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Quantification of Galectin-7 and Its Localization in Adult Mouse Tissues[1]

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We developed a method to quantify galectin-7 extracted from adult mouse tissues by Western blot analysis. More than 0.5 ng of galectin-7 per mg of tissue was detectable by this method. The amounts of galectin-7 in tissues were determined as follows: skin, 62 ± 3 ng/mg; esophagus, 23 ± 8 ng/mg; stomach, 18 ± 6 ng/mg; anus, 13 ± 1 ng/mg; and tongue, 12 ± 2 ng/mg. This indicates that galectin-7 production coincides with the degree of stratification of the epithelia. Interestingly, we also detected significant amounts of galectin-7, 5.9 ± 1.4 and 2.7 ± 0.6 ng/mg, in the trachea and ovaries, respectively. Moreover, we found that galectin-7 is localized in the pseudostratified epithelium of the trachea and stromal epithelium of the ovaries by immunohistochemistry. Thus, galectin-7 protein might be produced primarily in stratified epithelia, but also in some wet epithelia, and plays a unique role in cell-mucus contact, or the growth of ovarian follicles.

Key words: galectin family, mouse galectin-7, ovarian follicle, stratified epithelium, tracheal epithelium.

Galectins comprise a family of animal lectins characteristically containing at least one common carbohydrate recognition domain (CRD) with an affinity for the β-galactosides of glycoconjugates (*1–3*). They are soluble, 14–36 kDa proteins, and at least 12 members (galectins-1 to -12) have been cloned in mammals so far (*4–6*). Among them, galectins-1, -2, -5, -7, -10, and -11 belong to the 14 kDa subfamily, which contains one CRD exhibiting sequence similarity of about 20–50% at the amino acid level. Galectin-1 is ubiquitously expressed in mammalian tissues and able to form a homodimer, and has been shown to mediate cell–cell and cell–matrix interactions, and to influence the growth or apoptosis of various cells (*2, 7*). It is localized not only in the intracellular cytoplasm or nucleus, but also in extracellular spaces such as the cell surface or extracellular matrix. Its biological functions are considered to be related to immunity, development, cellular adhesion and neoplastic transformation. Although other members of the 14 kDa subfamily, such as galectins-2, -5–7, and -10, have been identified in restricted tissues (*8–12*), their functions are largely unknown.

Galectin-7 was first cloned from human epidermis during the screening of molecular markers for keratinocyte differentiation (*10*). However, galectin-7 is primarily distributed in stratified epithelial cells of the epidermis with no relation to the differentiation of keratinocytes, hair follicles, esophagus, tongue, lips, corneas, or Hassal corps of the thymus (*11, 13*). These facts suggest that galectin-7 is a specific marker of all types of stratified epithelia. Moreover, the expression of galectin-7 mRNA has also been found in developing mouse embryonic gonads, both ovaries and testes (*14*). However, the function of galectin-7 in embryonic gonads is unclear, and there has been no report concerning the quantitative analysis of galectin-7.

In the present study, we determined the amounts of galectin-7 protein produced in adult mouse tissues by Western blot analysis. Also, we clarified the expression of galectin-7 in tracheal and ovarian epithelia by immunohistochemistry.

MATERIALS AND METHODS

Animals, and the Preparation of Mouse and Human Galectin-7—Eight-week-old ddN mice were obtained from the Experimental Animal Center of Kagawa Medical University. Mouse galectin-7 was purified from skin by affinity chromatograpy on a lactosyl-agarose column. Briefly, mouse skin (after removing hair) was homogenized in a 5-fold volume of 10 mM Tris–HCl (pH 7.2), 0.15 M NaCl, 2 mM EDTA, 1 mM DTT, and 5 mM benzamidine-HCl (extraction buffer) in a Polytron homogenizer. The homogenates were centrifuged at 25,000 rpm for 30 min in a Hitachi Ultra centrifuge 55P-72. The resulting supernatants were applied directly to a lactosyl-agarose column (2 ml; Seikagaku, Tokyo), and washed extensively with extraction buffer containing 0.01% CHAPS, but not benzamidine-HCl. The proteins absorbed on the affinity resin were eluted with 10 mM Tris–HCl (pH 7.2), 0.15 M NaCl, 1 mM EDTA, 1 mM DTT, and 0.01% CHAPS containing 200 mM lactose. All steps were performed at 4°C.

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Human galectin-7 was prepared as a recombinant protein in *Escherichia coli* cells. Human galectin-7 was expressed as a glutathione S-transferase (GST)-fusion protein by the method described previously (*15*). The GST-fusion galectin-7 was purified on a glutathione-Sepharose column (Amersham Pharmacia Biotech), and the eluate was digested with thrombin. The resulting GST-deleted galectin-7 was further purified by lactosyl-agarose affinity chromatography.

Antiserum—Antiserum was raised in Japanese white rabbits using recombinant human galectin-7 (rhgalectin-7). The rabbits were subcutaneously immunized with 200 μg of rhgalectin-7 emulsified in Freund's complete adjuvant (Difco Laboratories) every 2 weeks. After the fourth injection, the rabbits were bled, and the serum was stored at −80°C until use.

Preparation of Tissue Extracts, SDS-PAGE, and Western Blot Analysis—To determine the conditions for the efficient extraction of galectin-7, mouse skin was homogenized in 10 volumes of 80% methanol/20% 20mM Tris-HCl (pH 7.2), 0.15 M NaCl (80% methanol–20% TBS), TBS, and TBS/100 mM lactose (TBS-Lactose) or TBS/100mM lactose/1% Triton X-100 (TBS-Lactose-Triton) in a Polytron homogenizer. Soluble and insoluble fractions were obtained by centrifugation at 15,000 rpm for 10 min. The pellets were solubilized in TBS containing 2% SDS and 5% glycerol by vortexing and heating at 95°C for 5 min. The supernatants were treated with 2% SDS (final concentration) and heated at 95°C for 5 min.

