Spider Web Glue: Two Proteins Expressed from Opposite Strands of the Same DNA Sequence

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The various silks that make up the web of the orb web spiders have been studied extensively. However, success in prey capture depends as much on the web glue as on the fibers. Spider silk glue, which is considered one of the strongest and most effective biological glues, is an aqueous solution secreted from the orb weaving spider's aggregate glands and coats the spiral prey capturing threads of their webs. Studies identified the major component of the glue as microscopic nodules made of a glycoprotein. This study describes two newly discovered proteins that form the glue-glycoprotein of the golden orb weaving spider *Nephila clavipes*. Our results demonstrate that both proteins contain unique 110 amino acid repetitive domains that are encoded by opposite strands of the same DNA sequence. Thus, the genome of the spider encodes two distinct yet functionally related genes by using both strands of an identical DNA sequence. Moreover, the closest match for the nonrepetitive region of one of the proteins is chitin binding proteins. The web glue appears to have evolved a substantial level of sophistication matching that of the spider silk fibers.

Introduction

Spider silk has attracted scientists to study its unique mechanical properties and its potential to provide new biobased materials for numerous applications ranging from protective clothing to medical products.[1] However, one of the most intriguing spider biomaterials, yet one of the least explored, is the aqueous glue that coats the sticky-spiral threads of orb weaving spiders to retain prey in the web. It is considered one of the strongest and most effective biological glues.[2–5] The aqueous coat is secreted from the orb weaving spider's aggregate glands, and the gland's contents have been studied by several research groups.[3–5] Chemical analysis of this complex aqueous solution shows relatively high concentrations of water-soluble organic compounds related to neurotransmitters, free amino acids, small peptides, low concentrations of various inorganic salts, and glycoproteins. It has been suggested that the contents of this solution generate hygroscopic forces that may contribute to the thread stickiness, however, studies identified the actual glue of ecribellate spiders as microscopic nodules made of a glycoprotein.[5] To date, almost nothing is known about the molecular structure and function of this glycoprotein.

In this study, we describe two newly discovered genes that encode two subunits of the glue-glycoprotein of the golden orb weaving spider *Nephila clavipes*. We show that both putative glycoprotein subunits have unique repetitive domains that are individually expressed from opposite strands of an identical DNA sequence. Cloning of these glycoprotein genes may enable large-scale production, studying their biochemical traits and developing new biobased glue for numerous purposes.

Materials and Methods

cDNA Construction and Gene Cloning. Adult females of the golden orb weaving spider *Nephila clavipes* were collected in Florida and dissected to isolate their aggregate glands. Glands were immediately frozen in liquid nitrogen and kept in a −80 °C freezer until use. Intact mRNA (mRNA) was extracted from those glands, using Tri Reagent (#TR118, MRC, OH, U.S.A.) and oligo-T Dynabeads (#610.05, Dynal Biotech ASA, Oslo, Norway). This mRNA was promptly used to construct a directional cDNA (cDNA) library, using ZAP Express cDNA synthesis kit and ZAP Express cDNA Gigapack III Gold Cloning kit (#200403, #200451, Stratagene, CA). The cDNA library was mass-excised to produce pBK-CMV vectors that were transformed into *Escherichia coli* host strain cells (as described by manufacturer, Stratagene, CA). The library was then screened by colony lifting. Plasmids were extracted from randomly selected colonies (QIAprep, 27104, Qiagen, CA) and inserts were sequenced. One clone was isolated at relatively high frequency. This clone showed two possible reading frames on opposite DNA strands that translated into two highly repetitive and potentially glycosylated proteins. Oligonucleotides were individually designed toward the C-terminus of each potential gene (reading frame), based on this clone. The oligonucleotides are 5′CA-GAAGAACCCGAAACACCGAGTCCAGAAAC3′ toward the 3 prime end of *asg1* and 5′GTTTCTGGACTCGGTGTTTCGGGTTCTTCTG3′ toward the 3′ end of *asg2*. The oligonucleotides were used as primers to clone the C-terminus of each putative gene by Rapid Amplification of cDNA Ends Polymerase Chain Reaction (RACE-PCR), utilizing the cDNA library as a DNA template and a vector primer adjacent to the C-terminus of the inserted clone. PCR was carried out in a thermal cycler (MyCycler, Bio-Rad). The reactions were performed in a volume of 50 μL of PCR solution containing 2 μL of template, 10 pMol of each primer, and 45 μL of Platinum PCR SuperMix (#11306, Invitrogen, CA). The thermal cycler was programmed to 95 °C (5 min) followed by 35 cycles of 95 °C (15 s), 55 °C (45 s), and 72 °C (5 min), followed by 72 °C (30 min). RACE-PCR products were purified (QIAquick Gel Extraction kit, 28704, Qiagen, CA) and served as DNA templates in a further nested-PCR, utilizing the same primers and same conditions used in RACE-PCR. This strategy helped to amplify gene specific fragments and to avoid low concentration undetectable DNA fragments. Certain nested-PCR products were subcloned and sequenced. The resulting products were visualized on 1% TAE-agarose gel, cleaned (QIAquick Gel Extraction kit, 28704, Qiagen, CA), cloned into a pCR 4-TOPO vector (TOPO TA Cloning kit for sequencing, #K4530−20, Invitrogen, CA), and sequenced. New oligonucleotides were generated toward the N-terminus of each putative gene, based on the newly

