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Structural and Functional Characterization of a Novel Tumor-Derived Rat Galectin-1 Having Transforming Growth Factor (TGF) Activity: The Relationship between Intramolecular Disulfide Bridges and TGF Activity[1]

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Previously we demonstrated that overexpression of a β-galactoside binding protein, galectin-1, caused the transformation of BALB3T3 fibroblast cells [Yamaoka, K., Ohno, S., Kawasaki, H., and Suzuki, K. (1991) *Biochem. Biophys. Res. Commun.* 179, 272-279]. We have now studied the structure-function relationships between the sugar-binding activity and the mitogenic activity of galectin-1 purified from an avian sarcoma virus-transformed rat NRK cell line, 77N1. The purified galectin-1 (t-galectin-1) had potent mitogenic activity in BALB3T3 cells, but no sugar-binding activity. Treatment of t-galectin-1 with 2-mercaptoethanol decreased its mitogenic activity, but resulted in the appearance of a sugar binding activity. Chemical modification of sulfhydryl groups in purified t-galectin-1 with [^{14}C]-iodoacetamide suggested the presence of intramolecular disulfide bonds. MALDI-TOF mass spectrometric analysis of the native and reduced forms of the tryptic peptides from t-galectin-1 showed that t-galectin-1 has two intramolecular disulfide bonds (Cys2-Cys16 and Cys42-Cys60). These studies suggest that these intramolecular disulfide bonds of t-galectin-1 are essential for its mitogenic activity and that the different activities may be regulated by structural changes caused by intramolecular disulfide bond-breakage.

Key words: BALB3T3, disulfide bond, galectin, MALDI-TOF, transforming growth factor.

Alterations of growth factor production resulting in autocrine or paracrine stimulation of tumor cell growth is a common feature of transformed or tumor cells. Among these growth factors, transforming growth factors (TGFs) were originally defined as growth factors that confer a malignant phenotype on untransformed cells and that induce the cells to form progressively growing colonies in soft agar (*1, 2*). The TGFs are acid and heat stable, and presumably have disulfide bonds, since their activity is lost in the presence of dithiothreitol. All the initially isolated TGFs were from transformed cells (*3, 4*), but later it was established that some of them are present in normal tissues (*5, 6*) and cells (*7*), and they were presumed to play an important role in cell growth and differentiation. A correlation between oncogenes and the growth factors was also established (*8, 9*).

We have purified a TGF-like protein from an avian sarcoma virus-transformed rat NRK cell line, 77N1 (*10*), which showed stimulation of DNA synthesis and the promotion of anchorage-independent growth of BALB3T3 A31 cells (*11*). Since many properties of this protein are distinguishable from those of TGFα (*12-14*) and TGFβ (*6*), we initially termed it TGFγ2 (*15*). TGFγ2 is a heat- and acid-labile protein with a molecular mass of approximately 14 kDa. It does not compete with epidermal growth factor (EGF) for receptor binding and does not require the coexistence of EGF for colony formation (*11*).

Protein sequencing (*16*) and cDNA transfection studies indicated that TGFγ2 is identical to 14-kDa galectin-1 (*17*), although purified TGFγ2 has no sugar-binding activity under non-reducing conditions, but shows potent mitogenic activity on A31 cells (*18*). Therefore, we temporarily named it tumor-derived galectin-1 (t-galectin-1). The native t-galectin-1 is a monomer and can be extracted by sonication without lactose under non-dissociative conditions.

Galectins have been found in a variety of species from invertebrates (*19, 20*) to mammals (*21*). These lectins are also involved in a variety of biological functions (*22-29*), many of which are ascribed to their common feature, a specific and reversible carbohydrate-binding activity (*25*). Structural aspects of studies on galectin-1 indicated that galectin-1 is a dimer, but disulfide bridges are not involved.

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Abbreviations: ASV, avian sarcoma virus; DMEM, Dulbecco's modification of Eagle's medium; DTT, dithiothreitol; EGF, epidermal growth factor; FBS, fetal bovine serum; MALDI-TOF; matrix-assisted laser desorption/ionization time-of-flight; NRK, normal rat kidney; TGF, transforming growth factor.

This type of lectin has free sulfhydryls and undergoes inactivation in the absence of a thiol-reducing agents (*30, 31*). These facts led us to examine the correlation between structure and function of t-galectin-1 and 14-kDa galectin-1.

In this study, we examined the role of thiol groups in the mitogenic activity and sugar-binding activiy of t-galectin-1. By the reduction of t-galectin-1 with 2-mercaptoethanol, mitogenic activity was decreased and the sugar-binding activity was detected. These reciprocal effects of the reducing agent suggest that the physiological functions of this bifunctional molecule depend on the conformational changes caused by the formation and breakage of these intramolecular disulfide bonds. To obtain further structural evidence of the relationship between mitogenic and sugar-binding activities of t-galectin-1, we have determined the localization of the disulfide pairing in t-galectin-1 and have shown that this sulfide bond is required for the appearance and maintenance of the mitogenic activity.

