understand the discrepancy between our results and conclusions and those of Gong *et al.* [7].

Several intracellular binding partners of Gal3 have been identified: (a) general transcription factor TFII-I (P.G. Voss and J.L. Wang, manuscript in preparation); (b) anti-apoptosis factor Bcl-2 [26,27]; (c) cysteine- and histidine-rich protein Chrp [28]; (d) Gemin4 [19], a component of a macromolecular complex responsible for the assembly of the snRNPs; (e) B-cell specific transcription coactivator OKA-B [29]; (f) coactivator of the Wnt signaling pathway β -catenin [30]; (g) cytokeratin [31]; (h) the calcium- and phospholipid-binding protein synexin [32]; (i) Sufu (Suppressor of fused), a negative regulator of the Gli family of transcription factors [33]; and (j) the thyroid-specific transcription factor TTF-1 [34]. Where there is available evidence, only the COOH-terminal CRD of Gal3 has been implicated in these interactions. To the best of our knowledge, there has been no report of an interaction involving the ND with an

identified partner. In this regard, it may be interesting to note that differential scanning calorimetry studies suggest that the ND of Gal3 has a very low melting temperature ($\sim 39^{\circ}\text{C}$), compared to the globular CRD which has a melting temperature of $\sim 56^{\circ}\text{C}$ [12]. This implies that the ND may not be folded tightly until it interacts with a ligand. On this basis, the identification of a binding partner that interacts with the PGAYPG motifs of the ND will be of great interest, not only in terms of the splicing reaction but also in terms of the possibility of determining the structure of the ND.

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Figure 1. Fusion proteins containing glutathione S-transferase and galectin-3 sequences of varying lengths

Panel A: Human galectin-3 cDNA (hGal3) was engineered into the pGEX 5X-2 vector bearing the *Schistosoma japonicum* glutathione S-transferase (GST) sequence. The numbers along the left-hand side indicate lane assignments in panels B-E. The names of each construct are listed beside the lane assignments. Numbers in parentheses indicate the galectin-3 amino acids included in each construct. N denotes the amino terminus; C denotes the carboxyl terminus. The rectangles represent the GST protein, and bars indicate the portions of galectin-3 included in the fusion protein (with amino acid residues listed above each bar). The approximate molecular weights of each construct are listed at the right. Panel B-E: Each construct was

expressed and the fusion protein was purified, subjected to SDS-PAGE, and analyzed by silver staining (panel B) and by immunoblotting with anti-GST antibodies (panel C), with mouse monoclonal NCL-GAL3 (panel D), and with rat monoclonal anti-Mac-2 (panel E). Approximately 35 ng of each purified fusion protein were electrophoresed. The numbers on the left indicate the positions of migration of molecular weight markers.

Figure 2. Peptide inhibition of immunoblotting of galectin-3 by two monoclonal antibodies directed against galectin-3

Equal amounts of purified recombinant GST-human galectin-3 (panels A and B) or HeLa cell nuclear extract (panels C and D) were subjected to SDS-PAGE. The antibodies used for immunoblotting are listed on the left. The triangle above each panel signifies increasing concentrations of peptide tested as inhibitors of blotting by an antibody. 18-mer, PGAYPGQAPPGAYPGQAP (2.5–250 nM); 9-mer, PGAYPGQAP (2.5–250 nM). 14-mer, MADNFSLHDALSGS (0.07–7 nM); mt 14-mer, MADNFALHDALSGS (0.07–7 nM).