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discovered C-terminus sequences. Those were used as primers for RACE-PCR and nested PCR as described above. One oligonucleotide, 5′GGTGTAGTTGTTGGTTCTTCCG3′, was designed to clone the 5′ end of *asg1*. Two oligonucleotides, 5′CCACTAAACCTTCCAT-TAAG3′ and 5′CCTAACATCTTCCGGTAATTCATCG3′, were designed to clone the 5′ end of *asg2*. All samples were sequenced at the University of Wyoming sequencing facility. Sequence analysis was carried out using NCBI-BLAST (http://blast.ncbi.nlm.nih.gov/Blast.cgi) and EXPASY tools (http://ca.expasy.org/tools/).

Northern Blots. The oligonucleotides that were designed toward the C-terminus of each gene (complementary to *asg1* and complementary to *asg2*) were DIG-labeled (DIG oligonucleotide 3′-end labeling kit, second generation, #03353575910, Roche Applied Science, IN, U.S.A.) and used as probes in Northern blot analysis, utilizing total RNA and mRNA that were purified from different spider silk glands and tissues of *N. clavipes* (using intact glands and RNA extraction methods as described above). Northern blots were carried out according to DIG manufacturer protocols and reagents (Roche Applied Science, IN, U.S.A.). Specific reagents we used include: 2% agarose gel in 1× MOPS buffer containing 2% formaldehyde, positively charged nylon membranes (#11209272001), hybridization solution "DIG Easy Hyb" (#1603558), DIG wash and block buffer set (#11585762001), Anti-Digoxigenin-AP Fab fragments (#11093274910), and CDP-Star (#11685627001).

Biochemical Analysis of Glycoprotein. Uncontaminated fresh orb webs were obtained by maintaining spiders individually in cages for up to 8 months. Webs were collected daily on sterile glass rods and stored at −20 °C until analyzed. Each extraction included 60−70 webs. Collected rods coated with webs were ground by mortar and pestle and incubated in a minimal volume of extraction buffer (6 M guanidine HCl, 0.7 M β-mercaptoethanol, 0.2 M Na_2HPO_4) with protease inhibitor cocktail (#P2714,, Sigma, MO, U.S.A.) for 24 h at 4 °C with gentle agitation. Extracts were then centrifuged for 30 min at 4000 rpm (J-6M, Beckman Coulter, Germany). Supernatants were recentrifuged for 5 min at full speed (Microfuge18, Beckman Coulter, Germany) to discard insoluble web contents. Protein extraction samples were dissolved in sample buffer (without β-mercaptoethanol as it is already present in the extraction buffer), boiled for 10 min, and then resolved on a 12% mini-SDS-PAGE. Approximately 200 μL was loaded per well in a concentration of 0.15 μg of soluble protein extract/μL (30 μg/lane). Gels were run for 5 h at 60 V in Tris-glycine buffer with 0.01 M of sodium thioglycolate. The gels were stained with glycoprotein-specific staining techniques (Pro-Q Emerald 300, Molecular Probes, OR, U.S.A.). Gels were also stained using total protein stains such as SYPRORuby (Molecular Probes, OR, U.S.A.) to ensure the purity of the glycoprotein band. Positive bands (using glycoprotein-specific stain) were cut out from the gel and homogenized in 20% acetonitrile. Samples were then vortexed for 15 min, and acetonitrile was added to 50% of the solution after vortexing. Samples were centrifuged for 5 min and the supernatants were collected. The gel pellet was subjected to 3−4 cycles of extraction as described above (supernatants were combined). Gel purified glycoprotein solution was dialyzed against double distilled water at 4 °C for 18 h, then lyophilized, resuspended in 0.75% Triton X-100, 0.4% β-mercaptoethanol, and 0.1% SDS, and homogenized ultrasonically using a frequency of 20 kHz/sec for 5 min (Sonicator 3000, Misonix, NY) on ice. Samples were boiled for 15 min before being subjected to deglycosylation.