MATERIALS AND METHODS

Cells and Galectin-1—77N1 cells, a clonal isolate of ASV B77-transformed rat NRK (normal rat kidney) cells (*10*), were grown in Dulbecco's modification of Eagle's medium (DMEM) (Sigma Chemical, St. Louis, MO) supplemented with 5% fetal bovine serum (FBS) (Boehringer Mannheim, Mannheim, Germany). The 77N1 cells were scraped with a rubber policeman, collected by centrifugation at $450 \times g$ for 10 min after washing three times with saline, and stored at −20°C until use. BALB3T3 clone A31 cells were maintained in DMEM supplemented with 10% FBS. A31-1 cells that express rat galectin-1 were generated by stable transfection of rat galectin-1 cDNA into A31 cells using an expression vector described previously (*18*), and grown in DMEM supplemented with 5% FBS. Purified human placenta galectin-1 (*32*) and recombinant human galectin-1 (*33*) were a generous gift from Drs. K. Kasai and J. Hirabayashi, Teikyo University.

Preparation of Cell Extracts—Cells were sonicated in 10 mM Tris-HCl, pH 7.4, containing 1 mM phenylmethylsulfonyl fluoride (Sigma), and the homogenates were centrifuged at $100,000 \times g$ for 1 h. The supernatant was used as the cell extract and stored at −20°C until use.

Purification of t-Galectin-1—The details of the purification have been described elsewhere (*11*). Briefly, 77N1 cell extracts were fractionated by ion exchange column chromatography and the active fractions were precipitated by the addition of 50-95% ammonium sulfate. The precipitates were subjected to chromatofocusing separation followed by DEAE Sephadex A-25 column chromatography (Pharmacia LKB Biotechnology). Active materials recovered in the flow-through fractions were lyophilized and desalted with a PD-10 column (Pharmacia LKB Biotechnology) and concentrated prior to use for biological studies. For chemical characterization, a reverse phase column (μRPCC2/C18SC2.1/10) with the SMART system (Pharmacia LKB Biotechnology) was used for purification.

Treatment of t-Galectin-1 with 2-Mercaptoethanol—t-Galectin-1 (6.5 μg in 100 μl of 10 mM Tris-HCl, pH 7.4) was treated with 5 mM 2-mercaptoethanol overnight at 4°C. After treatment, samples were directly added to the DNA synthesis assay medium at the dose indicated in the legend to Fig. 4. The addition of 10% (v/v) of 5 mM 2-mercaptoethanol to the medium had no effect on the assay for DNA synthesis.

Assay for DNA Synthesis—A31 cells were seeded onto a 96-well microtiter plate (Millipore, Bedford, MA) at 3×10^4 cells per well in 100 μl of DMEM supplemented with 0.5% FBS and grown for 24 h. The test sample was then added to each well. After 16 h, the cells were pulse-labeled with 5 kBq [^{125}I]iododeoxyuridine (dUrd) (NEN Research Products, Wilmington, DE) per well for 4 h, and the rate of DNA synthesis was estimated in duplicate by measuring the acid-insoluble radioactivity.

Antiserum Preparation—Polyclonal rabbit antiserum was raised against a synthetic peptide corresponding to the COOH-terminal sequence 120-134 of rat lung galectin-1, MAADGDFKIKCVAFE (*17*). The peptide included Cys at the NH_2 terminal for conjugation to keyhole limpet hemocyanin (KLH) protein. The immunization schedule for the rabbit consisted of subcutaneous injections at several sites of KLH-coupled synthetic peptide as follows: (1) 2.5 mg in Freund's complete adjuvant (1 : 2, v/v) on day 0, (2) a boost of 2.0 mg of KLH-coupled synthetic peptide in Freund's incomplete adjuvant (1 : 2, v/v) on day 14, and (3) a further boost of 1.5 mg on day 28. Animals were bled after 2 weeks. The titer of the antiserum was determined by immunoblot analysis using cell extracts of 77N1 cells. This antiserum reacted with a single polypeptide (M_r 14,000) in extracts of 77N1 cells, and the antibody was designated as anti-gal14 antibody.

Immunoblot Analysis—Samples were reduced and were electrophoresed on 15-25% polyacrylamide minigels by the method of Laemmli (*35*), and then transferred to a polyvinylidene difluoride sheet (Immobilon; Millipore) by electrophoresis. The filter was blocked by incubation in milk-TBS (20 mM Tris-HCl, pH 7.4, containing 0.9% NaCl and 0.1% Tween 20) and subsequently treated with anti-gal14 antibody for 1 h, and further treated with a "Blotting Detection Kit" (Amersham Int. plc, UK). M_r values were determined by comparison with prestained protein standards (Rainbow markers RPN755, Amersham).

Immunoadsorption—The ability of the anti-gal14 antibody to react with t-galectin-1 was tested by immunoadsorption experiments. To prepare the immunoadsorbent, 500 μl of the anti-gal14 antibody or preimmune serum was mixed with 500 μl of 0.1 M sodium phosphate buffer, pH 7.1, containing 3 M NaCl and 100 μl of a slurry of protein A Sepharose CL-4B and rotated end-over-end overnight at 4°C. The reaction mixture was spun down and washed with 0.1 M sodium phosphate buffer five times. The IgG-gel slurry conjugate thus obtained was added to 5 μg of purified t-galectin-1 and the mixture was rotated end-over-end overnight at 4°C. The reaction mixture was centrifuged at $13,000 \times g$ for 10 min, and the resulting supernatant was assayed for DNA synthesis in BALB3T3 A31 cells as described. As a control, 100 μg of the immunizing peptide was also added to an identical sample.

