Identification of the Motifs and Amino Acids in Aggrecan G1 and G2 Domains Involved in Product Secretion†

Chris Kiani, Liwen Chen, Vivian Lee, Peng-Sheng Zheng, Yaojiong Wu, Jianping Wen, Liu Cao, Mark E. Adams, Wang Sheng, and Burton B. Yang*

Sunnybrook & Women's College Health Sciences Centre and Department of Laboratory Medicine and Pathobiology, University of Toronto, Toronto, Canada

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ABSTRACT: Members of the large aggregating chondroitin sulfate proteoglycans are characterized by an N-terminal fragment known as G1 domain, which is composed of an immunoglobulin (IgG)-like motif and two tandem repeats (TR). Previous studies have indicated that the expressed product of aggrecan G1 domain was not secreted. Here we demonstrated that the inability of G1 secretion was associated with the tandem repeats but not the IgG-like motif, and specifically with TR1 of aggrecan. We also demonstrated that the G2 domain, a domain unique to aggrecan, had a similar effect on product secretion. The sequence of TR1 of G1 is highly conserved across species, which suggested similar functions played by these motifs. In a yeast two-hybrid assay, TR1 interacted with the calcium homeostasis endoplasmic reticulum protein. Deletion/mutation experiments indicated that the N-terminal fragment of TR1, in particular, the amino acids H^2R^4 of this motif were key to its effect on product secretion. However, the N-terminal 55 amino acids were required to exert this function. Taken together, our study suggests a possible molecular mechanism for the function of the tandem repeats in product processing.

The large aggregating chondroitin sulfate proteoglycans include aggrecan, versican (also known as PG-M), neurocan, and brevican (*1*−*5*). Members of this family share many similar features (*6*, *7*). These proteoglycans contain a central core protein to which glycosaminoglycan (GAG)[1] chains are covalently linked posttranslationally. The core protein of each molecule contains a signal peptide, an amino-terminal globular domain known as G1 domain, a carboxyl-terminal globular domain known as the G3 domain (or selectin-like domain) and a large sequence situated between G1 and G3 for modification by GAG chains, mainly chondroitin sulfate (CS) chains (*8*, *9*). The G1 domain is composed of one IgG-like motif and two proteoglycan tandem repeats. This domain has a high homology with link protein (*10*−*12*). G3 is composed of an alternatively spliced epidermal growth factor (EGF)-like motif(s), a carbohydrate recognition domain (CRD), a complement binding protein-like (CBP) motif and a short tail.

Aggrecan, in addition to these features, has some extra motifs (*1*, *13*). It contains a G2 domain, which is similar to the two tandem repeats of the G1 domain and link protein. An interglobular domain (IGD) is situated between the G1 and G2 domains. Situated between G2 and G3 is a large extended region for GAG chain attachment, which occurs on the serine of serine-glycine dipeptides. This extended region is further divided into two domains: one for attachment of keratan sulfate (KS) and one for attachment of CS chains (*1*, *6*), although species-specific differences in GAG modification and the GAG attachment region have been reported (*14*−*16*). Compared to other members of this family, aggrecan contains significantly more potential sites for GAG chain attachment, and thus, its molecular mass is much greater. These GAG chains account in large part for the unique properties of aggrecan in cartilage development (*17*−*19*). The importance of GAG modification in cartilage development was initially observed in the chicken mutant nanomelia, a lethal genetic disease. In this disease, the chicken aggrecan gene contains a point mutation resulting in a premature stop codon on the N-terminal side of the G3 domain (*1*). The truncated aggrecan core protein is neither modified by GAG chains nor secreted to the cartilage matrix (*1*, *19*, *20*). These studies suggested that aggrecan G3 domain was involved in GAG modification and product secretion (*21*). Since then, the role of the G3 domain in product processing has been extensively studied. It has been reported that the G3 domain enhances GAG modification and product secretion (*21*−*26*), and that the G1 domain product accumulates in the cytoplasm and is not secreted (*21*, *22*). Recently, we also demonstrated that a construct containing the G2 domain was not secreted (*22*). However, how G1 and G2 affect product processing is not clear. It is likely that the G1 domain interacts with unknown cellular components, thus inhibiting product secretion. This study was designed to characterize in detail how the G1 and G2 domains affect product secretion, and to investigate the biological function of this effect on product processing.

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* Correspondence to Burton B. Yang, Research Building, Sunnybrook & Women's College Health Sciences Centre, 2075 Bayview Avenue, Toronto M4N 3M5 Canada. Tel: (416) 480-5874; Fax: (416) 480-5737; E-mail: Burton.Yang@sw.ca.

[1] Abbreviations: GAG, glycosaminoglycan; CS, chondroitin sulfate (chain attachment sequence); TR, tandem repeat; IgG, immunoglobulin-like motif; ER, endoplasmic reticulum; DMEM, Dulbecco's modified Eagle's medium; FBS, fetal bovine serum; PBS, phosphate-buffered saline; PCR, polymerase chain reaction; PAGE, polyacrylamide gel electrophoresis.

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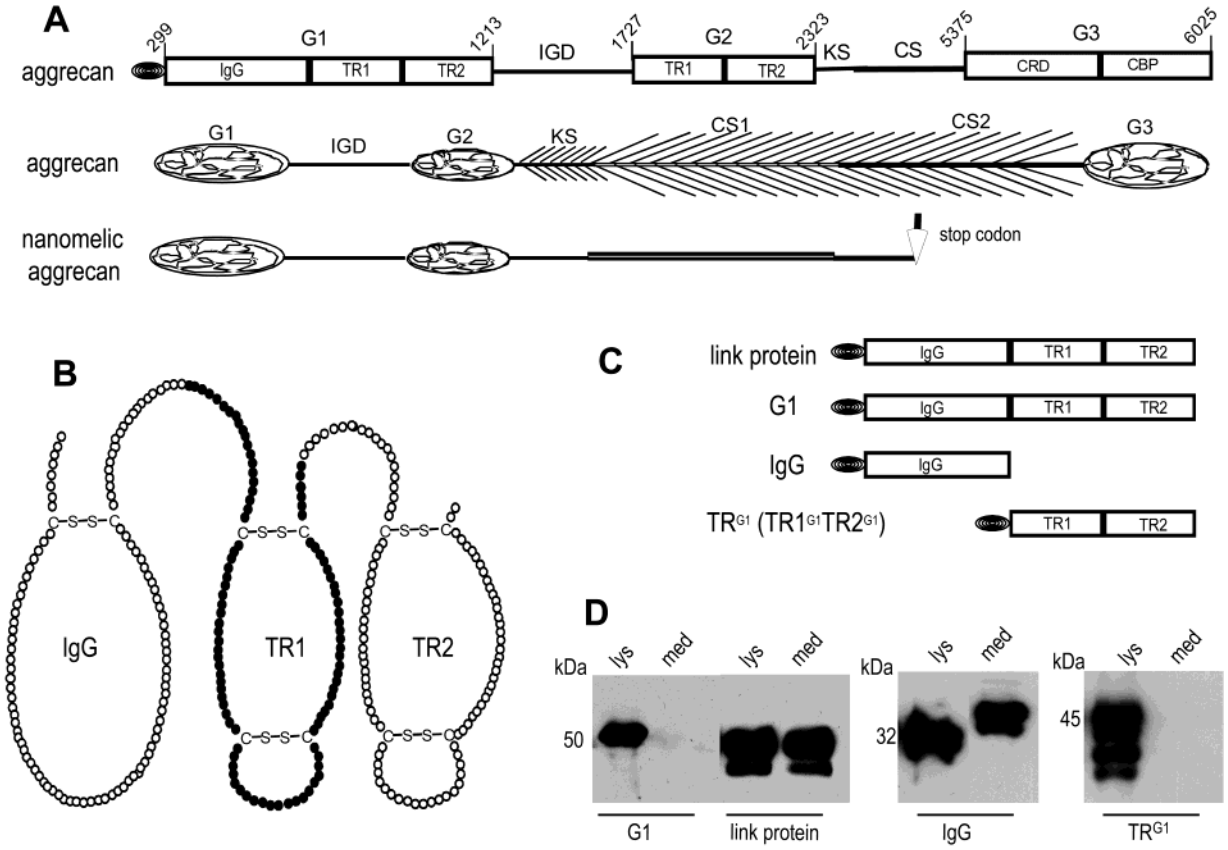

FIGURE 1: IgG, but not tandem repeats, is secreted to culture medium. (A) Diagram of the structures of aggrecan (wild-type and nanomelic). IgG, immunoglobulin-like motif; TR, tandem repeat; IGD, interglobular domain; KS, the fragment of the core protein modified by keratan sulfate chains; CS, the fragment modified by chondroitin sulfate chains; CRD, carbohydrate recognition domain; CBP, complement binding protein. The signal peptide added to all constructs was obtained from link protein (nucleotides 1−180). Numbers above the schematic correspond to nucleotides in the sequence of full-length aggrecan. (B) Structure of the aggrecan G1 domain. Each circle represents one amino acid residue. The IgG-like motif and the second tandem repeat (TR2) are shown as open circles, while the first tandem repeat (TR1) is shown as closed circles, where C represents a cysteine residue, and S−S represents a disulfide bond. (C) Four recombinant constructs were generated and used in COS-7 cell transfection. (D) Cell lysate and culture medium from COS-7 cells transfected with link protein, G1, IgG and TR^{G1} ($TR1^{G1}TR2^{G1}$) were subjected to SDS−PAGE on a 10% gel. The separated proteins were then transblotted onto a nitrocellulose membrane and probed with the monoclonal antibody 4B6. The products of IgG were synthesized (32 kDa in lysate) and were secreted to the culture media (40 kDa) at high levels. The core protein of link protein was also well secreted to the culture medium, but neither the G1 product nor the $TR1^{G1}TR2^{G1}$ (47 kDa) were secreted.

