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Cross-linking of galectin 3, a galactose-binding protein of mammalian cells, by tissue-type transglutaminase

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Abstract The 30 kDa β-galactoside-binding protein of baby hamster kidney (BHK) cells [Mehul et al. (1994), J. Biol. Chem. 269, 18250–18258] homologous to galectin 3, a widely distributed mammalian lectin, has been found to be a substrate for tissue type transglutaminase, as shown by the incorporation in a calcium- and time-dependent manner of 5-(biotinamido) pentylamine in the presence of guinea pig liver transglutaminase. The amino-terminal domain of hamster galectin 3, which is a repetitive sequence rich in glutamine, tyrosine, glycine and proline, is also an excellent substrate. A single lysine residue in the N-terminal domain is an essential requirement for transglutaminase-mediated oligomerization, and two equivalent glutamine residues present in identical sequence repeats within this domain appear to be involved as amine acceptors in cross-linking reactions. Transglutaminase-mediated cross-linking of galectin 3 to itself or to matrix components may be one mechanism for stablisation of a multivalent binding form of the lectin in cell secretions or in extracellular matrices.

Key words: Galectin; Cross-linking; Transglutaminase

1. Introduction

Galectin 3 is a name recently proposed [1] for a family of ~30–35 kDa β-galactoside-binding proteins of rat, human, mouse and hamster cells that share extensive sequence homologies, as well as similar physical properties and carbohydrate-binding specificities [2,5]. One striking feature of galectin 3 is an N-terminal domain consisting of a repeat sequence of on average nine amino acid residues rich in tyrosine, proline, glycine and glutamine, the function of which is unknown. Collagenase-treatment of galectin 3 selectively degrades the N-terminal domain leaving a C-terminal fragment that retains full carbohydrate-binding activity [5–8]. However, multivalency as assessed by haemagglutination or by positive cooperativity of lectin binding to multivalent glycoprotein ligands [7–9] requires the N-terminal domain. Chemical cross-linking studies indicate that the human [7,8] and hamster [5] lectin and the recombinant N-terminal domain of the latter [5] can associate to form intermolecular aggregates in a concentration-dependent manner, raising the possibility that a switch to multivalency of binding may be regulated through self-association of lectin molecules mediated mainly through the N-terminal domain. We show here that hamster galectin 3 and specifically the N-terminal domain is a substrate for tissue-type transglutaminase, a calcium-dependent acyltransferase which catalyses the formation of ε-(γ-glutamyl) lysine isopeptide cross-links between certain polypeptides and the conjugation of amines to protein-bound glutamine residues [10–12].

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Abbreviations: BHK, baby hamster kidney; TG, transglutaminase; PAGE, polyacrylamide gel electrophoresis; PCR, polymerase chain reaction; EHS, Englebreth-Holm-Swarm.

2. Experimental

2.1. Materials

Guinea pig liver transglutaminase [13] was obtained (1.9 Units/mg protein) from Sigma Corp. was stored (1.9 units/mg protein, 2 Units/ml) at −20°C. The biotin-labelled amine, 5-(biotinamido) pentylamine was obtained from Pierce Warriner, Chester, UK. Streptavidin horse-radish peroxidase was obtained from Vector Laboratories, Peterborough, UK. Matrigel, a basement membrane preparation from murine EHS tumour, was obtained from Collaborative Research Inc., Bedford, MASS, USA. Other reagents unless specified otherwise were purchased from Sigma Corp. Synthetic peptides Pep1 and Pep2 (Fig. 1) were supplied by the Peptide Synthesis facility at N.I.M.R.