We have developed protocols for the deglycosylation of purified glycoprotein solutions, adapted to glycoprotein from spider silk. Glycoprotein solutions, purified from SDS-PAGE bands, were used for deglycosylation. Protocols include enzymatic analyses based on E-DEGLY and PP0200 deglycosylation kits (Sigma, MO, U.S.A.) that contain the *N*-linked glycoprotein cleavage enzyme PNGase F and the *O*-linked glycoprotein cleavage enzymes endo-*O*-glycosidase, α-2(3,6,8.9)-neuraminidase (Sialidase A), β-1,4-galactosidase, and β-*N*-acetylglucosaminidase. Approximately 100 μg of gel-extracted glycoprotein were used in each reaction. Enzymes were directly added to
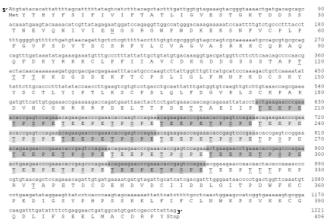

Figure 1. Nucleotide and amino acid sequence of the cloned spider glue glycoprotein ASG1. The 1221-bp clone contains an open reading frame encoding a 406 amino acid protein with a calculated molecular weight of 45221.7 Da and an isoelectric point of 4.22. The most likely potential *O*-glycosylation sites (threonine or serine) and an *N*-glycosylation site (aspargine) are underlined. DNA and protein repeats are shaded. Gene sequence has been deposited to GeneBank under accession number EU780014.

the solution and the reaction was incubated for 24 h at 37 °C. Samples of deglycosylation reactions were then resolved on 12% mini-SDS-PAGE. The gels were stained with glycoprotein-specific staining techniques as described above. Gels were also stained by total protein stain. Differential and distinct bands stained by total protein staining were cut out from the gel and the glycoprotein was then extracted from the gel as described above. Gel purified glycoprotein solution was dialyzed and lyophilized (as described above). Proteins were subjected to trypsin digestion. Samples were then analyzed by mass spectrometry carried out by Thermo Electron Corporation (San Jose, CA) to obtain peptide sequences.

Results

As a first step toward a molecular characterization of the glycoprotein from spider silk sticky coat, we constructed and screened a *N. clavipes* aggregate gland cDNA (cDNA) library. We discovered a frequently occurring DNA clone encoding a previously unknown repetitive and likely glycosylated protein domain. In addition, this clone had an open reading frame on the opposite strand of the same DNA that could encode another unknown spider protein. Oligonucleotides based on each DNA strand were used in rapid amplification of cDNA ends polymerase chain reaction (RACE-PCR) to determine whether both putative proteins were indeed expressed in the aggregate gland tissue and if their full length clone existed in the cDNA library. As shown in Figures 1 and 2, two complete genes named Aggregate Spider Glue (*asg1*, *asg2*) were cloned that encode two different potentially glycosylated proteins (ASG1 and ASG2, respectively). Both proteins have multiple high potential *O*-glycosylation sites and one possible *N*-glycosylation site.

Each protein has a unique repetitive domain of 11 10-amino acid repeats. Predictably, both proteins share the same highly repetitive 351-bp DNA that encodes their repetitive protein domains on opposite strands (Figure 3).