Assay for Inhibition of Soft Agar Colony Formation—One milliliter of 0.7% Noble agar (Difco Laboratories, Detroit, MI) in DMEM with 5% FBS was poured as a basal agar layer in a 35-mm plastic plate. After the basal layer was solidified at room temperature, a top layer was made by pouring 1 ml of 0.35% agar in DMEM with 5% FBS containing 1×10^3 rat galectin-1 cDNA-transfected A31

cells (A31-1) with anti-gal14-IgG or control IgG onto the basal layer, and allowed to solidify at room temperature. The cultures were then incubated at 37°C in a humidified atmosphere of 5% CO_2 in air for one week and viable colonies were stained with 1 ml of iodonitrotetrazolium per plate overnight at 37°C (*34*). The colony efficiency was calculated for each sample by counting the number of colonies with a diameter greater than 0.05 mm under a microscope.

Detection of Free Sulfhydryl Groups in t-Galectin-1 and Recombinant Human 14-kDa Galectin-1—Purified t-galectin-1 (500 ng) and recombinant human 14-kDa galectin-1 (250 ng) were treated with dithiothreitol (2.5 mM) for 2 h at 50°C. Both samples as well as non-reduced controls were carboxymethylated with [^{14}C]iodoacetamide (4.7 nmol; 2 kBq) (NEN Research Products) for 20 min at room temperature, and electrophoresed on 15-25% polyacrylamide gels. The radioactivity incorporated into the 14-kDa band was measured by radioluminography using a Fujix bioimage analyzer, BAS2000 (Fuji Photo Film).

Asialofetuin Column Chromatography—An asialofetuin column was prepared by the coupling of asialofetuin to CNBr-activated Sepharose 4B (Pharmacia LKB Biotechnology), and equilibrated with phosphate-buffered saline (PBS) with or without 10 mM 2-mercaptoethanol. Native and 10 mM 2-mercaptoethanol-treated purified t-galectin-1 was passed through an affinity column (2-ml bed volume, 4°C). After the unbound materials were washed out of the column with PBS, the absorbed materials were eluted with PBS containing 100 mM lactose. The flow-through fractions and lactose-eluted fractions were examined by immunoblot analysis with the anti-gal14 antibody.

Proteolytic Cleavage and Dithiothreitol Treatment of t-Galectin-1—For reducing conditions, t-galectin-1 was treated before enzymatic digestion with dithiothreitol (molar ratio 1 : 50) under nitrogen gas at 50°C for 4 h. TPCK-treated trypsin (Sigma) was added to 100 μl of 0.1 M sodium bicarbonate buffer (pH 8) containing intact or dithiothreitol-treated t-galectin-1 in a substrate-to-enzyme ratio of 20 : 1 (w/w) and incubated at 37°C under nitrogen gas for 18 h. The reaction was abolished by the addition of glacial acetic acid and the sample was lyophilized. The lyophilized sample was dissolved in 2 : 1 0.1% aqueous TFA/acetonitrile prior to use.

Mass Spectrometry—The intact t-galectin-1 as well as its enzymatic degradation products were analyzed by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF) using a Vision 2000 (Finnigan MAT, San Jose, CA) equipped with an N_2-laser (337 nm, 3 ns pulse duration). Laser beam irradiances were in the mid 10^6 W/cm^2 range, about 2-3 times higher than the threshold for obtaining ions from the mixture of the digest. Ions were accelerated to a kinetic energy of 5 keV and postaccelerated to 12 keV (25 keV for the intact t-galectin-1) prior to detection. All spectra were taken in the reflection mode, and no deflection of low mass ions was used. The spectra represent the accumulation of 40 single laser shots. They were calibrated externally by a standard sample (bovine insulin, 5,733.6 Da, or equine cytochrome *c*, 12,360.1 Da) that was prepared on the same target. The matrix used was 2,5-dihydroxybenzoic acid (DHB) dissolved in a 2 : 1 mixture of 0.1% aqueous TFA and acetonitrile at a concentration of 10 mg/ml. For MALDI analysis, 1 μl of the sample solution was mixed with an equal volume of the matrix solution on the target.

RESULTS

Characterization of Antibody against Rat Galectin-1 (Anti-gal14 Antibody)—The reactivities of the purified 14-kDa t-galectin-1 molecule, human placenta galectin-1, and cell extracts from 77N1 and A31-1 cells with rabbit anti-gal14 antibody were assayed by an immunoblotting technique. As shown in Fig. 1, a 14-kDa polypeptide band was observed in every treated sample (Fig. 1). The antiserum also reacted with bands corresponding to proteins of approximately 30 and 42 kDa, which might represent dimer and trimer of human placenta galectin-1, as shown in Fig. 1, lane 2. The anti-gal14 antibody has no species specificity and reacted with both rat and human galectin-1. The immunoblotted 14-kDa galectin-1 could be displaced in the presence of an excess amount of the synthetic peptide that was used to induce anti-gal14 antibody. Preimmune rabbit serum failed to react with this band (data not shown). The same result was obtained with the antiserum raised against purified t-galectin-1 (data not shown). These results suggest that t-galectin-1 and galectin-1 are immunologically identical.

Next we examined whether anti-gal14 antibody could inhibit the t-galectin-1-induced DNA synthesis activity of A31 cells. When anti-gal14 antibody conjugated to Protein A Sepharose CL-4B was incubated with t-galectin-1, DNA synthesis was reduced to 30% of that of the control in which t-galectin-1 was incubated with Protein A Sepharose CL-4B gel slurry alone (Fig. 2). Preimmune serum conjugated to Protein A Sepharose CL-4B did not reduce the mitogenic activity of t-galectin-1. The addition of the synthetic peptide, which was used as immunogen, did not interfere with the mitogenic activity of t-galectin-1 in the DNA synthesis assay.