2.2. Recombinant proteins

Characterization and bacterial expression of a full-length cDNA clone (i1) of hamster galectin 3, and purification, collagenase treatment and N-terminal sequencing of recombinant lectin has been described [5]. cDNA corresponding to amino acid residues from 1 to 125 of the hamster galectin sequence starting at the initiating methionine and ending at Met^{125} was generated by PCR, expressed as a fusion protein with glutathione S-transferase and the N-terminal lectin fragment was purified by affinity chromatography on a glutathione-based matrix followed by thrombin cleavage of the fusion protein [5]. The predicted amino acid sequence of the product (Fig. 1) contains six extra amino acids GSPGRP originating from the thrombin cleavage site of the fusion protein [5]. The same strategy was used to prepare a mutant N-terminal fragment containing a $Lys^{114} \rightarrow Ala^{114}$ substitution. Briefly, a PCR product was obtained using BioTaq polymerase (Bioline, London, UK), primers 5′-TCCCCGGGTCGACCCATGGCAGACGGTTTTTCGC-3′ and 5′-ATGAATTCTTACATTCGAGGCATGACTCCTCCAGCCAAGGGCAGCGCA TA-3′ (double underline indicates Ala codon) and i1 cDNA clone as template using the following conditions: one cycle for 3 min at 94°C, 10 cycles of denaturation at 94°C for 30 s, annealing at 50°C for 1 min, extension at 72°C for 1 min, 20 cycles at 94°C for 30 s, 57°C for 1 min, 72°C for 1 min and finally 1 cycle at 72°C for 5 min. The PCR product giving a single 370 bp band was subcloned into the pGex-2T plasmid (Pharmacia) using *SmaI–EcoRI* restriction sites and used to transform *E. coli* DH 5α [5]. A positive transformant was picked and the mutated nucleotide sequence was verified by dideoxy-chain termination sequencing using internal primers as before [5]. This transformant was induced by IPTG, fusion protein was identified by Western blotting of bacterial extracts and purification of mutant N-terminal fragment was carried out exactly as described for wild-type fragment [5]. Fragments were concentrated to approximately 1 mg/ml using Amicon Centricon 3 membranes (Amicon) and stored at 2°C without freezing.

2.3. Antibodies

Polyclonal antibodies raised in rabbits against purified BHK cell galectin 3 were obtained as described [4,5]. Rabbit antibodies against synthetic peptide P2 (Fig. 1) were raised by subcutaneous injection of peptide P2 coupled to keyhole limpet haemocyanin in complete Freund's adjuvant at five sites. Following six booster injections in

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incomplete Freund's adjuvant antisera were collected seven days after the final boost injection. Goat anti-rabbit immunoglobulin coupled to horse-radish peroxidase was purchased from ICN Biochemicals, Cal., USA.

2.4. SDS–Polyacrylamide gel electrophoresis and blotting

Electrophoresis using 10% or 15% polyacrylamide gels and antibody blotting was carried out as described [14]. Biotinylated proteins were detected after electrophoresis by incubation of filters with streptavidin-peroxidase conjugate (2 μg/ml in 10 mM HEPES buffer, pH 7.2) at room temperature for 2 h. Bands were visualized using 3-amino-9-ethyl carbazole (Sigma) as substrate.

2.5. Transglutaminase incubation

Reaction mixtures containing recombinant protein (10 μl of 0.1 mM solutions), 500 mM Tris-HCl, pH 6.8–0.15 M NaCl (15 μl), 0.15 M $CaCl_2$ (1 μl), 5-(biotinamido) pentylamine (5 μl of a 10 mM solution) or water (5 μl) and guinea pig liver transglutaminase (4 μl) were incubated for various periods at 37°C. In some experiments synthetic peptides Pep1 or Pep2 (Fig. 1) were added (5 μl of 10 mM solutions) to reaction mixtures. Following incubation, the reaction mixtures were mixed with 10 μl of five-fold strength Laemmli sample buffer containing 5% 1-mercaptoethanol and boiled at 100°C for 5 min before SDS–polyacrylamide gel electrophoresis.