RESULTS

Identification of the Motif in Aggrecan G1 Domain That Is Incapable of Secretion. To dissect the motif in aggrecan G1 domain that is responsible for the inhibition of product secretion (for domain compositions of aggrecan and the G1 domain see Figure 1A,B), we generated a number of recombinant constructs carrying either IgG or the tandem repeats: G1, IGD, TR^{G1} (or $TR1^{G1}TR2^{G1}$). Link protein is similar to the G1 domain and was thus used as a control (*1*, *27*). The structures of these constructs are shown in Figure 1C. To compare amounts of secreted products, we transfected COS-7 cells with these constructs. Western blot analysis indicated that the G1 product was synthesized but not secreted to culture medium (Figure 1D). This confirms previous findings (*21*, *22*). As a positive control, link protein, which is structurally similar to the aggrecan G1 domain, was also expressed and analyzed. High levels of this product were observed in the culture medium. The IgG motif, the largest motif in the aggrecan G1 domain, was also highly expressed and secreted (Figure 1D). The products observed in the culture medium were larger than those in the cell lysate, indicating glycosylation of the product in the medium, as there are two potential sites for *N*-linked glycosylation in the IgG motif (*1*). In contrast, the tandem repeat domains (TR^{G1}) were not secreted (Figure 1D). If the product was not detected in the medium, total proteins were concentrated (100−500-fold), and the concentrated samples were analyzed on Western blot to ensure no trace amounts of product were present.

Our results indicated that, despite the resemblance between the G1 domain and link protein, the two peptides have different effects: aggrecan G1 product is not secreted, while link protein is well secreted. Since our evidence indicated that the inability of product secretion is related to the tandem repeats, we investigated these motifs in more detail. Alignment of amino acid sequences from different species indicates that the sequences of each motif are highly conserved between species. For example, TR1 and TR2 motifs of the G1 domain (i.e., $TR1^{G1}$ and $TR2^{G1}$), TR1 and TR2 motifs of the G2 domain ($TR1^{G2}$ and $TR2^{G2}$) and TR1 motif of link protein ($TR1^{LP}$) exhibit 95.7, 94.2, 88.8, 88.2, and 97.2% homology, among human, rat, bovine, murine, and chicken sequences, respectively (Figure 2A). Notably, human, rat, cow, and mouse share 100% homology in the TR1 motif of the G1 domain. However, the homology between different motifs is relatively low in each species,

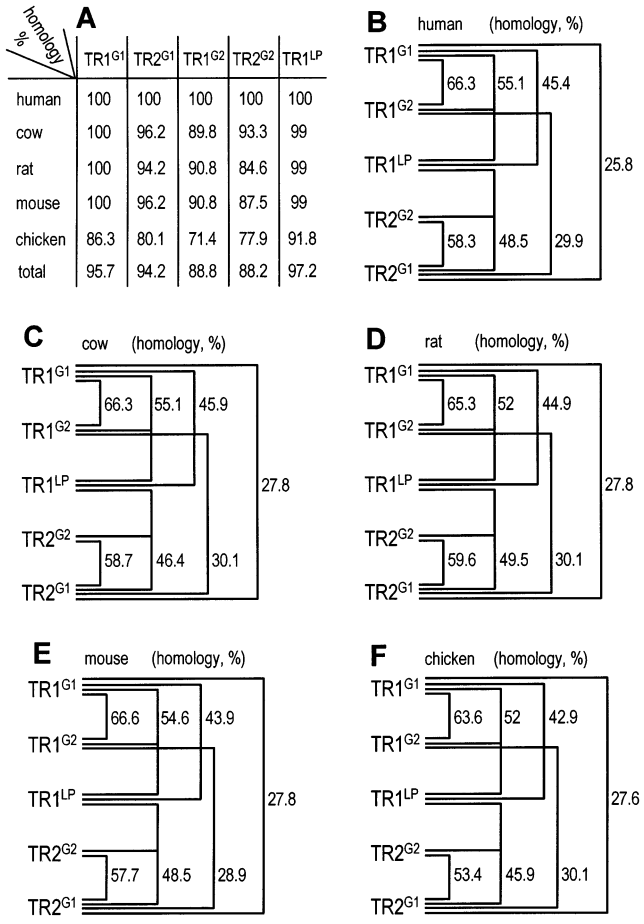

FIGURE 2: Amino acid homology of TR1 and TR2 motifs. (A) Sequence analysis indicated that each motif is highly conserved across species. The sequence of each species was compared to the human sequence; the total homology 95.7, 94.2, 88.8, 88.2, and 97.2% is the homology of all species. The formula, homology% = [(total number of amino acids − number of different amino acids) ÷ total number of amino acids] × 100%, was used to calculate sequence homology. Among human (B), cow (C), rat (D), mouse (E), and chicken (F), the patterns of conservation are very similar in the order of (from high homology to low homology) $TR1^{G1}$ and $TR1^{G2}$ (63.6−66.6%) > $TR2^{G1}$ and $TR2^{G2}$ (53.4−59.6%) > $TR1^{G1}$, $TR1^{G2}$ and $TR1^{LP}$ (52−55.1%) > $TR1^{LP}$, $TR2^{G2}$ and $TR2^{G1}$ (45.9−49.5%) > $TR1^{G1}$ and $TR1^{LP}$ (42.9−45.9%) > $TR1^{G2}$ and $TR2^{G1}$ (28.9−30.1%) > $TR1^{G1}$ and $TR2^{G1}$ (25.8−27.8%).

and the patterns of homology are very similar (Figure 2B−F). Interestingly, among all species, the TR1 motif of G1 domain and TR1 motif of link protein, both of which are involved in hyaluronan binding and were thus expected to be more homologous, share, in fact, low degrees of homology (42.9−45.9%). In addition, the TR1 and TR2 motifs of the G1 domain, both of which are involved in hyaluronan-binding, exhibit the lowest level of homology (25.8−27.6%). On the other hand, the TR1 motifs in G1 and G2, which have no known functional similarity, share the highest degree of homology (63.3−66.3%) among the five motifs compared (Figure 2B−F). This suggests that the TR1 motifs might share some similar functions, while the TR1 and TR2 motifs in the same domain may play different roles despite their hyaluronan binding activity.

These data have led to the speculation that individual tandem repeats of the G1 and G2 domains may play different roles in product secretion. In light of this, we designed experiments to determine which tandem repeats affect product secretion. Recombinant constructs $IgGTR1^{G1}$, $IgGTR2^{G1}$, $TR1^{G1}$, $TR2^{G1}$, $TR1^{G1}G3$, and $TR2^{G1}G3$ were generated (Figure 3A). As a control, we used a G3 construct generated previously (*28*, *29*). These constructs were expressed in COS-7 cells, and the products were analyzed as above. The $TR1^{G1}$ product was not secreted (Figure 3B). Similar results were observed when the $TR1^{G1}$ motif was combined with other domains, whose products were otherwise well secreted: the $IgGTR1^{G1}$ and $TR1^{G1}G3$ products were not secreted. As a control, G3 was expressed and secreted as expected (Figure 3B). The $TR2^{G1}$ product was observed in the medium at high level. The observed sizes of the $TR2^{G1}$ (33 kDa in cell lysate and 38 kDa in medium) were greater than the sequence of the $TR2^{G1}$ peptide backbone and the leading peptide would have predicted (∼17 kDa). This suggested glycosylation had occurred, as this motif contains many potential sites for glycosylation. The $TR2^{G1}$ motif was then linked with the IgG-like motif or the G3 domain of aggrecan, producing the $IgGTR2^{G1}$ and $TR2^{G1}$-G3 constructs. Immunoblotting showed that products of both $IgGTR2^{G1}$ and $TR2^{G1}G3$ were well secreted to culture medium (Figure 3B).

$TR1^{G1}$ Binds to ER Protein and Affects Processing of Mini-Aggrecan. The finding that G1 retards product secretion suggests that this domain may interact with some cellular components and affects product processing. To investigate this, we used the tandem repeats ($TR1TR2^{G1}$) as bait in pGBKT7 plasmid (producing a construct pGBKT7-TR1TR2; Figure 4A) to screen a human brain cDNA library in a yeast two-hybrid experiment. After clone selection and DNA sequencing, we obtained a clone (pACT2-ER) with a sequence identical to the cDNA of 2655 bp encoding calcium homeostasis endoplasmic reticulum protein (GeneBank nucleotide access number NM_006387). Cloned from human erythroleukaemia cells, the ER protein is located at the ER membrane where it modulates intracellular calcium mobilization (*30*). In addition, this protein has ER retention signals at the C-terminus and several potential domains for macromolecular assembly, such as the histidine repeats and serine/arginine-rich region for protein−protein interactions (*30*). Our clone has an insert of 2 kb. It contains a C-terminal coding region of 660 bp and a 3′ noncoding region of ∼1.3 kb. The serine/arginine-rich region (Ser^{705} to Ser^{785}) is located within the clone pACT2-RT that we isolated.