The overexpression of galectin-1 by BALB3T3 A31 cells

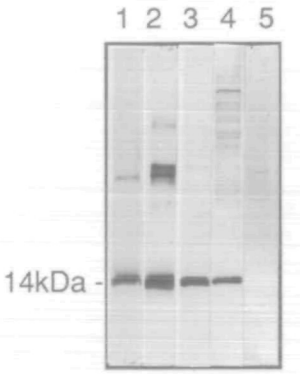

Fig. 1. Specificity of rabbit anti-14-kDa galectin serum. Antiserum was raised against a synthetic peptide fragment of the 14-kDa rat galectin-1 as described in "MATERIALS AND METHODS." Samples were reduced prior to SDS-PAGE on 15-25% polyacrylamide minigels. The polypeptides were electrotransferred to Immobilon membranes and immunoblotted with anti-gal14 antibody. 1, purified t-galectin-1 from 77N1 cells (300 ng), 2, human placenta galectin-1 (300 ng), 3, cell extract of A31-1 cells (20 μg); 4, cell extract of 77N1 cells (20 μg); 5, the same as lane 4 treated with the synthetic peptide (10 μg), corresponding to the C-terminal 15 amino acids of rat galectin-1 that was used for raising the antiserum, during antiserum treatment.

induces a transformed phenotype, which shows anchorage-independent growth in soft agar and tumorigenicity in nude mice (*16*). We examined whether anti-gal14 antibody exerts any effect on the soft agar colony formation of A31-1 cells. After 7 days of treatment with the anti-gal14 antibody, A31-1 cells showed about 40% inhibition of the anchorage-independent growth (Fig. 3). Under the same conditions, there was no such reduction in A31-1 cells treated with preimmune serum-IgG. These data suggest that the anti-gal14 antibody neutralized the action of the synthesized rat galectin-1, which might be secreted from the cells where it could cause autocrine-like growth stimulation of tumor cells.

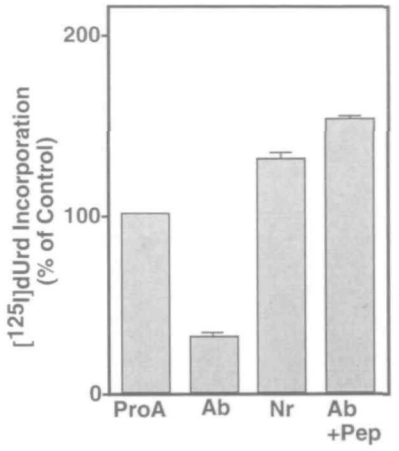

Fig. 2. **Adsorption of t-galectin-1 by anti-gal14 antibody as shown by DNA synthesis activity.** This was determined on the supernatant obtained by preincubation of the samples with antiserum or preimmune serum conjugated with Protein A ProA, t-galectin-1 adsorbed by Protein A Sepharose CL-4B only; Ab, t-galectin-1 adsorbed by anti-gal14 antibody; Nr, t-galectin-1 absorbed by normal serum, Ab+Pep, t-galectin-1 absorbed by anti-gal14 antibody plus peptide (immunoadsorption of t-galectin-1 in the presence of excess peptide representing amino acids 120-134 of rat galectin-1)

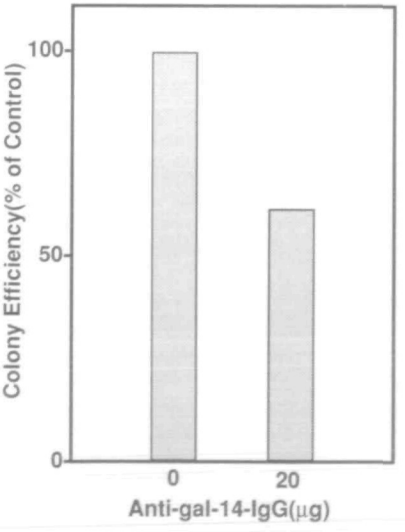

Fig. 3. Inhibition of soft agar colony formation by anti-gal14 antibody. The soft agar colony assay was performed as described previously (*11*). Briefly, A31-1 cells (1×10^3 cells/plate) were grown in soft agar medium with anti-gal14 antibody-IgG at the indicated dose or with preimmune serum IgG for a week and colony efficiency was determined The data are expressed as a percent of preimmune control IgG (20 μg/plate).

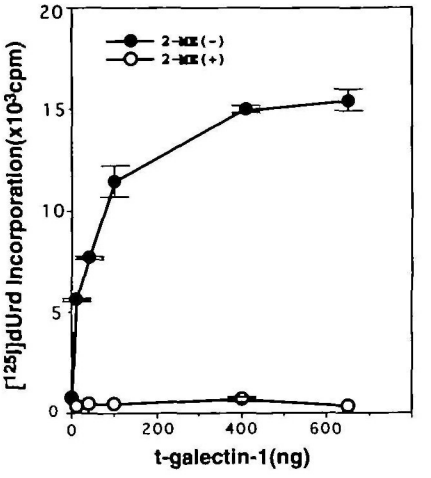

Fig 4 **Effect of 2-mercaptoethanol (2-ME) on the DNA synthesis activity of t-galectin-1.** Dose-response curve of the t-galectin-1 dependent DNA synthesis in 3T3 target cells (3×10^4 cells). The target cells were treated with 2-ME-treated or untreated t-galectin-1 at the indicated dose and were then assayed for [^{125}I]dUrd incorporation as described in "MATERIALS AND METHODS." The data represent the averages of duplicate determinants The incorporation of labeled material by a 5% FBS-stimulated well was 18,539±308 cpm.