2.6. Gel filtration

A reaction mixture containing recombinant N-terminal fragment rCBP-N (100 μl, 0.1 mM solution), 500 mM Tris-HCl, pH 6.8, 150 mM NaCl (150 μl), 150 mM $CaCl_2$ (10 μl), 5-(biotinamido) pentylamine (50 μl, 10 mM solution) and guinea pig liver transglutaminase (40 μl) was incubated overnight at 37°C and applied to a column (2 cm × 55 cm) of Biogel P60 100–200 mesh (Biorad) equilibrated with 10 mM HEPES buffer, pH 7.2, 150 mM NaCl. Fractions (2 ml) were collected at 2°C and aliquots (40 μl) were analyzed for protein by the bicinchoninic acid (BCA, Pierce) assay. Selected fractions were mixed (1 ml aliquots) with Streptavidin Agarose beads (100 μl, 10% suspension in 10 mM HEPES buffer pH 7.2, 150 mM NaCl) and incubated at 2°C overnight. The beads were washed three times with buffer, suspended in Laemmli sample buffer (50 μl) and boiled at 100°C for 5 min. Aliquots (30 μl) were subjected to SDS–polyacrylamide gel electrophoresis followed by Streptavidin-peroxidase blotting. In control experiments a sample of rCBP-N (100 μl, 0.1 mM solution) was applied to the Biogel P60 column and analyzed as above.

3. Results

Incubation of pure recombinant hamster galectin 3 [5] with guinea pig liver transglutaminase (TG) in approximately 20:1 molar ratio catalyzed the incorporation of 5-(biotinamido) pentylamine into the lectin as detected by reducing SDS–PAGE and Western blotting with Streptavidin peroxidase conjugate. A single band of M_r = 30,000 expected for hamster galectin 3 [4] was biotinylated in a time-dependent manner (Fig. 2), and was dependent on calcium ions since addition of EDTA abolished the incorporation (result not shown). When a parallel set of mixtures but lacking the biotinamine probe was analyzed by SDS–PAGE and Western blotting with antibodies raised against hamster galectin 3 [4,5] we observed a time-dependent appearance of an antibody-reactive M_r = ~60,000 component on SDS–PAGE consistent with a dimer (Fig. 2).

The N-terminal domain of hamster galectin 3 [5] contains five glutamine residues in a proline-, tyrosine- and glycine-rich repetitive sequence (Fig. 1). When recombinant N-terminal fragment extending through to residue Met^{125} was treated with transglutaminase oligomerization occurred as shown by immunoblotting of reaction mixtures with anti-lectin antibodies (Fig. 3A). After 1–2 h incubation products up to hexamers could readily be detected (Fig. 3A) and longer time (16 h) gave higher oligomers that failed to enter the resolving SDS–polyacrylamide gel (result not shown). The incorporation of the biotinamine probe into the N-terminal fragment occurred together with oligomerization (Fig. 3A). A large-scale incubation mixture containing 100 μg of N-terminal fragment plus biotinamine was fractionated after long-term treatment with transglutaminase by Biogel P60 chromatography (Fig. 4A). The peak protein fractions eluted ahead of those of a control mixture containing N-terminal fragment not treated with transglutaminase. In the latter case the peak fractions were eluted with a α-lactalbumin standard (M_r = 14,200) consistent with an apparent monomeric size. Column fractions from the transglutaminase-containing reaction mixture, analyzed by SDS–PAGE under reducing conditions and blotting with Streptavidin-peroxidase, showed (Fig. 4B) the fractionation of biotinylated polypeptides of various sizes. The earlier eluting fractions contained increasing amounts of the most highly cross-linked products, the largest of which migrated on SDS–PAGE with M_r = ~75,000–90,000 consistent with their point of elution from Biogel P60 just ahead of a BSA (M_r = 64,000) standard.

Two identical repeats within the N-terminal domain of hamster galectin 3 contain glutamine residues Q^{47} and Q^{56} (Fig. 1). Peptides containing one (Pep1) or two (Pep2) repeats were synthesized and examined for their ability to act as acyl donors in transglutaminase-catalyzed reactions. Reaction mixtures were set up containing N-terminal fragment, the biotinamine probe, transglutaminase and an excess (about 30-fold) of either Pep1 or Pep2 and the products formed after incubation at 37°C were analyzed by SDS–PAGE and blotting either with Strep-