We further confirmed the binding of pGBKT7-TR1TR2 with pACT2-ER using yeast two-hybrid color development assay. This assay used the plasmid pGBDC1, another plasmid in the yeast two-hybrid system. Three constructs, pGBDC1-TR1TR2, pGBDC1-TR1, and pGBDC1-TR2, were made to examine which tandem repeat bound pACT2-ER. Color development indicated that pACT2-ER interacted with pGBDC1-TR1TR2 and pGBDC1-TR1 but not with pGBDC1-TR2 compared to vector control (Figure 4B).

We then investigated whether the interaction of the tandem repeats (particularly TR1) with a cellular component (e.g., the calcium homeostasis ER protein) is important for product processing. If so, overexpression of TR1 would affect processing of a product containing TR1. The mini-aggrecan was thus coexpressed with $TR1^{G1}$, $TR2^{G1}$ or the control vector pcDNA3. The experiment indicated that the mini-aggrecan was well expressed, but its secretion was

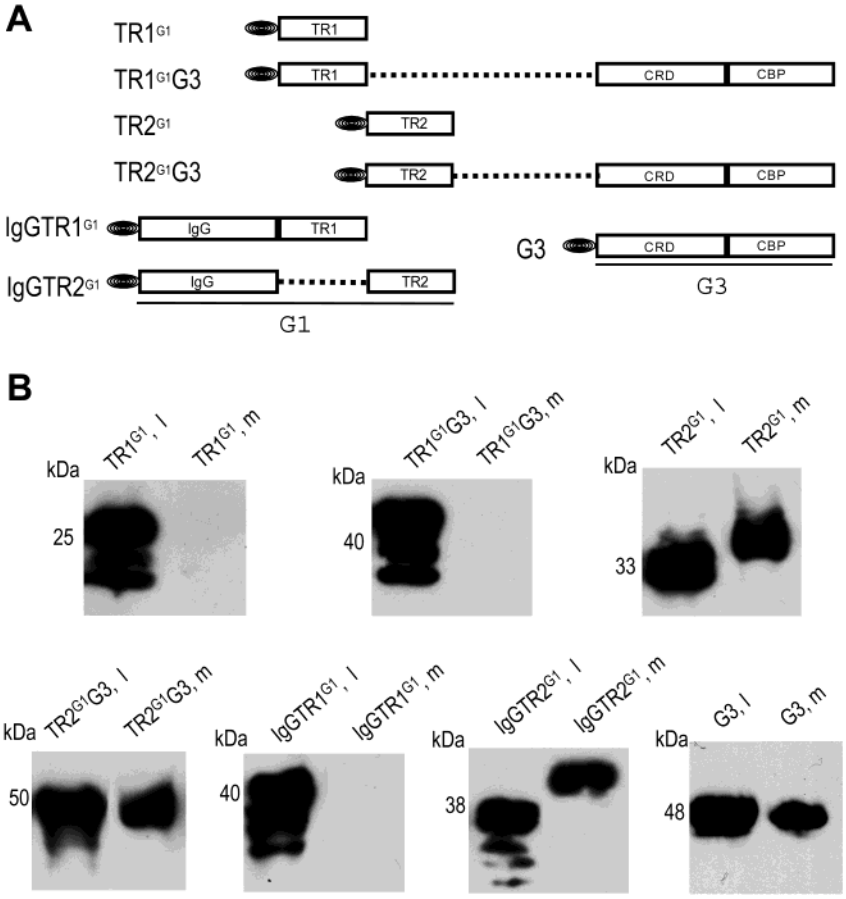

FIGURE 3: Products containing $TR1^{G1}$ are not secreted. (A) Six constructs containing one of the G1 tandem repeats were transiently expressed in COS-7 cells. Aggrecan G3 construct was used as a control. (B) Cell lysate and culture medium were prepared and analyzed on Western blot probed with 4B6. G3 (48 kDa), $TR2^{G1}$ (33 kDa), $IgGTR2^{G1}$ (38 kDa), and $TR2^{G1}G3$ (50 kDa) were well synthesized and secreted. However, neither the products of $TR1^{G1}$ (25 kDa), nor the TR1-associated products, $TR1^{G1}G3$ (42 kDa) and $IgGTR1^{G1}$ (40 kDa), were secreted to the culture medium.

inhibited when coexpressed with $TR1^{G1}$ but not with $TR2^{G1}$ (Figure 5).

The Tandem Repeats of the G2 Domain Function Similarly to G1 in Product Secretion. The second globular domain in aggrecan, G2, differs from G1 in that it is composed only of two tandem repeat motifs and lacks an IgG-like motif. As shown by the homology analysis, the G2 tandem repeats are conserved across species but are less homologous to the TRs of G1 or to each other. Only the TR1 motif of the G2 domain shares high degrees of homology with the TR1 motif of the G1 domain. Seven constructs, G2, $TR1^{G2}$, $TR2^{G2}$, $TR1^{G2}G3$, $IgGTR1^{G2}$, $TR2^{G2}G3$ and $IgGTR2^{G2}$, were generated to test their effects on product secretion (Figure 6A). These constructs were expressed in COS-7 cells, and cell lysate and culture medium were analyzed on Western blot. Two products, $TR1^{G2}G3$ and $IgGTR1^{G2}$, were not secreted (Figure 6B). Consistent with the results from G1 domain, the $TR2^{G2}$ product and $TR2^{G2}$-associated products ($TR2^{G2}$-G3 and $IgGTR2^{G2}$) were secreted. Thus, in both globular domains, the first tandem repeat, $TR1^{G1}$ and $TR1^{G2}$, seems to incapacitate product secretion. While the second tandem repeat, $TR2^{G1}$ and $TR2^{G2}$, allowed product secretion, their apparent molecular mass appeared to be different (i.e., $IgGTR2^{G2} < IgGTR2^{G1}$). This suggested different degrees of glycosylation.

Identification of the Amino Acids in the TR1 Motif That Account for the Incapability of Product Secretion. To further investigate how the TR1 motif of the G1 domain failed to be secreted, seven constructs shown in Figure 7A (aligned with the construct $TR1^{G1}$) were generated. Among these seven constructs, four of them ($TR^{G1}K \rightarrow Q$, $TR1^{G1}mu1$, $TR1^{G1}mu2$, and $TR1^{G1}mu3$) contain amino acid mutations. These amino acids are 100% conserved in the TR1 motifs of the G1 and G2 domains across all species analyzed, and we expected that these amino acids might be important in product processing. Two constructs were truncated: one lacked the N-terminal nine amino acids ($TR1^{G1}N9del$), and the other lacked the C-terminal 15 amino acids ($TR1^{G1}$-C15del). The seventh construct $TR^{G1}\Delta381$ contains a deletion in the middle of the two tandem repeats due to the presence of two *Kpn*I restriction endonuclease sites in the same reading frame. COS-7 cells were transiently transfected with these seven constructs. Analysis of culture medium and cell lysate on Western blot probed with 4B6 indicated that only the products of $TR1^{G1}N9del$ were secreted, suggesting that the N-terminal nine amino acids played an important function in product secretion (Figure 7B).

To further narrow down the N-terminal sequence of amino acids affecting product secretion, five constructs, G1N4del, G1N4mu, $TR1^{G1}N4del$, $TR1^{G1}N4mu$ and $TR1^{G1}HRmu$, were generated. There are four amino acids (FHYR), which are identical in the TR1 motifs of the G1 and G2 domains across all five species analyzed. In G1N4del and $TR1^{G1}N4del$, we deleted these four amino acids (FHYR) from the N-terminus

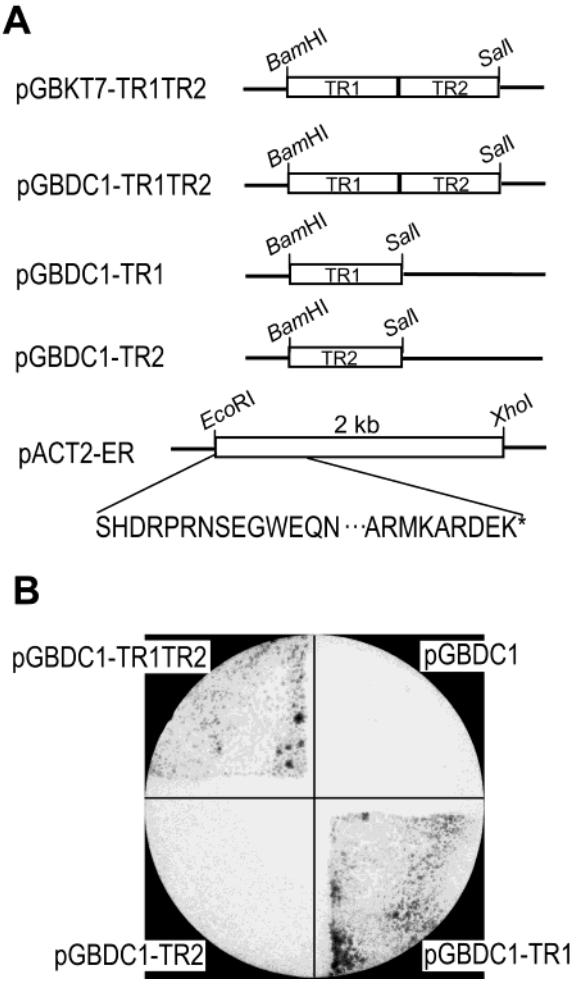

FIGURE 4: Interaction of $TR1^{G1}$ with ER protein. (A) Schematic of ER protein clone, pACT2-ER, isolated using $TR1TR2^{G1}$ as the bait in the construct pGBKT7-TR1TR2, and generation of yeast two-hybrid constructs pGBDC1-TR1TR2, pGBDC1-TR1 and pGBDC1-TR2. The ER protein clone (2 kb insert) contained 660 bp coding sequence encoding the C-terminal 220 amino acids and ~1.3 kb 3′ noncoding sequence. (B) In yeast two-hybrid assay with color development, the ER protein clone interacted with pGBDC1-TR1TR2 and pGBDC1-TR1, but not with pGBDC1-TR2, compared to the pGBDC1 vector control.