Using Northern blot analysis we have examined the expression of *asg1* and *asg2* in the aggregate glands, other silk glands such as major ampullate, minor ampullate, flagelliform, tubuliform, and in mRNA (mRNA) extracts from the spider's body. Results revealed that both *asg1* and *asg2* mRNAs were exclusively expressed in the aggregate glands and were not detected in other spider glands or tissues. One probe detected
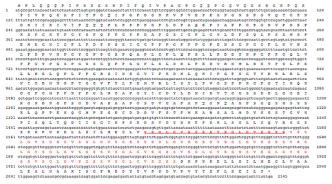
Figure 2. Nucleotide and amino acid sequence of the cloned spider glue glycoprotein ASG2. The 2145-bp clone contains an open reading frame encoding a 714 amino acid protein with calculated size of 71548.3 Da and an isoelectric point of 4.10. The most likely *O*-glycosylation sites (threonine or serine) and an *N*-glycosylation site (asparagine) are underlined. The DNA and protein repeat is red underlined and that region is in red. Gene sequences have been deposited to GeneBank under accession number EU780015.
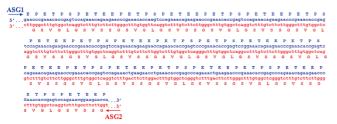

Figure 3. DNA and protein sequences of the repetitive regions of both proteins. The repetitive domains of ASG1 (amino acids 208−324) and ASG2 (amino acids 545−660) are transcribed and translated on two different open reading frames from opposite strands of the same DNA sequence.
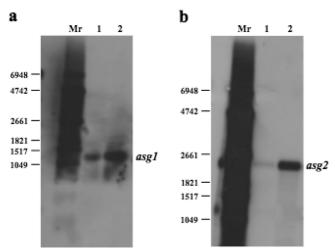

Figure 4. Northern blot analysis shows the transcripts of *asg1* and *asg2* in the aggregate glands. Oligonucleotides were designed to be complementary to the repetitive DNA of each gene and were DIG-labeled. For each probe, two samples of aggregate glands of RNA were used: total RNA (lane 1) and mRNA (lane 2). Mr: DIG-labeled RNA marker indicating sizes in bp (RNA1 #11526529910, Roche Applied Science, IN). (A) Hybridization with a probe complementary to *asg1* detected an approximate 1200 bp transcript. (B) Hybridization with a probe complementary to *asg2* detected an approximate 2200 bp transcript.

an mRNA of 1.2 kb (Figure 4a) and the other detected an mRNA of 2.2 kb (Figure 4b).

In our biochemical studies we extracted and purified a glycoprotein directly from spider orb webs. Using specific glycoprotein detection methods, we found this protein to be the
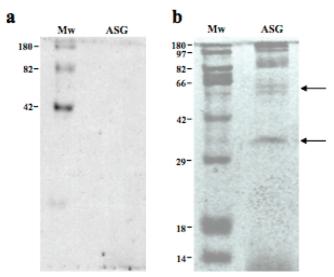

Figure 5. SDS PAGE gel of web glue glycoproteins. The 12% SDS-PAGE shows results of deglycosylation of the spider's glycoprotein purified from orb webs. M_w: Molecular weight marker indicating sizes in kDa (CandyCane, Molecular Probes, OR, U.S.A.), ASG: deglycosylated spider's glycoprotein. (A) Staining with highly specific glycoprotein stain clearly shows that no glycoprotein band can be detected which indicates that no glycosylated glycoprotein remained in the solution after the enzymatic treatment. (B) Staining with total protein dye shows two apparent deglycosylated glycoprotein bands (approximately 65 kDa upper band and 38 kDa lower band, arrowed). Higher molecular size bands were shown to be remains of the deglycosylation enzymes in the solution that was loaded on the gel.

only glycoprotein associated with the aqueous coat of the spider silk web after SDS-PAGE. This purified glycoprotein from the initial SDA-PAGE was deglycosylated and rerun on SDS-PAGE, which showed two bands of approximate sizes of 65 and 38 kDa (Figure 5).

Staining with highly specific glycoprotein stain clearly shows that no glycoprotein band can be detected at this stage, which indicates that no glycosylated glycoprotein remained in the solution after the enzymatic deglycosylation. Staining with total protein dye shows the two deglycosylated glycoprotein bands. Higher molecular weight bands on the gel were shown to be remains of the deglycosylation enzymes in the solution that were loaded on the gel and are not related to any of the spider's proteins. Additionally, the larger protein (65 kDa), detected after deglycosylation, was cut out of the gel, purified, and subjected to mass spectrometry analysis. Peptides derived from this analysis show identities to the translated protein ASG2. Two of the peptides found were Gly-Ser-Ser-Val-Ser and Gly-Leu-Gly-Val, which appear as one repeating sequence (GSSVS-GLGV) in ASG2, and the Ala-Gly-Pro-Gly-Thr peptide also appears in ASG2.