Figure 3. The effect of antibody addition on the splicing of pre-mRNA and on spliceosome assembly
 Panel A: Effect of NCL-GAL3 on the splicing reaction; Panel B: Effect of anti-Mac-2 on the splicing reaction. In both panels, the splicing activity of NE (no additions) is shown in lane 1; the effect of anti-Sm antibodies (1:23 dilution of human autoimmune serum) is shown in lane 8. In both panels, the concentrations of the Gal3-specific antibody tested were: lane 2, ~8 µg/ml; lane 3, ~10 µg/ml; lane 4, ~17 µg/ml; lane 5, ~21 µg/ml; lane 6, ~23 µg/ml; and lane 7, ~24 µg/ml. The cell-free splicing assay was carried out using ^{32}P -labeled MINX pre-mRNA substrate. Products of the splicing reaction were analyzed by electrophoresis through a 13% polyacrylamide-urea gel system, followed by autoradiography. The positions of migration of the pre-mRNA substrate, the splicing intermediates (exon 1 and lariat-exon 2), and the products

(mature RNA and lariat intron) are indicated between the two panels. Panel C: Time course of spliceosome assembly in the presence of two different concentrations of NCL-GAL3. Panel D: Time course of spliceosome assembly in the absence and presence of anti-Mac-2. In both panels, splicing reaction mixtures containing ^{32}P -labeled MINX pre-mRNA were sampled at various times (min) indicated and were analyzed by electrophoresis through non-denaturing gel system, followed by autoradiography. The regions of migration of early complexes (H- and E-complexes) and active spliceosomes (A- and B-complexes) are indicated between the two panels.

Figure 4. Comparison of the effect of addition of synthetic peptides on the splicing activity of nuclear extract

Panel A: Effect of peptides containing three iterations, two iterations, and a single iteration of the PGAYPGQAP motif. Lanes 1–3: 27-mer at 100, 300, and 600 μ M. Lane 4: NE control (no addition). Lanes 5–8: 18-mer at 100, 300, 600, and 1000 μ M. Lane 9–12: 9-mer at 100, 300, 600, and 1000 μ M. Panel B: Effect of the 27mer-S peptide, containing scrambled sequence of the same amino acid composition (YPGGGQPPAPQYPGPPAAAGYAGPQPA). Lane 1: NE control (no addition). Lanes 2–4: 27mer-S at 100, 300 and 600 μ M. All reactions contained 32 P-labeled MINX pre-mRNA substrate and products of the splicing reaction were analyzed by electrophoresis through a 13% polyacrylamide-urea gel, followed by

autoradiography. The positions of migration of pre-mRNA substrate, splicing intermediates, and RNA products are highlighted on the right.

Figure 5. Comparison of the effect of GST-hGal3 (1–100) and GST on pre-mRNA splicing
 Panel A: autoradiogram of the splicing assay. The proteins were tested at concentrations of 10, 100, and 200 μM. All reactions contained ^{32}P -labeled MINX pre-mRNA substrate and products of the splicing reaction were analyzed by electrophoresis through a 13% polyacrylamide-urea gel, followed by autoradiography. The positions of migration of pre-mRNA substrate, splicing intermediates, and RNA products are highlighted on the right. Panel B: Dose-response curve of the effects of the proteins on product formation, derived from the experiment shown in panel A.

Figure 6. The effect of the 27-mer peptide on the kinetics of the splicing reaction

Panel A: autoradiogram of the splicing assay. The 27-mer was tested at a concentration of 600 μ M. All reactions contained 32 P-labeled MINX pre-mRNA substrate (5000 c.p.m.) and products of the splicing reaction were analyzed by electrophoresis in polyacrylamide-urea gels, followed by autoradiography. Panel B: Time course of formation of products in the presence and absence of the 27-mer peptide, derived from the experiment shown in panel A.

Figure 7. The effect of the 27-mer peptide on the kinetics of spliceosome assembly

Panel A: autoradiogram of the non-denaturing gel. The 27-mer was tested at a concentration of 600 μ M. All reactions contained 32 P-labeled MINX pre-mRNA substrate (5000 c.p.m.) and were analyzed in non-denaturing polyacrylamide gels, followed by autoradiography. The positions of migration of the H-, A-, B-, and C-spliceosomal complexes are highlighted on the right. Panel B–E: Quantitation of the data shown in panel A, for H-, A-, B-, and C-complexes, respectively.