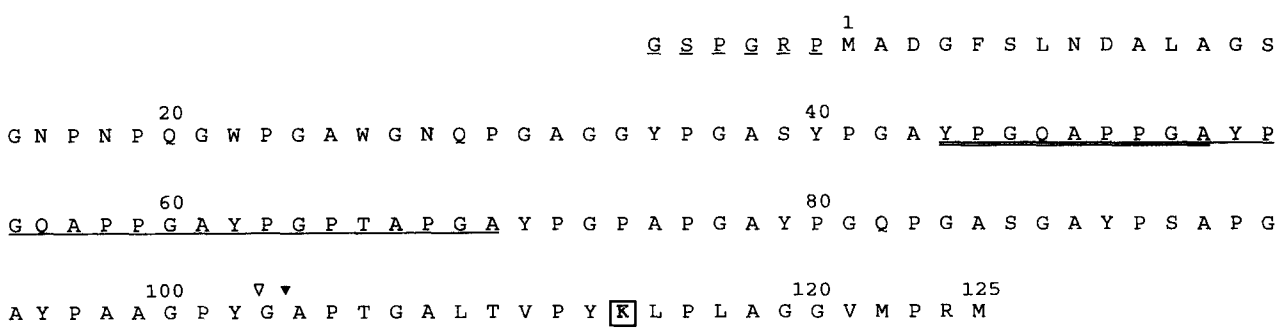

Fig. 1. Amino acid sequence of recombinant N-terminal fragment of hamster galectin 3. Residues are numbered from the initial methionine to Met^{125}. Lys^{114} is boxed. Six additional residues underlined at the N-terminal derive from the thrombin cleavage site of the fusion protein (see Ref. 5 for details). Synthetic peptides Pep1 (residues 44–52) and Pep2 (residues 44–70) are underlined. The N-termini of carbohydrate-binding fragments generated by digestion of galectin 3 with bacterial collagenase (▽) and by endogenous proteolysis (▼) are indicated.

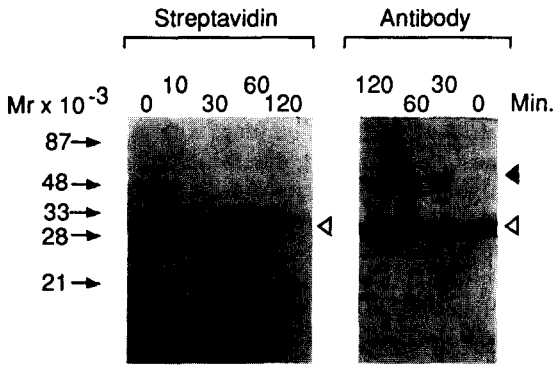

Fig. 2. Transglutaminase catalyzed cross-linking of hamster galectin 3. Equal amounts of hamster galectin 3 were incubated with TG with (left hand panel) or without (right hand panel) a biotinamine probe for the times indicated. After reducing SDS–15% PAGE the separated products were blotted with either Streptavidin-peroxidase or with anti-galectin 3 serum followed by second antibody-peroxidase. Monomeric (open arrow heads) and dimeric (filled arrow head) galectin 3 are indicated. The migration of protein standards is indicated at left hand margin.

tavidin or with antibodies raised against Pep2. This antibody reacted with whole galectin and the N-terminal fragment in immunoblotting after SDS–PAGE and the specific reactions were blocked by 200 μg/ml of Pep2 (Fig. 5). Minor cross reactions with unidentified polypeptides in both recombinant galectin 3 and N-terminal fragemnt were not blocked. In the transglutaminase reaction mixture containing Pep2 a major biotinylated band strongly reactive with anti-Pep2 antibodies was detected migrating more slowly than the biotinylated monomeric species (Fig. 3). This band was absent in control mixture lacking Pep2. The difference in migration of the biotinylated monomer and the new band (about 2,000–3,000) is consistent with the expected size of a cross-linked conjugate of N-terminal fragment (M_r = 13,000) and Pep2 (M_r = 2,500). In the Pep1-containing mixture a closely spaced doublet representing the biotinylated monomeric species appeared after immunoblotting (Fig. 3C) suggesting that Pep1 containing only 9 amino acid residues was also conjugated with the N-terminal fragment by transglutaminase. Interestingly although the extent of cross-linking appeared to be rather similar in control or peptide-containing reaction mixtures as detected by the biotinylation probe Streptavidin, reactivity of the cross-linked products, particularly trimers and pentramers, to the anti-Pep2 antibodies was considerably enhanced in the mixture containing Pep2 (Fig. 3C, compare tracks 1 and 3). Presumably, oligomers containing the synthetic peptide react more strongly with the antibodies than the corresponding oligomers containing only the N-terminal fragment. The reason for the apparently weak incorporation of Pep2 into dimers and tetramers (Fig. 3C, track 1) remains to be determined.