We identified the spider glue glycoproteins in another orb-weaving spider, *Araneus gemmoides*. We were able to clone a portion of the repetitive domain from this species. Comparison of sequences from both species shows 92% identity for ASG1 and 91% identity for ASG2. As shown in Figure 6, both ASG1 and ASG2 from *N. clavipes* have an insert of five amino acids that does not appear in *A. gemmoides*.

Discussion

This study was designed to determine the molecular components of the glue that coats the spiral capture prey threads of

A

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N. clavipes    EPETPSPETEEPETPSPETEEPETPSPETEEPETPSPETPSPETEEPETPSPETEEPETP
A. gemmoides   EPETLKPETKEPETLRPETEEPETPSPETEEPETP-----SPETEEPETPSPETEEPETP
               ****  *** *** ****  *******************    *****************

N. clavipes    SPETEEPETPSPETEEPETPSPETEEPETPSPETEEPTTPKPRVTAPETDCDENDVDCI
A. gemmoides   SPETEEPETPSPETEEPETPSPETEEPETPSPETEEPTTPKPRVTAPETDCDENDVDCI
               **********************************************************

N. clavipes    IDDLGITPDWF
A. gemmoides   IDDLGITPDWF
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B

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N. clavipes    GSSEVSGLGVSGSSEVSGLGVSGSSEVSGLGVSGSSEVSGLGVSGSSEVSGLGVSGSSEVSGLGVS
A. gemmoides   GSSEVSGLGVSGSSEVSGLGVSGSSEVSGLGVSGSSEVSGLGVSGSSEVSGLGVS
               **************************************************    ******* ****

N. clavipes    GLGVSGSSEVSGLGVSGSSEVSGLGVSGSSEVSGLGVSGS
A. gemmoides   G-----SGSSEVSGLSVSGSFVSGFSVSGS
               *    ************** **** *** ****
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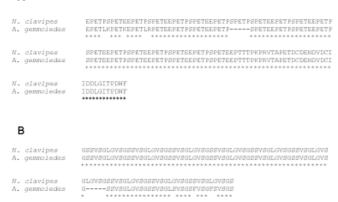
Figure 6. Comparison (alignment) of partial repetitive domains of ASG1 and ASG2 from *N. clavipes* and *A. gemmoides*. (A) ASG1 shows 92% identity between species. (B) ASG2 shows 91% identity between species.

orb weaving spiders. Three arguments support the premise that the two glycoprotein subunits we have identified and cloned function as key components of the spider glue. First, the fact that both *asg1* and *asg2* mRNAs were exclusively expressed in the aggregate glands and were not detected in other spider glands or tissues suggests that ASG1 and ASG2 are not products of housekeeping genes and are involved only in the function of the aggregate glands. Both of these mRNAs were also present in high abundance as would be expected if they were the major products of the gland. Moreover, one probe detected an mRNA of 1.2-kb as expected from the size of the cloned DNA of *asg1* (Figure 1) and the other detected an mRNA of 2.2 kb also as expected from the size of cloned *asg2* (Figure 2). Because *asg1* and *asg2* share the same repetitive DNA sequence (Figure 3) and the mRNA transcript of one is partially antisense to the transcript of the other and could prevent its translation, it is possible that there is a separation by time or space of their expression.

Further sequence analysis shows no additional sequence homologies between the two proteins. In addition both the N-terminal and C-terminal sequences of both proteins show no relevant homology to the highly conserved N- and C-termini of spider silk proteins.

Second, deglycosylation of the only glycoprotein identified and purified from spider webs and biochemical analysis of the deglycosylation products demonstrates that the spider glue glycoprotein indeed functions as a combination of two subunits with approximate sizes (38 and 65 kDa) predicted from the size of the deduced ASG1 and ASG2 sequences (Figures 1 and 2). Peptide sequences derived from the 65 kDa deglycosylation product, originally derived from the spider web purified glycoprotein, show full identities to the translated protein ASG2. These results strengthen the premise that ASG2 functions as a key component in the spider web glue. Additionally, glycoproteins that were extracted and deglycosylated from two other species of orb weaving spiders, *A. gemmoides* and *Argiope trifasciata* showed the same pattern of two distinct bands of similar molecular weights (results not shown) as shown here (Figure 5), which correlates with our hypothesis that the spider glue nodules are made of two different glycoproteins. The smaller 38 kDa subunit was not analyzed due to insufficient material for reliable MS analysis.