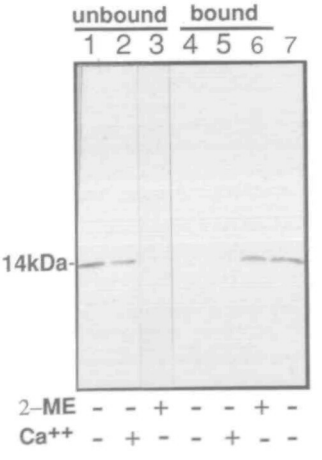

Fig 5 **Sugar binding activity of t-galectin-1 in the presence or absence of 2-mercaptoethanol.** PBS 100 μl containing 1 mg of t-galectin-1 was incubated in the absence or presence of 10 mM of 2-mercaptoethanol (2-ME) for 1 h at 4°C. The samples were then applied to an asialofetuin-Sepharose 4B column (0 7×3 cm), pre-equilibrated with PBS alone or PBS containing 10 mM 2-mercaptoethanol Native or 2-mercaptoethanol-treated t-galectin-1 solution was then added to the column pretreated with either PBS alone or 2-mercaptoethanol, respectively. Unbound fractions were collected and the column was washed in PBS until OD_{280} was less than 0.002. Bound materials were eluted with 0.1 M lactose. Unbound and lactose-eluted fractions were electrophoresed and immunoblotted as described in "MATERIALS AND METHODS." 1-3 were unbound, 4-6 were bound 1 and 4, no 2-mercaptoethanol, 2 and 5, with Ca^{2+}; 3 and 6, with 2-mercaptoethanol; 7, unbound fraction from fetuin-Sepharose column.

Intramolecular disulfide bridges are indispensable for transforming growth factor activity.

Galectin-1 is known to require reducing agents to maintain its hemagglutination as well as sugar binding activities, neither of which requires divalent cations. To examine the role of thiol groups, we first examined the effect of thiol reagents, such as 2-mercaptoethanol, on the mitogenic activity of t-galectin-1. Figure 4 shows that the treatment of t-galectin-1 with 2-mercaptoethanol resulted in a complete loss of its mitogenic activity, while untreated t-galectin-1 enhanced DNA synthesis activity in a dose-dependent manner. Previously, we reported that β-galactoside inhibits the hemagglutination activity of this molecule under reducing conditions, but not its mitogenic activity (*18*).

In order to analyze further the reciprocal relationship of these two activities, we examined the sugar binding activity of purified t-galectin-1 by means of an asialofetuin binding assay. In the absence of 2-mercaptoethanol, t-galectin-1 did not bind to the asialofetuin column, as shown in Fig. 5. In contrast, in the presence of 2-mercaptoethanol, t-galectin-1 was bound to the column and eluted with the lactose-containing buffer. The eluate was immunoblotted by anti-gal14 antibody in the presence of 2-mercaptoethanol, giving a 14-kDa band corresponding to galectin-1. Using the asialofetuin column, we also found that t-galectin-1 did not require Ca^{2+} for sugar binding as do other galectins. These results strongly suggest that under reducing conditions, t-galectin-1 has a diminished mitogenic activity, but gains a sugar binding activity, and that the mitogenic activity of t-galectin-1 is not mediated *via* lectin activity. It is very likely that these two contrasting activities of t-galectin-1 can be ascribed to different molecular determinants.

To clarify whether the cysteinyl residues exist in a free or linked state in native t-galectin-1, we measured the sulfhydryl content in the t-galectin-1 molecule, using a radiolabeled sulfhydryl reagent, [^{14}C]iodoacetamide. No significant labeling of native t-galectin-1 was observed under non-reducing conditions, indicating an absence of free sulfhydryl groups at least on the surface of the molecule (Fig. 6). Upon sample reduction, however, the 14-kDa monomeric form of t-galectin-1 was strongly labeled with [^{14}C]iodoacetamide, suggesting that all the disul-

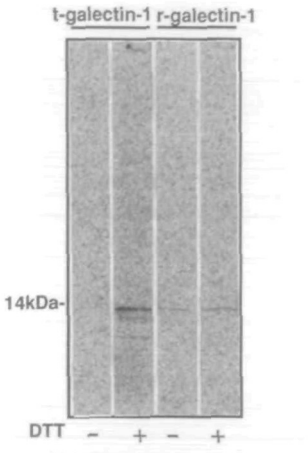

Fig. 6. **Incorporation of [^{14}C]iodoacetamide, a sulfhydryl reagent, into non-reduced or reduced t-galectin-1 and recombinant human galectin-1 (r-galectin-1).** The reaction products were separated by SDS-PAGE and visualized by autoradiography. For autoradiography, the dried gels were exposed to a Fuji Film imaging plate in the BAS2000 bioimage analyzer for 2 days DTT, dithiothreitol