of the TR1 motif. In G1N4mu and $TR1^{G1}$N4mu, these four amino acids were mutated to ILNS, and in $TR1^{G1}$HRmu, H and R were mutated to LS as shown in Figure 8A. These truncation/mutation constructs were transiently expressed in COS-7 cells. Product analysis on Western blot indicated that all constructs were well-expressed and secreted to culture medium (Figure 8B). However, when the truncation/mutation was manipulated in the $TR1^{G1}$ construct, producing $TR1^{G1}$-N4del, $TR1^{G1}$N4mu, and $TR1^{G1}$Hrmu, we observed a shift of the bands in the secreted products, suggesting glycosylation of the products. As a result, the products were well secreted. On the other hand, when the truncation/mutation was manipulated in the G1 construct, producing G1N4del, G1N4mu constructs, little shift of the bands was detected, and product secretion was less efficient as compared with the above three constructs. Nevertheless, product secretion was still evident compared with the parental construct G1 (Figure 1D). This suggested that the first four amino acids FHYR were involved in product secretion.

We then generated two mutant constructs, pGBKT7-TR1Ndel4 and pGBKT7-TR1Ndel4, in yeast expression

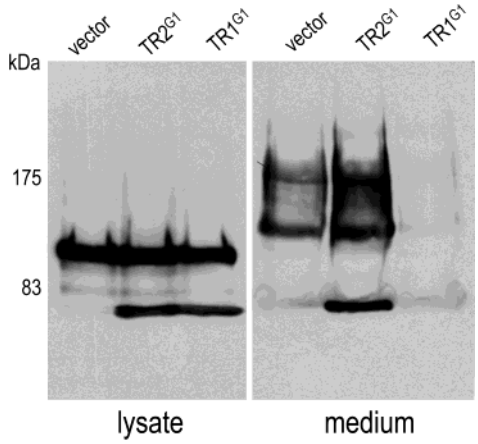

FIGURE 5: Secretion of the mini-aggrecan is inhibited by TR1. The mini-aggrecan was coexpressed with $TR1^{G1}$, $TR2^{G1}$ or a control vector in COS-7 cells. Culture medium and cell lysate were subjected to electrophoresis in 7% gel, followed by Western blot analysis probed with 4B6. Coexpression with $TR1^{G1}$ inhibited secretion of the mini-aggrecan.

vector (Figure 8C). Binding analysis using yeast two-hybrid assay indicated that products of both mutant constructs still interacted with the product containing ER fragment (Figure 8D). This result suggested that another factor might be involved in the inhibition of product secretion, and the ER protein appeared to exert only part of the inhibitory effect.

We further tested if these four amino acids are responsible for the lack of product secretion, we linked these four amino acids to the N-terminal of aggrecan G3 domain (producing FHYR-G3). Product analysis indicated that this chimeric product was secreted (data not shown). It indicated that these four amino acids were not sufficient to modulate product secretion. We hypothesized that a longer fragment in the TR1 N-terminal sequence was required to modulate product secretion.

To test our hypothesis, we generated six constructs containing the IgG motif linked to the N-terminal fragments of the TR1 motif of increasing sizes (from 10 to 55 amino acids; Figure 9A). A 45-amino acid fragment of the TR1 N-terminal which contained a point mutation ($C^{45} \rightarrow P^{45}$) was also generated and linked to IgG (IgG45aamu). These constructs were transiently expressed in COS-7 cells as above. Analysis of these products indicated that IgG linked to the N-terminal 10 or 20 amino acids of $TR1^{G1}$ was well secreted. The constructs containing the 35 or 45 amino acid fragments were partially secreted, and mutation of the cysteine apparently had no effect. IgG linked to the N-terminal 55 amino acid fragment was not secreted (Figure 9B). Our results demonstrated that the amino acids in the N-terminal fragment of TR1 had additive effects on product secretion. Although the first four amino acids FHYR were important in this functional role, a fragment containing 55 amino acids was required to completely inhibit secretion of the IgG motif.

DISCUSSION

The large aggregating chondroitin sulfate proteoglycans are a family of glycoconjugates with a central core protein to which GAG side chains are covalently linked posttranslationally. Aggrecan, a structural proteoglycan of this family, is expressed in cartilage and is responsible for its resilience

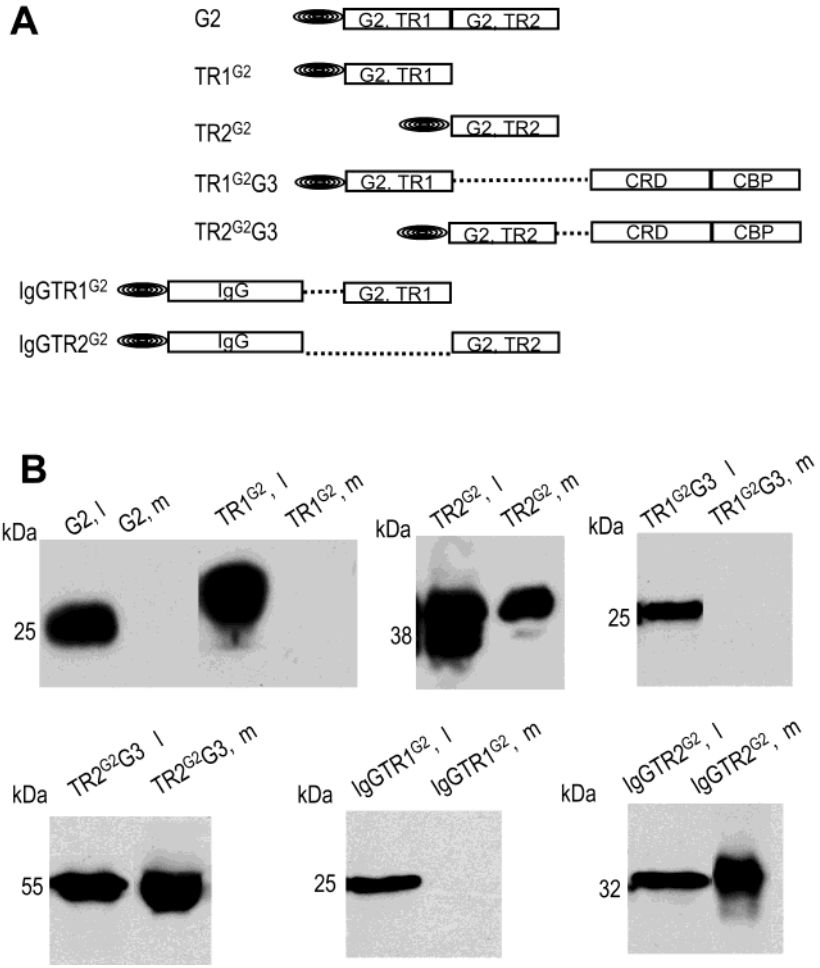

FIGURE 6: G2 exhibits similar function in product secretion. (A) Seven constructs were generated as shown in the figure and transiently expressed in COS-7 cells. (B) Cell lysate (l) and culture medium (m) were analyzed on Western blot. All constructs were well expressed. $TR2^{G2}$ and $TR2^{G2}$-associated products ($TR2^{G2}G3$ and $IgGTR2^{G2}$) were well secreted, while $TR1^{G2}$ and $TR1^{G2}$-associated products ($TR1^{G2}$-G3 and $IgGTR1^{G2}$) were not secreted to culture medium.

and load-bearing properties. Since its partial cloning, the structure and expression of aggrecan has been extensively studied (*17*, *31*−*34*). The core protein of aggrecan is composed of three globular domains (G1, G2, G3). A large extended region (CS) is situated between G2 and G3 for GAG chain attachment that occurs on the serine of serine-glycine dipeptides present in this region (*1*, *13*). As aggrecan is such a large proteoglycan, it would be difficult to study its processing with full-length aggrecan. In fact, studies have indicated that each aggrecan domain has a unique functional role in product secretion (*21*−*24*, *26*, *35*). This has allowed us to examine the role of each domain/motif in different combinations without changing its effect. In this report, we demonstrated that the function of each motif is consistent in all types of combinations, and changing restriction endonuclease sites in the ligation linkage (creating some non-native amino acids) did not alter its property. It seems likely that amino acids in the domain junctions do not change the folding of the domain nor the interactions with intracellular proteins such as chaperones. This property also allowed us to test the role of each motif in product synthesis and secretion in different types of cells, most of which would otherwise not be able to produce a native full-length aggrecan. These domain constructs have been successfully expressed in chondrocytes, mesenchymal cells, fibroblasts, CHO cells, and COS-7 cells (*21*, *22*, *24*, *29*, *36*, *37*). In our studies, we have not observed obvious differences among cell types in product properties such as product synthesis, secretion, GAG chain attachment, and glycosylation (*22*, *28*, *29*, *36*, *38*, *39*).