We considered next the nature of the acyl acceptor in the N-terminal fragment, which in proteins or polypeptides is most commonly the ε-amino group of lysine. The N-terminal fragment contains just one lysine residue K^{114} (Fig. 1) as deduced from the cDNA sequence [5]. In order to confirm this assignment in the protein we took advantage of the susceptibility of the N-terminal domain of galectin 3 to bacterial *Arthrobacter iophagus* collagenase [5]. N-terminal amino acid sequencing of the carbohydrate-binding fragment obtained after collagenase treatment by affinity chromatography on asialofetuin gave an unambiguous sequence starting at Gly^{103} and ending at Arg^{124}: Lys^{114} was clearly identified in this sequence (result not shown). Similar analysis of a carbohydrate-binding fragment obtained by affinity chromatography after endogenous degradation of galectin 3 isolated from BHK cells [5] gave an unambiguous amino acid sequence starting at Ala^{104} and ending at Met^{122} and again giving a clear Lys signal at position 114 (result not shown). To determine whether this residue acts as an acyl acceptor, a site-directed mutant was made in which the lysine was replaced by an alanine. The mutant $K^{114}\rightarrow A^{114}$ fragment incorporated the biotinamine probe to comparable extent as the normal K^{114} fragment (Fig. 3B) showing that the glutamine acyl donors were functional. However, transglutaminase-catalyzed cross-linking of the $K^{114}\rightarrow A^{114}$ mutant was blocked (Fig. 3B).