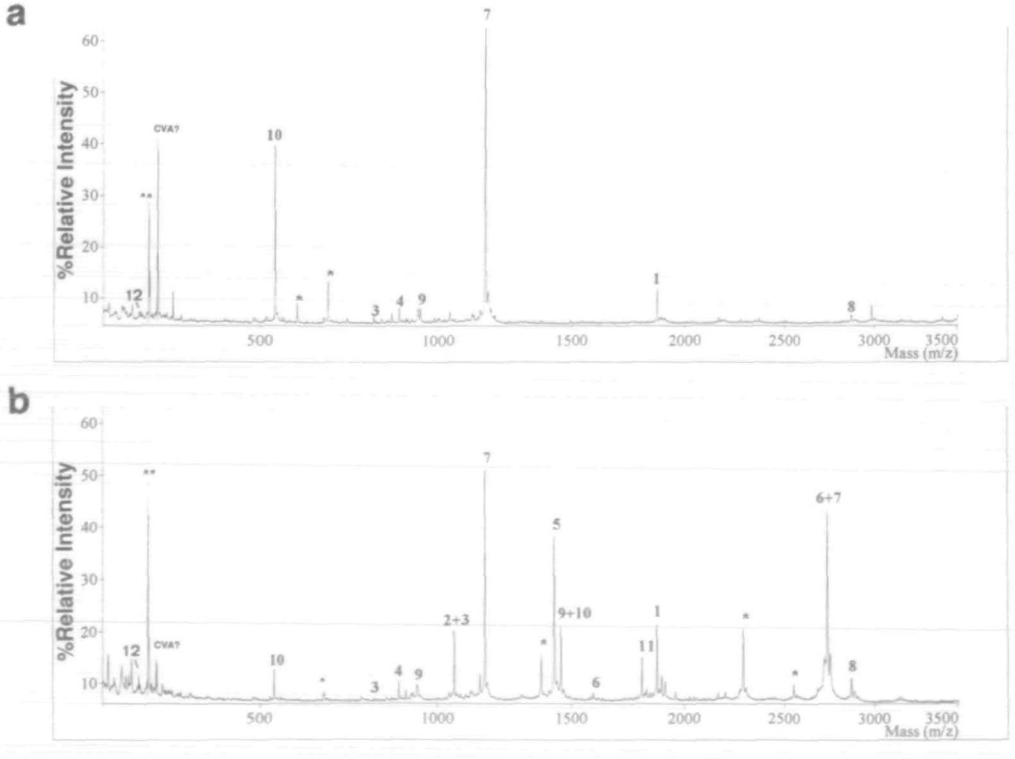

Fig. 7 **Positive ion MALDI-TOF mass spectra of tryptic digests of t-galectin-1.** a, the MALDI spectrum of the tryptic digest of the native disulfide-linked peptide; and b, the MALDI spectrum of the same digest after reduction with dithiothreitol as described in "MATERIALS AND METHODS." Numbers represent peptide fragments described in Table I (T omitted). *, and **, signals from trypsin and other components of the reaction mixture, such as matrix, respectively.

TABLE I. **Average molecular mass [$(M+H)^+$ ions] in MALDI-TOF spectra of trypsin digested t-galectin-1 either untreated (native) or treated with DTT.** Cys containing peptides are marked by an (*). For the theoretical calculations of the peptides all Cys have been assumed to contain free sulfhydryl groups (no disulfide bridges).

Fragment sequence	Theor. calculation $(M+H)^+$ (Da)	Observed peaks		Peptide assignment
		Native (Da)	DTT treated (Da)	
1-18	1,873.3	1,872.1	1,873.9	T1*
19-20	274.2	Overlapping with matrix peak		T2
21-28	800.9	801.4	801.0	T3
29-36	878.1	878.5	878.6	T4
37-48	1,430.6	—	1,431.5	T5*
49-63	1,591.8	—	1,592.8	T6*
64-73	1,165.2	1,166.1	1,165.9	T7
74-99	2,873.3	2,873.2	2,873.5	T8*
100-107	943.1	943.4	943.3	T9
108-111	533.6	534.2	533.9	T10
112-127	1,804.1	—	1,804.3	T11
128-129	260.4	260.3	260.3	T12
130-134	568.6	—	—	T13*

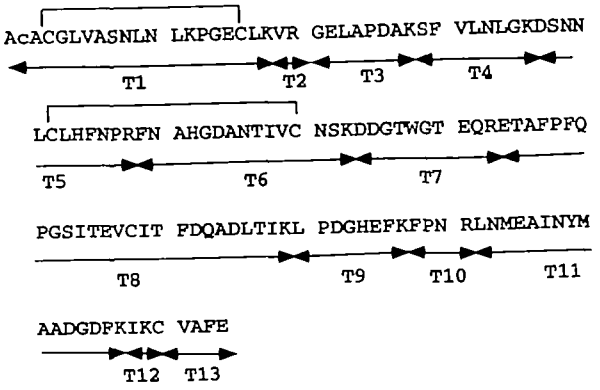

Fig. 8. **Amino acid sequence of rat lung 14-kDa lectin (*17*) (galectin-1).** The tryptic peptides are designated as T1 to T13. The N-terminal alanine is acetylated, as determined in this study.

fide bonds in the molecule were broken and easily labeled. On the other hand, recombinant human galectin-1, which has a lectin activity, was labeled under both reducing and non-reducing conditions, indicating that this molecule contains free sulfhydryl groups. In these experiments, the labeling of recombinant human galectin-1 was weaker under non-reducing conditions than under reducing conditions, possibly due to some cysteinyl residues buried inside the molecule (*17*).