We have previously observed that aggrecan G1 and G2 domain constructs were expressed but not secreted (*22*). This observation was confirmed in this study, and we further characterized these domains to determine which motifs could account for these effects. We demonstrated that, expressed individually, the product of the IgG-like motif of G1 was well secreted. Only in the presence of the two tandem repeats was the G1 product not secreted. Interestingly, when the IgG-like motif and the tandem repeats were expressed individually, the products of both constructs were heavily modified by sugar moieties, as indicated by the increase in the size of the products. When these motifs were expressed as one construct, G1N4del or G1N4mu (Figure 8B), the products were only slightly modified by glycosylation. It was also true when $TR2^{G1}$ and $TR2^{G2}$ were linked with the G3 domain. One explanation may be that, when the peptides are small, potential sites for GAG chain attachment and glycosylation are exposed to modifying enzymes. It is not clear how these sites are modified on native aggrecan expressed by chondrocytes. As aggrecan contains a large number of potential sites for GAG chain attachment and glycosylation, and mutation of one potential site would not change the molecular

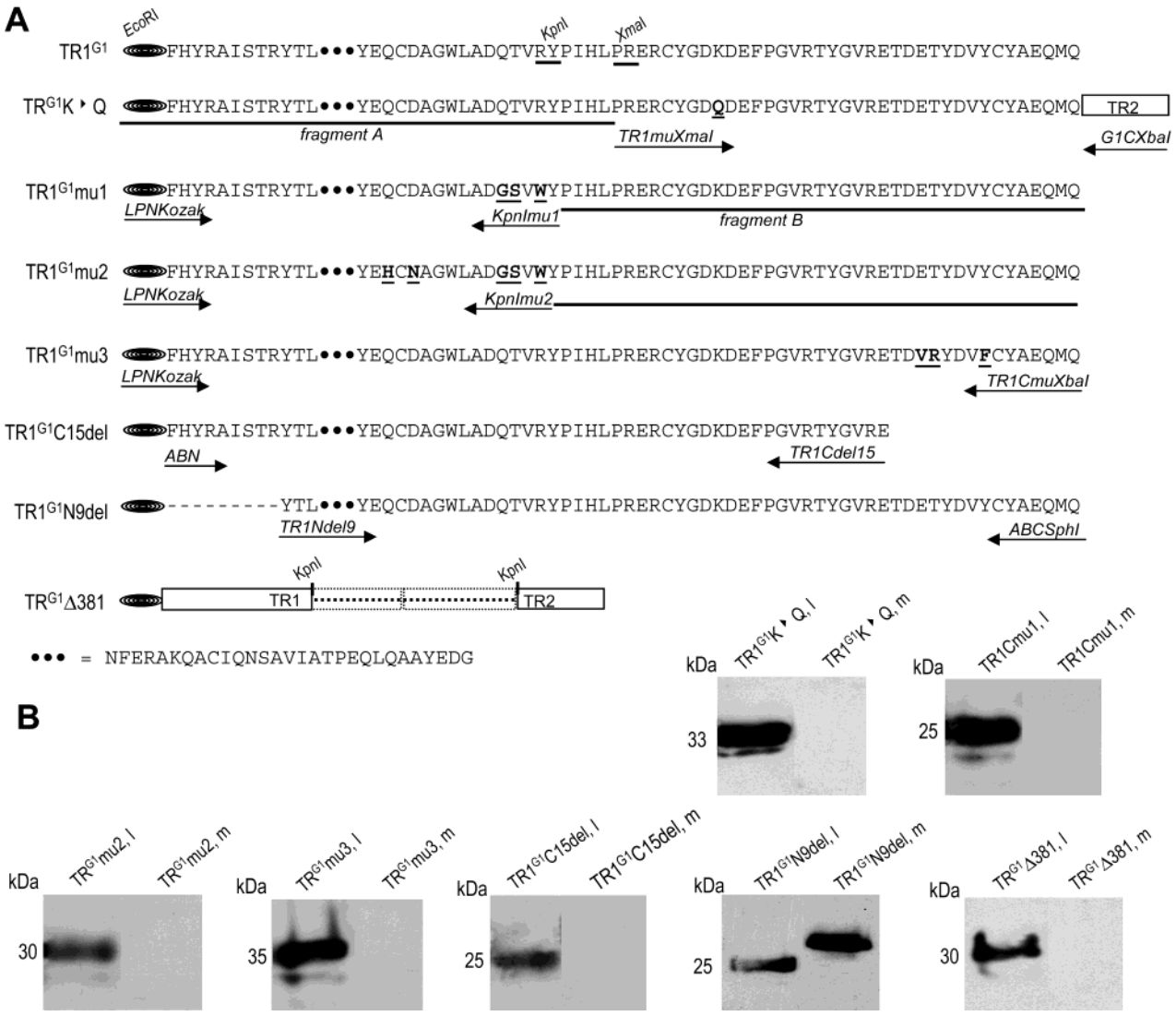

FIGURE 7: Deletion of the N-terminal nine amino acids allows product secretion. (A) seven constructs (shown aligned with the TR1^{G1} sequence) were produced to study the effect of the C- and N-terminal fragments and some conserved amino acids on product secretion. Strategy and primers are shown. For example, construct TR1^{G1}K → Q was produced by linking two fragments in *Eco*RI/*Xba*I-digested pcDNA3: fragment A was obtained by digesting the TR1^{G1} construct with *Eco*RI/*Xma*I, and the other fragment was synthesized with two primers TR1mu*Xma*I and G1C*Xba*I in a PCR. TR^{G1}Δ381 was generated by deletion of the *Kpn*I-digested fragment, which does not change the reading frame of the construct. The construct TR1^{G1}mu3 was synthesized with two primers LPNKozak and TR1Cmu*Xba*I, and so on. (B) COS-7 cells were transiently transfected with these constructs. Cell lysate (l) and culture medium (m) were analyzed on Western blot probed with 4B6. Among these products, only the products of TR1^{G1}N9del were secreted to the culture medium.

mass significantly, the large size of aggrecan means it is impossible to examine how each potential site is modified.

Further examination indicated that only one of the two tandem repeats of the G1 domain was involved in modulating product secretion. These two tandem repeats are structurally similar and both are known to bind hyaluronan (*33*, *40*). No other function has been claimed for these two tandem repeats. Our study has revealed a novel functional role for the tandem repeats: we have shown that aggrecan TR1^{G1}, and not TR2^{G1}, retards secretion. The function of the two tandem repeats in the processing of other members of the large aggregating chondroitin sulfate proteoglycan family (including versican, neurocan, and brevican) has not been tested. However, we speculate that the first tandem repeat in the G1 domain of these proteoglycans may have a similar effect on product secretion, as the TR1 sequences of these molecules are highly conserved (data not shown). Indeed, we have shown that the tandem repeats of versican also retarded product secretion, although they have not yet been individually tested (*39*). The amino acid sequences of the first tandem repeat of aggrecan G1 domain exhibits 100% homology across all species examined except chicken, which is only 86.3% homologous to the other species.

We hypothesized that the amino acid residues involved in hyaluronan binding must be conserved among aggrecan, versican, and link protein, and are likely to be conserved between the two tandem repeats of the G1 domain. On the other hand, the amino acid residues involved in product processing must be conserved among the first tandem repeat of versican and of aggrecan G1 and G2 domains, but they are less conserved among link protein, the first and second tandem repeats of versican and the first and second tandem repeats of the G1 and G2 domains of aggrecan. On the basis of these hypotheses, we designed a series of point mutations to pinpoint the amino acid residues involved in product processing. With this strategy, we were able to identify two