Third, ASG1 is likely to be highly glycosylated (25 *O*-glycosylation and 1 *N*-glycosylation sites, Figure 1) and its repetitive domain shows sequence similarities to mucin repetitive domains, which are known to be highly glycosylated.[6] This protein has a high proportion of charged amino acids in the nonrepetitive region which could aid in retention of water as is seen in the web droplets. The ASG2 repetitive domain shows similarities with elastin and flagelliform spider silk protein repetitive sequence domains, and its N-terminal region sequence is similar to other structural proteins such as collagen. In particular it possesses a very high proportion of proline. It is therefore likely, based on structure−function analysis, that ASG2, when functioning as an oligomer, possesses some level of elasticity. ASG2 is also predicted to be glycosylated (16 *O*-glycosylation and 1 *N*-glycosylation sites, Figure 2). Both traits are expected from the spider's sticky-coat-glycoprotein to act as glue. Highly glycosylated proteins, such as mucins are known to have adhesive traits,[6] whereas the elasticity would help in the creation of expanding nodules as previously described for the spider's glycoprotein.[5] Moreover, the upstream nonrepetitive sequence of the ASG1 protein shows substantial similarities to chitin-binding proteins (25−30% identity, 50−55% similarity), which suggests that this protein also has chitin binding traits that would be highly useful in its function of retaining insects in the web.

We predict that the glycoproteins of the spider glue are *O*-glycosylated (Figures 1 and 2), as previously described, and this is consistent with our biochemical analysis of the proteins from the web that match our cDNAs. Tillinghast et al.[4] purified and studied a glycoprotein from orb webs of the spider *Argiope aurantia* and studied its morphology and biochemical traits. They found that it has an apparent high molecular weight (>200 kDa) and has a residue of *N*-acetylgalactosamine *O*-linked to threonine. They also showed that the glycoprotein shares several characteristics with mammalian secretory mucins. Their electron microscopic examination of the glycoprotein preparation revealed poly disperse linear macromolecules exhibiting considerable flexibility as we also predict in our study.

Although it has been previously shown in other species that opposite strands of the same DNA can encode for two independent proteins,[7,8] to our knowledge it has never been described for highly repetitive domains in two proteins assumed to be functionally dependent. Furthermore, ASG2 shows similarities to flagelliform spider silk. Evolutionarily it is known that the aggregate glands and their contents evolved later than other silk glands and spider silk proteins.[3] This suggests that the repetitive glue DNA originally was derived from a spider silk gene and has evolved to its current form via gene duplication. The RNAs transcribed from each DNA strand then independently led to the evolution of two genes expressed in the aggregate glands, both involved in the generation of glycoprotein nodules that function as glue. Moreover, partial repetitive domains of *asg1* and *asg2* have been cloned from another orb weaving spider, *A. gemmoides*. This species is thought to have separated from *N. clavipes* over 100 million years ago, yet the repetitive domains of ASG1 and ASG2 from both species show 91−92% identity (Figure 6).

N. clavipes domains have an insert of five amino acids that do not exist in *A. gemmoides* genes, which suggests that the original DNA of the genes had already existed in their ancestor's genome and mutation in the DNA independently appeared in *N. clavipes* after separation of species. Despite this mutation, the high similarities of the current proteins between species suggests that they are highly important for the survival of orb weaver spiders, in functioning as glue, necessary to retain prey in the web.

The reason for the perfect DNA identity between the two genes in their repetitive regions is unclear. However the fact that both species examined have maintained the identity of these sequences in the two genes despite more than 100 million years of separation indicates its importance. This is particularly true because the five amino acid insertion or deletion between the two species is seen in both genes of that species.

The results of this study, together with comparisons to previous studies of the putative spider glue glycoprotein, contribute novel biological and evolutionary knowledge of these specific genes and their partner proteins. Such knowledge is essential for further biological and chemical studies on the structure and function of these proteins as well as to further studies on their carbohydrate moieties. Once the cloned genes are overexpressed in systems such as insect or bacterial cell cultures, large-scale production of the glycoprotein can be used to develop a new biobased glue for a variety of purposes.

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