MALDI-TOF Mass Spectrometry—The presence of intramolecular disulfide bridges was also confirmed by mass spectrometric analysis. Figure 7 shows MALDI-TOF spectra of the tryptic digests of native and DTT-treated t-galectin-1. Trypsin cleaves at the C-terminal side of lysine and arginine, unless they are not followed by a proline. As the sequence of rat galectin-1 (*17*) contains 8 lysine (the lysine at position 12 is linked to proline) and 4 arginine residues, 13 fragments are expected if there is no intramolecular disulfide bridge (Fig. 8). Table I gives an overview of the theoretical molecular weights of the fragments [$(M+H)^+$ ions] and the peaks that were observed in the respective MALDI-TOF spectra. t-Galectin-1 contains 6 Cys in the fragments T1, T5, T6, T8, and T13. As can be seen, for fragment T1 a mass increase of 1.8 Da was measured after DTT-treatment. T1 contains two Cys at the positions 2 and 16. Therefore, upon the reduction by DTT two free sulfhydryl groups were created, revealing the Cys2-Cys16 linkage in the native state of t-galectin-1. The fragments T5 and T6 could not be observed in the case of the native sample. Further, no signal of a combination of both peptides (3,019.28 Da, if bound by a disulfide bridge only, or 3,001.28 Da, if additionally linked by a peptide bond) was detected. Although the exact reason is not known, it can be assumed that the hydrophobicity of fragment T6 is responsible for the suppression of the signal of the combined peptides. However, after the reduction of the sample both fragments could clearly be observed in the MALDI-TOF spectrum; peptide T6 showed a lower intensity, as expected. This indicates that a disulfide bridge exists between Cys42 (T5) and Cys60 (T6) that was cleaved by the DTT-treatment. Fragment T8 was detected for both the native and the reduced sample, therefore it contains a free sulfhydryl group. Fragment T13 was not observed for either digest. However, instead a signal at about 290 Da was detected in both cases, close to the amino acid sequence of CVA [T13 without FE, $(M+H)^+ = 568.6$ Da $- 276.5$ Da $= 292.1$ Da]. Moreover, when analyzing the intact, entire t-galectin-1, a molecular weight was measured that corresponded to the loss of the last two amino acids (14,472.5 Da) from the theoretically expected sequence (14,763.7 Da, calculated on the assumption that two disulfide bridges exist). Although the measured masses do not completely agree with the theoretical molecular weights, both experimental findings indicate that the last two amino acids in t-galectin-1 are either cleaved or truncated. As the Cys130 in T13 has no counterpart left in the sequence to be linked to, it can be concluded that it contains a free sulfhydryl group. The suggestion of two disulfide bridges out of 6 cysteine residues in t-galectin-1 is also supported by the mass difference of the intact native and the DTT-treated sample, measured in successive MALDI-TOF analyses with both samples side by side on the same target. The measured average molecular weights of 14,472.5 Da for the native and 14,476 Da for the reduced t-galectin-1 differ by a mass of 3.5 Da, corresponding to the creation of 4 free sulfhydryl groups after reduction by DTT.

Generally, when analyzing complex mixtures such as enzymatic digests by MALDI, differences in the properties of the fragments such as hydrophobicity and inclusion into the matrix crystals influence the observed relative intensities of the fragments (as the illustrated MALDI spectra actually reflect). Therefore, some tryptic fragments might be difficult to detect or way not appear in all spectra. Especially when working at lower sample concentrations, some fragments might be suppressed. In the presented spectra, for example, the peptide T11 could be observed with a relatively low signal intensity in one of the DTT-treated samples only. Most probably this is due to an occasionally slightly higher concentration or better purification of the sample. However, since the theoretical MW was confirmed for T11 by the measurement, it is very unlikely that its amino acid sequence differs from that assumed. It does not contain a Cys. In the case of the peptide T5, however, a suppression of this single peptide in the untreated spectrum can be excluded because of its high signal intensity in the DTT-treated spectrum. It is very

likely that T5 is in fact not observed in the untreated spectrum because it is bound to the quite hydrophobic fragment T6 by a disulfide bridge.

DISCUSSION

TGFγ2 (t-galectin-1) was first observed in culture medium conditioned by ASV-transformed rat cells, 77N1 (*10*). t-Galectin-1 is a polypeptide growth factor that shows significant structural homology with galectin-1 derived from rat lung (*17*). Galectin-1 is a lectin whose only known function is to bind galactosides. The sequence identity of t-galectin-1 and rat lung galectin-1 clearly indicates that they are identical or closely related proteins. Previously, we also postulated that the stable expression of an animal lectin, rat galectin-1, in mouse BALB3T3 fibroblast cells resulted in the acquisition of a transformed phenotype (*18*). Galectins are the most common of the vertebrate lectins that are dimeric proteins (*36, 37*). They have neither hydrophobic signal sequences, nor intramolecular disulfide bonds, even if multiple cysteine residues are present.