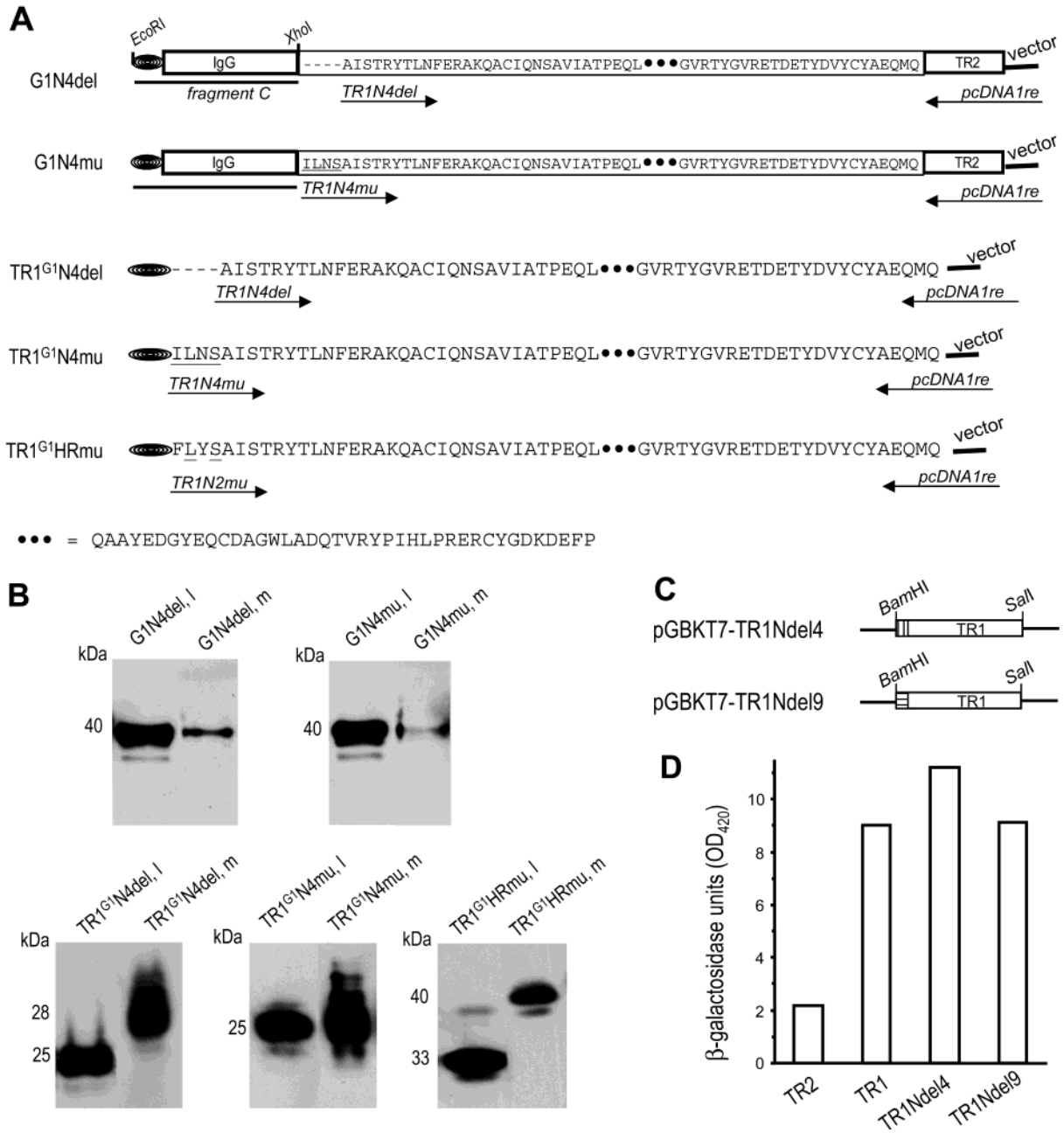

FIGURE 8: Mutation of the amino acids H^2R^4 abolishes TR1's effect on product secretion. (A) The N-terminal four amino acids (FHYR) were deleted or mutated in four constructs—two based on a G1 backbone and the other two based on $TR1^{G1}$ backbone, producing G1N4del, G1N4mu, $TR1^{G1}$N4del, and $TR1^{G1}$N4mu. In a fifth construct, $TR1^{G1}$HRmu, two amino acids were mutated (H → L, R → S). Deletion/mutation of these four amino acids or mutation of H and R is shown. Primers used for PCR are also shown. (B) These deletion/mutation constructs were transiently expressed in COS-7 cells. Product analysis on Western blot probed with 4B6 indicated that all constructs were well-expressed and secreted to culture medium (m). Some secreted products were larger in size than those in cell lysate (l) suggesting glycosylation of those products. (C) Diagram of two mutant constructs generated in yeast expression vector pGBKT7. (D) In liquid assay of yeast two-hybrid interaction, pGBKT7-TR1 (TR1), pGBKT7-TR1Ndel4 (TR1Ndel4), and pGBKT7-TR1Ndel9 (TR1Ndel9) exhibited binding activity with pACT2-ER compared to the background produced by pGBKT7-TR2 (TR2) construct.

amino acid residues (His and Arg) in the N-terminal of the first tandem repeat that play an important role in retarding product secretion. However, to fulfill this function, the N-terminal 55 amino acids are required.

Our studies provide a basis for understanding the biological activity of the aggrecan G2 domain, a domain which contains two tandem repeats. G2 is unique to aggrecan and not found in any other chondroitin sulfate proteoglycan. It is homologous to the TR motifs of aggrecan G1 domain and also to link protein but, though the tandem repeats of G1 have been shown to bind to hyaluronan, G2 is incapable of this (*33*, *34*, *40*−*42*). Using expression and immunoblotting techniques, our study has shown that G2, like G1, fails to be secreted in the presence of the first tandem repeat.

Why would aggrecan contain two elements (G1 and G2) that hinder product secretion? It is highly probable that they function as double check-points in the processing of aggrecan. As aggrecan contains significantly more GAG chains than the other chondroitin sulfate proteoglycans, and aggrecan contains an extra G2 domain, it is logical to suggest that their effects on product secretion are related to glycosylation. Retarding product secretion may allow sufficient

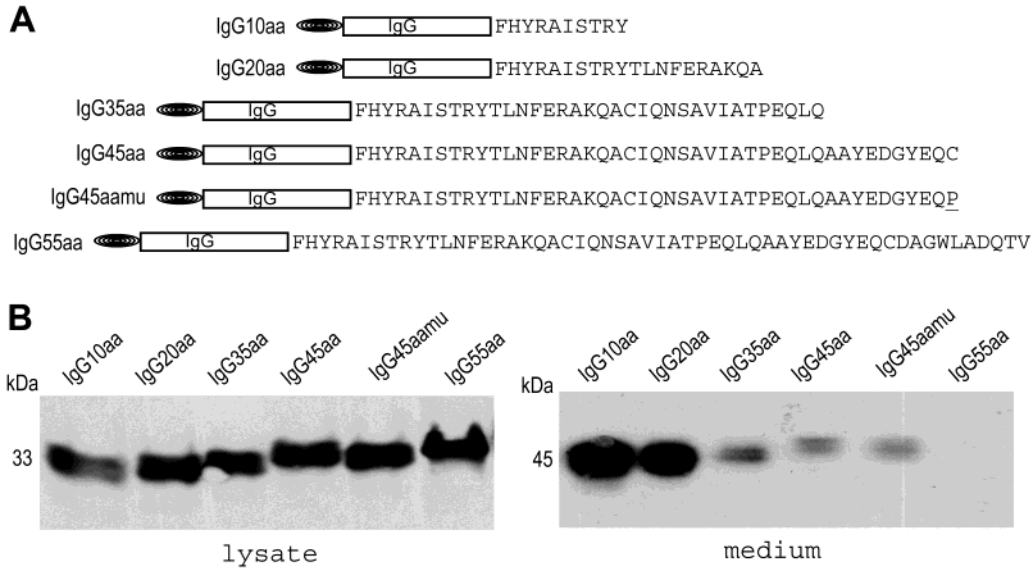

FIGURE 9: IgG-like motif linked to a fifty-five amino acid TR1 fragment is not secreted. (A) Six constructs containing the IgG motif linked to N-terminal fragments of the TR1 motif or a point mutation ($C^{45} \rightarrow X^{45}$) of the TR1 (TR1 fragment size of 10, 20, 35, 45, and 55 amino acid residues) were generated and transiently expressed in COS-7 cells. (B) Analysis of these products on Western blot probed with 4B6 indicted that IgG containing the N-terminal 20 amino acids was well secreted. Addition of 35 or 45 amino acids allowed partial secretion of IgG, while mutation of the cysteine had no effect. IgG linked to the N-terminal 55 amino acids was not secreted.

glycosylation at the ER, and this may be required for aggrecan processing. If this cannot be achieved, the immature products will be subjected to degradation for recycling.

Although the mechanism by which TR1 affects product secretion is still not fully understood, our finding that the calcium homeostasis endoplasmic reticulum protein interacted with the TR1 motif may shed some light on how TR1 affects aggrecan processing. This interaction may be key to aggrecan processing. There are at least two potential hypotheses to account for this: (i) Some ER proteins may bind mutated or mis-folded core proteins and retain them within the cell for the purpose of quality control. (ii) The interaction of ER proteins with the TR1 motif may allow sufficient glycosylation of aggrecan core protein. There are numerous reports to support the former explanation. For example, BiP is often associated with nonsecretable or misfolded mutants, and either retards substrate transportation or targets substrates for degradation through the Sec61p channel (*43*−*45*). The lectin chaperones are also well positioned to determine the fate of proteoglycans; a proteoglycan is either recognized as immature (to be retained in the ER allowing for further folding or directed to the degradation pathway through binding to BiP) or recognized as mature and transported (*46*). This is also supported by our observation that some products, if not secreted, were degraded without retention, and only trace amounts of degraded products could be detected in the cell lysate (data not shown). Our results seem to favor the latter. The interaction of TR1 with some ER proteins may be an important step in product glycosylation and GAG modification. Without this interaction, the core protein is not properly modified, and the unmodified product cannot be secreted.