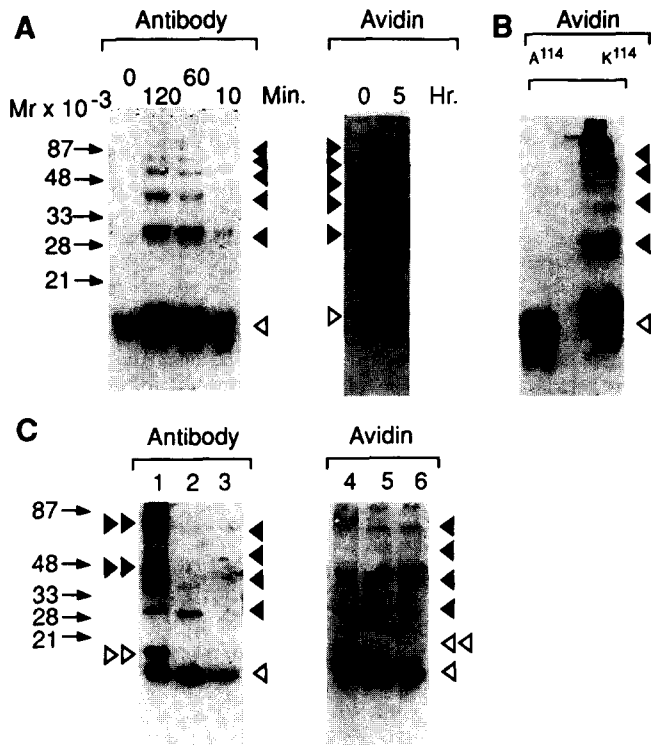

Fig. 3. Transglutaminase-catalyzed cross-linking of N-terminal fragment of hamster galectin 3. (A) Equal amounts of fragment were incubated at 37°C with TG minus (left hand panel) or plus (right hand panel) a biotinamine probe for the times shown and after reducing SDS–15% PAGE the separated products were blotted with anti-hamster galectin 3 serum or with Streptavidin. The migration of protein standards are shown at margin. The position of monomeric N-terminal fragment is indicated by the open arrow head. Oligomeric species are indicated by filled arrow heads. (B) Equal amounts of normal Lys^{114} fragment or mutant Ala^{114} fragment were treated for 5 h at 37°C with TG in presence of biotinamine and analyzed as in panel A. (C) Equal amounts of normal Lys^{114} fragment were mixed with an excess of synthetic peptide Pep1 (tracks 2 and 5) or Pep2 (tracks 1 and 4) or no synthetic peptide (tracks 3 and 6) and incubated with TG and biotinamine for 5 h at 37°C before SDS–15% PAGE and blotting with anti-Pep2 antiserum (tracks 1–3) or with Streptavidin (tracks 4–6). Open arrow heads, galectin fragment monomer; filled arrow heads, galectin fragment oligomers. Conjugates containing Pep2 are shown by double arrow heads.

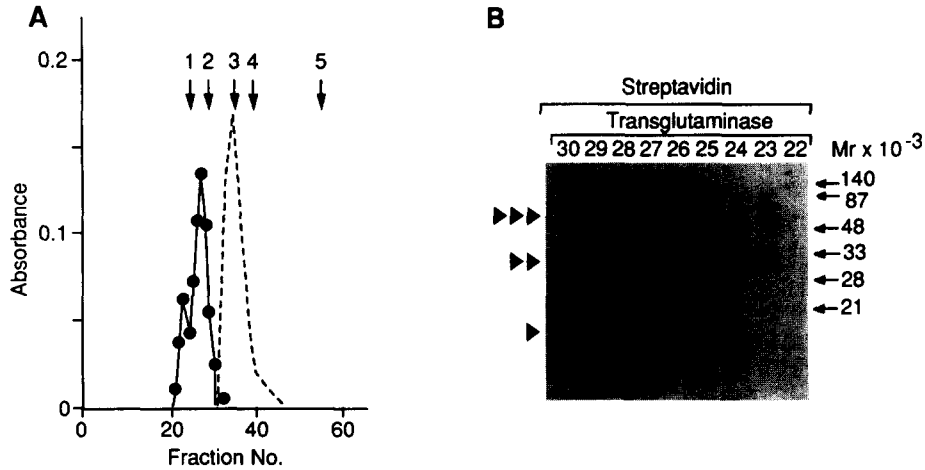

Fig. 4. Gel filtration of transglutaminase products. (A) Gel filtration of the N-terminal fragment of hamster galectin 3 before (broken line) and after (solid line) transglutaminase-catalyzed cross-linking in presence of biotinamine. The elution of BSA (64 kDa), β-lactoglobin (36 kDa), α-lactalbumin (14.2 kDa), cytochrome *c* (12.4 kDa) and synthetic peptide Pep2 (2.5 kDa) are shown (arrows 1–5 respectively). (B) SDS–15% PAGE of selected column fractions (22–30) from gel filtration chromatography followed by blotting with Streptavidin. The migration of monomeric, dimeric and trimeric species are indicated by arrow heads.

4. Discussion

Tissue-type transglutaminase is widely distributed in most cell types as a monomeric M_r = 77,000 protein which is predominantly cytoplasmic although it can be expressed at the cell surface [15–19] to initiate extracellular functions, one of which is believed to be stabilization of cell interactions with matrix [20,21]. Tissue transglutaminase has a rather selective affinity for the glutamine residues in proteins that can act as acyl donors in cross-linking reactions [11]. By contrast the nature of the primary amine which functions as acyl acceptor is less restrictive, and the probe 5′-(biotinamido) pentylamine has been used previously as a specific reagent to detect transglutaminase substrates in whole cells [22]. Our data shows that galectin 3 joins a relatively short list of proteins containing glutamine residues functional as acyl donors in tissue transglutaminase-catalyzed reactions, including cytoskeletal and plasma membrane proteins such as actin [23], lens crystallins [24], lipocortin [25] and extracellular matrix proteins such as fibrinogen [26], fibronectin [27], collagen [28,29], nidogen [29] and osteopontin [30,31].