In this study, we have demonstrated that intramolecular disulfide bridges are indispensable for the transforming growth factor activity of t-galectin-1, and that the protein has both TGF and lectin activities. We also showed that the binding of anti-gal14 to galectin-1 inhibits its growth stimulating activity, suggesting that the antibody inhibits the access of galectin-1 to membrane receptors and blocks the signal transduction for DNA synthesis. Furthermore, the addition of anti-gal14 antibody to a culture of A31-1 cells resulted in the prevention of colony formation in soft agar, a characteristic that is usually exhibited by transformed and tumorigenic cells. Lotan *et al.* (*38, 39*) suggested that galectin-1 may be related to the colony-forming activity of certain tumor cells in soft agar. Though galectin-1 has no signal sequence, several researchers have reported that it may be secreted and found extracellularly (*40-43*) where it subsequently activates a mitogenic pathway and thus autocrine stimulation of cell growth. Storage of galectin-1 requires the addition of reducing agents to maintain the molecule's hemagglutination activity (*44*). Clerch *et al.* (*17*) reported that Cys2 of rat 14-kDa lectin was the most reactive among 6 cysteine residues, suggesting that Cys2 is exposed on the surface of the lectin molecule and is most readily oxidized. They also suggested that the thiol-reducing reagent added for stabilization of the lectin seems to protect the native conformation against random formation of disulfide bonds. In addition, the TGF activity of t-galectin-1 is completely abolished by treatment with 2-mercaptoethanol. This in turn implies that the formation of a disulfide bond is essential to the functioning of this molecule. Furthermore, the disulfide linkages in t-galectin-1 have been examined by a combination of reduction with dithiothreitol and iodoacetamide radiolabeling. Free cysteinyl residues were not detected in the native form of t-galectin-1 by carboxymethylation, suggesting that intramolecular disulfide bonds are indispensable for mitogenic activity. Regarding the sugar binding property of t-galectin-1, we could not detect any binding to an asialofetuin column under non-reducing conditions. This might be related to the fact that it was purified as a monomer. Intramolecular disulfide bridges might prevent the formation of the dimer (*36, 37*).

There are at least two possible explanations for the present observation that the TGF and lectin activities are mutually independent. One is that galectin-1 has a single conformation that requires different molecular determinants for each activity. The other is that the galectin-1 protein exists in two distinct conformations that are mutually convertible. For example, galectin-1 may exist as a monomer for TGF activity and a dimer for lectin activity. The difference in the sensitivity to reducing agents may support the latter explanation that galectin-1 exists in two distinct molecular conformations that depend on inter- or intramolecular disulfide bridges. It is tempting to speculate that galectin-1 is "stored" in a latent state bound to galactoside in normal cells and that some physiological alterations of the cells lead to the "release" of the TGF form of the molecule. These alterations may include an increase in t-galectin-1 protein synthesis, a secretion of, or a decrease in the number of galectin-1 binding galactoside molecules, or changes in galactoside-mediated cell-cell interactions. Such a model can explain the abundant expression of galectin-1 in a variety of tissues and cells, where it potentially acts as a TGF.

The intramolecular disulfide bond was also confirmed by MALDI-TOF mass spectrometry of tryptic digests of purified t-galectin-1. Two of the disulfide linkages were confirmed by these studies. The first 4 cysteine residues are joined as Cys2-Cys16 and Cys42-Cys60, leaving the 5th and 6th Cys residues unpaired. In addition, the anti-gal14 antibody reacted equally with t-galectin-1 and galectin-1, suggesting that the C-terminal cysteine residue (Cys130) might be free. These results, combined with the results from studies of the effects of reducing agent on the TGF activity of t-galectin-1, indicate that the two disulfide-linked loops are essential for the mitogenic activity of t-galectin-1.

It has been reported that the oxidation of Cys residues of human 14-kDa lectin, which prevents them from being involved in disulfide formation, could be a key process in the inactivation of this molecule. In the oxidative inactivation of bovine galectin-1, the N-terminal Cys2 and the C-terminal Cys130 are turned out to form an intramolecular disulfide bond, and this causes a conformational change in the molecule, resulting in a substantial loss of sugar binding activity (*45*). It is widely accepted that disulfide bonds in the majority of immunoglobulin (Ig)-like domains stabilize the final conformation and thus are essential to the function (*46*), as they are in t-galectin-1. t-Galectin-1, which has been shown to behave as a TGF, is stabilized by a putative Cys-Cys disulfide bond.

The amino terminal amino acid of t-galectin-1 is blocked. The mass of the tryptic fragment 1-18 (T1), determined by MALDI-TOF mass spectrometry was 1,871.1 Da. The calculated molecular mass of the peptide ion without a blocking group is 1,828.26 Da; the mass difference of 42.8 Da corresponds quite closely to an acetyl group. We conclude that the N-terminal alanine is blocked by an acetyl group, as has been reported for other galectin-1s (*17, 45*).

Diverse carbohydrate-binding proteins of cell surfaces, the cytoplasm, and extracellular matrices are important in the specification of cell recognition and cell-ligand interactions (*25, 37, 47, 48*). The presence of a bifunctional galectin-1 protein may also explain the mechanism of

contact inhibition. When normal cells grow, galectin-1 behaves as a growth factor. However, when cells come in contact with each other, the galectin-1 may be transformed into a lectin with a simultaneous loss of TGF activity. The involvement of cell surface glycoproteins in the inhibition of the growth of human fibroblast cells has been suggested (*49*). Wieser and Oesch (*50*) have shown that the inhibition is effective only when the terminal galactose residue is present, which is quite consistent with the idea that t-galectin-1 stimulates cell growth and that the stimulation is regulated by the extent of cell-cell contacts. The fact that transformed cells express higher levels of galectin-1 than untransformed cells (*28*) is consistent with our overexpression experiments (*18*).

Autocrine or paracrine mechanisms for self-stimulation would confer obvious selective growth advantages on very early embryonic cells and could help to account for the explosive growth and multiplication of cells that occur during the earliest stages of embryogenesis. The sugar-binding latent form of galectin-1 has the ability to tightly and spatially control TGF activity, if galectin-1 is intracellularly reduced to prevent its acting as a TGF, and also if extracellular secretion makes disulfide bond formation possible, with the resulting expression of TGF activity.

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