Quality control proteins are known to recognize signals on the surface of a protein. This includes the carbohydrate moieties attached to the core proteins of proteoglycans, the KDEL peptide, detected at the C-terminus of many ER-retained proteins, and the C-terminal peptide of rhodopsin (*47*). Evolution may have placed such trafficking signals on the surface or at the N- and C-terminal fragments so that they are easily accessible to interacting proteins. The motif that is responsible for the inhibitory effect of product secretion is located in the middle of the G1 domain. This represents an entirely novel mechanism for protein trafficking. Investigation of the biological functions of G1 with ER proteins may elucidate new information on aggrecan processing.

EXPERIMENTAL PROCEDURES

Materials. Taq DNA polymerase, T4 DNA ligase, and restriction endonucleases were purchased from Boehringer Mannheim and New England Biolabs (NEB). Mammalian expression vectors (pcDNA1, pcDNA3, and pcR3.1), Unidirectional Mammalian TA Cloning kit and *Escherichia coli* strains MC1061 and TOP10F′, Lipofectin, DMEM growth medium, fetal bovine serum (FBS), and trypsin/EDTA were purchased from Invitrogen. Bacterial growth medium was from Difco. Prep-A-Gene DNA purification kits were from Bio-Rad, and prestained protein markers were from NEB. DNA mini-prep kit was from Bio/Can Scientific. ECL Western Blot Detection kit was from Amersham. Horseradish peroxidase (HRP)-conjugated goat anti-mouse IgG was from Sigma. DNA Midi-prep kit was from Qiagen Inc. Tissue culture plates (12-well, 6-well, and 100 mm) were from Nunc Inc. Human cDNA library and supplies for yeast two-hybrid assays were from Clontech. All chemicals were from Sigma.

Strategy for Gene Construction. In this study, a total of 41 constructs were used: 37 constructs were used for gene expression and the other four constructs were used for yeast two-hybrid binding assays. Four constructs—G1, G3, link protein and a mini-aggrecan—have been described by us previously (*22*, *28*, *29*, *38*) (for structures of aggrecan and the G1 domain see Figure 1A,B). Production of the remaining 33 constructs is described in detail below. All new constructs generated by PCR were verified by sequencing. All constructs used in this study were obtained from chicken sequences.

Table 1: Sequences and Restriction Endonuclease Sites of Oligonucleotides

oligonucleotide	sequence	restriction site
AIGN	5′ aaaggatccctgggaagctccctg	*Bam*HI
ABN	5′ aaactcgagggcgtcgtgttccactac	*Xho*I
AAC	5′ aaaaaagcatgctttcaccaggacctctat	*Sph*I
G1C	5′ aaaaaagcatgcatcaccactgtaacagat	*Sph*I
G3C	5′ aaaaaatctagagtgatggtgatggtgatgatgggtgggtctgtgcac	*Xba*I
ABC	5′ aaaaaagcatgcttgcatttgctctgcata	Sph*I*
AB′N	5′ aaactcgagggcaaagtcttctacgcc	*Xho*I
G2F	5′ aaactcgagggtgtggtgttccactac	*Xho*I
G2R	5′ aaatctagatctgaagcagaaagc	*Xba*I
G2TR1C	5′ aaaaaatctagactttagcctgtcgatgta	*Xba*I
G2TR2N	5′ aaaaaactcgagggtgaggtgtttttgcc	*Xho*I
G1TR2N	5′ aaaaaagcatgcggcaaagtcttctacgcc	*Sph*I
G1TR1C	5′ aaaaaaaagcttttgcatttgctctgcata	*Hind*III
LPNKozak	5′ aaaaaagaattcgccgccaccatggcaagtctactctttctg	*Eco*RI
G1N	5′ aaactcgagctgggaagctccctgaac	*Xho*I
TR1mu (K→Q)	5′ ctgccccgggagcgctgctacggtgaccag	*Xma*I
TR1Cdel15	5′ gggtctagactcaccgacaccgtaggtact	*Xba*I
TR1N4del	5′ gggctcgaggcaatctccacaaggtac	*Xho*I
TR1N2mu	5′ gggctcgagttcctctacagtgcaatctcc	*Xho*I
Kpnlmu1	5′ gatggggtaccagacagacccgtc	*Kpn*I
Kpnimu2	5′ gatggggtaccagacagacccgtcagccagccagccggcattgcagtg	*Kpn*I
TR145aaR	5′ gggtctagagcactgctcgtacccatc	*Xba*I
TR145aaRmu	5′ gggtctagaccactgctcgtacccatc	*Xba*I
TR135aaR	5′ gggtctagactgcagctgctcaggggt	*Xba*I
TR120aaR	5′ gggtctagaggcctgctttgccctctc	*Xba*I
TR110aaR	5′ gggtctagagtaccttgtggagattgc	*Xba*I
G3CApaI	5′ ggggggcccatgggtgggtctgtgcac	*Apa*I
TR1Cmu	5′ gggtctagattgcatttgctctgcatagcagaaaacatcatatcttac	*Xba*I
TR1N4mu	5′ gggctcgagatcctcaacagtgcaatctccaca	*Xho*I
G3N	5′ gggtctagagacctggcaaactgtgag	*Xba*I
G1CXbaI	5′ aaaaaatctagaatcaccactgtaacagat	*Xba*I
TR1Ndel9	5′ gggctcgagtacactttgaacttcgagggg	*Xho*I
pcDNA1re	5′ atttaggtgacactatagaatagg	
TR1N	5′ cccggatcctcccactacagagcaatc	*Bam*HI
AG1C	5′ aaagtcgacatcaccactgtaacagat	*Sal*I
TR2N	5′ aaaggatccggcaaagtcttctacgcc	*Bam*HI
TR1C	5′ cccgtcgacttgcatttgctctgcata	*Sal*I
TR1Ndel4	5′-cccggatccgcaatctccacaaggtac	*Bam*HI
TR1Ndel9	5′-cccggatcctacacttgaacttcgag	*Bam*HI

Cloning of TR^{G1}, TR1^{G1}, TR2^{G1}, and IgG in pcDNA1. Aggrecan G1 subdomains were PCR-amplified and cloned in the vector pcDNA1 via the following cloning strategy: using the ABN/G1C primer set (sequences for all primers used in this study are shown in Table 1) and G1 cDNA as a template, we PCR-amplified aggrecan G1 tandem repeats (TR^{G1} or TR1^{G1}TR2^{G1}). TR1^{G1}and TR2^{G1} were also PCR-amplified individually using G1 cDNA as the template and two primer sets: ABN/ABC and AB′N/G1C.

The reaction mixture (total volume of 100 μL) typically contained 200 μM dNTPs, 0.2 μg of each primer, 50 ng of template DNA, 2 units of *Taq* DNA polymerase and Mg^{2+}-containing buffer. The reactions were carried out at 94 °C for 5 min for one cycle, 94 °C (60 s), 55 °C (60 s), and 72 °C (60−120s depending on the size of DNA amplified) for 25 cycles and a final extension at 72 °C for 10 min. The protocol was standard for all reactions. Products were agarose gel-purified and digested with two appropriate restriction endonucleases at 37 °C overnight. The digested PCR products were purified and ligated into a plasmid vector. Upstream (5′) to the inserts, all constructs contained the link protein leading peptide sequence (LP60), which harbors the signal sequence and an epitope recognized by a monoclonal antibody 4B6 (*48*).

Similarly, the IgG-like motif of aggrecan G1 was also PCR-amplified using the primers AIGN/AAC and G1 cDNA as a template. The PCR products were gel-purified and digested with *Bam*HI/*Sph*I at 37 °C overnight. The digested PCR products were purified from agarose gel and ligated into a *Bam*HI/*Sph*I-digested pcDNA1 vector carrying the LP60 signal sequence 5′ to the insert. Constructs made in pcDNA1 vector were used to transform *E. coli* strain MC1061.

Cloning of TR1^{G2}TR2^{G2} (or G2), TR1^{G2}, and TR2^{G2} in pCR3.1 (TA Cloning System). Aggrecan G2 domain and its subdomains were cloned as follows: using the primer sets G2F/G2R, G2F/G2TR1C and G2TR2N/G2R, we PCR-amplified aggrecan G2 (TR1^{G2}TR2^{G2}), TR1^{G2} and TR2^{G2} using aggrecan G2 domain as the template. All PCR fragments were gel-purified and digested with their respective restriction enzymes. The digested PCR products were purified and ligated into an *Xho*I/*Xba*I-restricted pcDNA3 vector harboring LP60 5′ to the inserts. The ligation mixes were used as templates in three separate PCR reactions using the primer sets LPNKozak/G2R (for the constructs G2 and TR2^{G2}) and LPNKozak/G2TR1C (for the construct TR1^{G2}). We were then able to PCR amplify G2, TR2^{G2} and TR1^{G2} to be used in the Unidirectional TA Cloning kit. These fragments were ligated into the vector pCR3.1 at 14 °C overnight. The ligation mixes were used in transformation of *E. coli* strain TOP10F′. The resultant recombinant constructs (G2, TR2^{G2}, and TR1^{G2} in pCR3.1) were digested with *Eco*RI to confirm the proper orientation of the inserts in the vector.