The finding that transglutaminase catalyzed the limited formation of hamster galectin 3 dimers (Fig. 2A) shows that intermolecular cross-links are produced between the reactive glutamine residues and available primary amine groups in the polypeptide. Some evidence for disulphide-bonded dimerization of mouse [32,33] and human [8] galectin 3, which appeared to be reversible and dependent on lectin concentration, has been reported. Clearly the M_r = ~60,000 component we observe (Fig. 2A) in transglutaminase-treated hamster galectin 3 is not related to the disulphide-bonded dimers reported by others, inasmuch as it persisted after heating with reducing agent and SDS–polyacrylamide gel electrophoresis carried out under reducing conditions. Interestingly, samples of murine galectin 3 purified by affinity chromatography of mouse lung extracts on asialofetuin Sepharose were shown after SDS–PAGE under reducing conditions to contain in addition to the expected monomeric M_r = 35,000 lectin a minor component of M_r = ~60,000–70,000 [34]. Since this latter band clearly had carbohydrate-binding activity and reacted with an antibody against the pure M_r = 35,000 mouse galectin 3, it is a related molecule formed by cross-linking other than a disulphide bond. Such oligomerization would lead to multivalency of carbohydrate-binding activity and allow the lectin to form interconnections between reactive glycoproteins, to bridge between cells in agglutination reactions or possibly to mediate cell–matrix interactions, under conditions where monomeric lectin is ineffective or less effective for example at low concentrations of lectin.

The very efficient transglutaminase-catalyzed cross-linking of the hamster N-terminal fragment may be due to its unusual amino acid composition containing a high content of proline and glycine residues and which exists largely unstructured as shown by CD and tryptophan fluorescence spectroscopy [5]. The glutamine residues utilized by transglutaminase in other proteins are often also found in terminal polypeptide domains which are flexible and solvent accessible [35,36]. It seems very likely that one or both of the glutamine residues Q^{47} and Q^{56}, which our present data indicate function as acyl donors in cross-linking of the fragment, also are involved in transglutam-

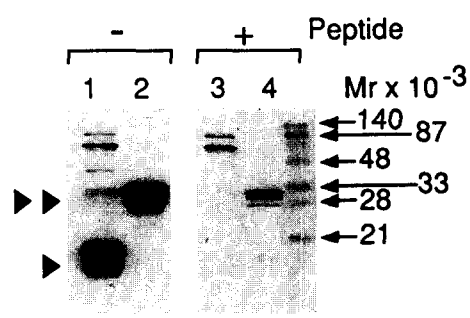

Fig. 5. Specificity of peptide antibodies. Rabbit polyclonal antisera were raised against synthetic peptide Pep2 (Fig. 1) and used for blotting of hamster galectin 3 (tracks 2 and 4) or the recombinant N-terminal fragment (tracks 1 and 3) in absence or presence of 200 μg of Pep2 as indicated. The migration of the fragment (single arrow head) and intact galectin 3 (double arrow heads) are shown.

inase-mediated reactions with the whole lectin, given the selectivity of the enzyme in choice of acyl donor. It is not so clear that the lysine residue K^{114}, functional as an acyl receptor in fragment cross-linking, also is important in dimerization of the intact lectin. For example, six out of nine lysine residues in seminal vesicle protein can be involved in cross-linking [37] although more selective use of a particular lysine residue is known in α B-crystallin [35]. In any event, K^{114} is so far unique to hamster galectin 3 and is replaced by either asparagine in human or aspartic acid in mouse and rat galectin 3 (see [5]). If transglutaminase-catalyzed cross-linking of intact galectin 3 is a general property across species other lysine residues present in the C-terminal, carbohydrate-binding domain of the molecule must be involved. Interestingly, five out of a maximum of nine (in the rat) lysine residues are perfectly conserved in hamster, mouse, rat and human galectin 3 (see [5]).

Galectin 3 binds in vitro to laminin of EHS tumour [14] and we have recently obtained evidence for its association with extracellular matrices such as Matrigel (unpublished results). It is interesting that tissue transglutaminase has also been detected in the pericellular matrix of several tissues [29,38] raising the possibility of transglutaminase-catalyzed cross-linking of galectin 3 with other matrix proteins. If this is the case, the presence of a carbohydrate-binding activity that is stably incorporated into the biomatrix could have interesting biological implications for cellular interactions with matrix.

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