Targeted Cloning of Different Aggrecan G1 Subdomains with Each Other and with the G3 Domain. Using the primer sets G1N/ABC and AB′N/G3C, we PCR-amplified IgGTR1^{G1} and TR2^{G1}G3 using G1 cDNA and G2G3 cDNA (already cloned by our group in the vector pCR3.1) as templates. The PCR products were gel-purified, digested with *Xho*I/*Sph*I, repurified, and ligated into *Xho*I/*Sph*I-restricted pcDNA1, 3′ of the LP60 sequence.

We also cloned TR1^{G1}G3 and IgGTR2^{G1} in pcDNA3 and pCR3.1, respectively. Using the primer set LPNKozak/G1TR1C and G1 cDNA as a template, we PCR-amplified the TR1^{G1} fragment. The PCR fragments were gel-purified and digested with the appropriate restriction enzymes. The digested PCR products were purified and ligated into a *Hind*III/*Eco*RI-restricted pcDNA3 harboring the aggrecan G3 domain 3′ to the ligated PCR inserts. Ligation was performed at 16 °C overnight and the ligation mix was used to transform *E. coli* strain TOP10F′.

To generate the IgGTR2^{G1} construct, we first PCR-amplified TR2^{G1} using the primer set G1TR2N/G1C and G1 cDNA as the template. The PCR products were gel-purified and digested with *Sph*I/*Xba*I. An IgG fragment was obtained from the IgG-pcDNA1 construct (described earlier) by digestion with *Eco*RI/*Sph*I, and these two fragments, IgG (*Eco*RI/*Sph*I) and TR2^{G1} (*Sph*I/*Xba*I), were ligated into pcDNA3 vector digested with *Eco*RI/*Xba*I. Ligation was performed at 16 °C overnight. The ligation mix was then used in a PCR reaction to amplify IgGTR2^{G1} using the primer set LPNKozak/G1C. The PCR products were cloned into pCR3.1 utilizing the Unidirectional TA Cloning kit. The resultant construct was digested with *Eco*RI to confirm the proper orientation of the inserted PCR product.

Linkage of G2 Motifs with IgG Motif or the G3 Domain. To generate the construct IgGTR1^{G2}, the LP60−IgG fragment was obtained from the construct G1N4mu by digestion with *Eco*RI/*Xho*I, while the TR1^{G2} fragment was obtained from the construct TR1^{G2} by digestion with *Xho*I/*Xba*I. These two fragments were inserted into *Eco*RI/*Xba*I-digested pcDNA3 vector. To generate the construct IgGTR2^{G2}, the LP60−IgG fragment was obtained as above, and the TR2^{G2} fragment was obtained from the TR2^{G2} construct by digestion with *Xho*I/*Xba*I. These two fragments were cloned as above producing IgGTR2^{G2}. To construct TR1^{G2}G3, a LP60-TR1^{G2} fragment was obtained from the TR1^{G2} construct by digestion with *Eco*RI/*Xba*I, and the G3 fragment was synthesized in a PCR using two primers, G3N and G3C*Apa*I, followed by digestion with *Xba*I/*Apa*I. These two fragments were cloned into the pcDNA3 vector, which had been digested with *Eco*RI/*Apa*I. To generate construct TR2^{G2}G3, the TR2^{G2}G3 fragment was amplified by PCR using two primers, G2TR2N and G3C*Apa*I, with the G2G3 construct as a template. The PCR products were digested and cloned into *Xho*I/*Apa*I-digested pcDNA3 vector harboring the LP60 moiety.

Site-Directed Mutagenesis and Truncation. Generation of these types of constructs is more complex, and it is described in the figure legends (Figures 7 and 8). These constructs include TR^{G1}K → Q, TR1^{G1}mu1, TR1^{G1}mu2, TR1^{G1}mu3, TR1^{G1}C15del, TR1^{G1}N9del, TR1^{G1}Δ381, G1N4del, G1N4mu, TR1^{G1}N4del, TR1^{G1}N4mu, and TR1^{G1}HRmu.

Addition of N-Terminal Fragments of TR1^{G1} to IgG Motif. Constructs IgG10aa, IgG20aa, IgG35aa, and IgG45aa were generated by PCR using the construct IgGTR1^{G1} as a template. Primers used were LPNKozak and one of the following: TR110aaR, TR120aaR, TR135aaR, or TR145aaR. The construct IgG45aamu (containing a point mutation at amino acid C^{45} of TR1^{G1}) was PCR-generated using the same template and the primers LPNKozak and TR145aaRmu. As the primer LPNKozak contains an *Eco*RI site and the other primers contain an *Xba*I site, the PCR products were digested with *Eco*RI/*Xba*I and cloned into *Eco*RI/*Xba*I-digested pcDNA3 vector. Due to the presence of a *Kpn*I site at amino acids 55−56 of TR1 motif, the construct IgG55aa was generated by releasing this fragment from TR1 construct by *Eco*RI/*Kpn*I digestion.

Generation of Yeast Two-Hybrid Binding Constructs. One or both tandem repeats of the G1 domain were inserted into the G1 domain were inserted into the plasmid pGBKT7 or pGBDC1 to generate four constructs for yeast two-hybrid studies: pGBKT7-TR1TR2, pGBDC1-TR1TR2, pGBDC1-TR1, and pGBDC1-TR2. Each bait fragment was amplified by PCR using two primers: TR1N and AG1C amplifying TR1TR2; TR1N and TR1C amplifying TR1; and TR2N and AG1C amplifying TR2. Two mutant constructs, pGBKT7-TR1Ndel4 and pGBKT7-TR1Ndel9, were generated using two pairs of primers: TR1Ndel4 and TR1C, and TR1Ndel9 and TR1C. All restriction enzyme-digested PCR fragments were inserted into the cloning sites *Bam*HI and *Sal*I, producing constructs named above.

Expression of Recombinant Constructs in COS-7 Cells. COS-7 cells were transiently transfected with the recombinant constructs using Lipofectin. Briefly, COS-7 cells were seeded to a six-well plate at 1.5×10^5 cells per well. The cells were allowed to attach and grow overnight in DMEM supplemented with 5% FBS and 100 units of penicillin/100 μg of streptomycin/2.5 μg of amphotericin (per milliliter). Cells were transfected at 70% confluence. Lipofectin (1 μL) was incubated with plasmid DNA (5 μg) for 15 min in 200 μL of DMEM followed by addition of 800 μL of DMEM. During the incubation, COS-7 cell cultures were rinsed with 2 mL of DMEM. The lipofectin−DNA mixture was applied to the rinsed cultures and incubated for 10 h. The DNA/Lipofectin mixture was replaced with 1 mL of DMEM supplemented with 5% FBS. The growth medium and cell lysate were harvested separately after 3 days.

In coexpression studies, COS-7 cells prepared as above were cotransfected with the mini-aggrecan and TR1^{G1}, TR2^{G1} or the control vector. Briefly, plasmid DNA (2 μg) containing mini-aggrecan was mixed with the plasmid DNA (3 μg) containing TR1^{G1}, TR2^{G1} or the control vector, followed by incubation and transfection as described above.

Western Blot Assays. Cell lysate and culture medium were subjected to SDS−PAGE electrophoresis as described earlier (*36*, *39*, *49*, *50*). Proteins separated in SDS−PAGE were transblotted onto a nitrocellulose membrane (Bio-Rad) in 1× TG buffer (Amresco) containing 20% methanol at 60 V for 2 h in a cold room. The membrane was blocked in TBST (10 mM Tris-Cl, pH 8.0, 150 mM NaCl, 0.05% Tween 20) containing 10% nonfat dry milk powder (TBSTM) for 1 h at room temperature, and then incubated at 4 °C overnight with monoclonal antibody 4B6 diluted in TBSTM. The membranes were washed with TBST (3 × 30 min) and then incubated for 1 h with HRP-conjugated goat anti-mouse antibody (diluted 1:50 000) in TBSTM. After washing as above, the bound antibodies were visualized with chemilu-

minescence (ECL kit).

Yeast Two-Hybrid Assay. To isolate proteins which bind to the tandem repeats, we employed Clontech Gal4 yeast two-hybrid system with chicken aggrecan $TR1TR2^{G1}$ as bait. Yeast strain JP-69−4A was cotransfected with pGBKT7-TR1TR2 and human brain cDNA library generated in the plasmid pACT2. Library screening and colony identification were performed according to the manufacturer's instructions. Sequencing of positive clones revealed a number of genes that may interact with pGBKT7-TR1TR2. Only one of them, containing part of the calcium homeostasis endoplasmic reticulum protein (ER protein), appeared to have a potential role in protein processing, and the effect of this clone, named pACT2-ER, was examined.

The tandem repeats were cloned into plasmid pGBDC1, producing pGBDC1-TR1, pGBDC1-TR2, and pGBDC1-TR1TR2, which were then coexpressed with pACT2-ER in JP-69−4A. The transfected yeast cells were grown on SD/-Trp/-Leu agar plates at 30 °C for 4−5 days. Ten yeast colonies were picked up from each cotransfection assay and inoculated onto agar plates of minimal SD base/-Trp/-Leu/his plus 5 mM 3-amino-1,2,4-triazole. After 3−4 days, cells on the agar plates were transferred onto a filter or to liquid cultures. Color development was performed with β-galactosidase to detect protein−protein interaction according to the manufacturer's instruction.

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