For determination of the tissue disribution of galectin-7, mouse tissues were fixed immediately after collection in ice-cold 80% methanol–20% TBS for 90 min, and then homogenized in 10 volumes of 80% methanol–20% TBS in a Polytron homogenizer. The insoluble total extracts were collected by centrifugation at 10,000 rpm for 10 min. The pellets were solubilized in TBS/2% SDS/5% glycerol, as described above.

Pure galectin-7 and mouse tissue extracts were subjected to 12.5% SDS-PAGE under reducing conditions. The proteins were electrically transferred to PVDF (polyvinylidene difluoride) membranes (Immobilon; Millipore), and the membranes were blocked with 5% nonfat milk-TBS for 1 h. Then, the membranes were incubated with the rabbit antiserum against galectin-7 at 1:200 in 1% nonfat milk-TBS for 12 h, and washed three times with 1% nonfat milk–TBS for 10 min. After incubation with the second antibody (peroxidase-conjugated anti–rabbit IgG; Amersham Pharmacia Biotech) in 1% nonfat milk–TBS for 1 h, the membranes were washed twice with 1% nonfat milk–TBS and then twice with TBS for 10 min each. The second antibody was detected by means of a chemiluminescence method using an ECL kit (Amersham Pharmacia Biotech).

Immunohistochemistry—Mouse tissues were fixed in 10% neutral-buffered formalin and embedded in paraffin for immunohistochemistry. Representative tissue sections were incubated for 30 min with 0.3% hydrogen peroxide in methanol to quench endogenous tissue peroxidase activity. Immunostaining was performed using anti–rhgalectin-7 antiserum (1:500 dilution). After overnight incubation at 4°C, the sections were washed and treated with biotinylated secondary anti–rabbit IgG, followed by streptavidin coupled to biotinylated peroxidase, according to the manufacturer's instructions (Nichirei, Tokyo). The immunohistochemical reactions were visualized using diaminobenzidine as a chromogenic peroxidase substrate. Sections were counterstained with hematoxylin after immunostaining.

RESULTS

Quantification of Mouse and Human Galectin-7 by Western Blot Analysis—We prepared rabbit antiserum against rhgalectin-7, and examined its specificity for galectin family members by Western blot analysis. Only weak or no cross-reactivity of the antiserum was observed on X-ray film by staining with ECL systems for other rhgalectins, *i.e.* -1, -2, -3, -4, -8, -9, and -10 (Fig. 1).

Next, the reactivity of the antiserum was quantitatively compared between native mouse galectin-7 and rhgalectin-7. Both proteins, *i.e.* human and mouse galectin-7, were subjected to SDS-PAGE and blotted onto PVDF membranes after electrophoresis. The membranes were treated with anti–rhgalectin-7 antiserum, and then the protein blots of galectin-7 were visualized as shown in Fig. 2A. Then, the staining intensities of the visualized blots were integrated using NIH image software. As a result, linear relationships were found in the semi-log plots between the amounts of both galectins-7 and the relative staining intensities, as shown in Fig. 2B. Moreover, the intensity curves for both mouse and human galectin-7 corresponded closely in the range of 0.25 to 20 ng.

Expression of Galectin-7 Protein in Adult Mouse Tissues—To extract galectin-7 efficiently from adult mouse tissues, we examined the conditions for extraction using mouse skin. Total skin proteins were prepared by 2% SDS extraction of precipitates after homogenization in 80% methanol–20% TBS. Soluble and insoluble proteins were divided into supernatant and precipitate fractions after homogenization with TBS, TBS-Lactose, or TBS-Lactose-Triton. Then, galectin-7 was examined in each fraction by

Fig. 1. **Specificity of rabbit anti–human galectin-7 antiserum for galectin family proteins.** Recombinant human galectins (rhgalectins) -1, -2, -3, -4, -8, -9, and -10 were prepared by the same method used to prepare rhgalectin-7 as described under "MATERIALS AND METHODS." The galectin proteins were subjected to SDS-PAGE (12.5% acrylamide gel) as follows: a, galectin-1 (500 ng); b, galectin-2 (500 ng); c, galectin-3 (500 ng); d, galectin-4 (500 ng); e, galectin-7 (10 ng); f, galectin-8 (500 ng); g, galectin-9 (500 ng); h, galectin-10 (500 ng/lane). After SDS-PAGE, the proteins were electrically blotted onto a PVDF membrane. The blots of the galectin family proteins were treated with rabbit anti–rhgalectin-7 antiserum, and the immunological cross-reactivity of the antiserum among galectin family members was examined by staining with an ECL system. Only a faint band of cross-reactive galectin-1 was visualized on the X ray film. The cross-reactivity for galectin-1 was estimated to be less than 0.05%.

Western blotting (Fig. 3-A1). As shown in Fig. 3-A2, galectin-7 was most effectively extracted by direct SDS treatment of the precipitates after homogenization in 80% methanol–20% TBS. In contrast, the extraction of galectin-7 was insufficient using other buffers, *i.e.* TBS, TBS-Lactose, and TBS-Lactose-Triton. Therefore, we chose a conventional method involving 80% methanol–20% TBS for homogenization of all tissues examined.

First, in order to examine the expression of the galectin-7 protein in adult mouse tissues, total proteins obtained from 500 μg equivalent of tissue were subjected to SDS-PAGE and Western blot analysis. For skin, esophagus, tongue, stomach, anus, trachea, and ovary, a protein band of galectin-7 was detected, as shown in Fig. 3B. However, no galectin-7 band was detectable for small and large intestine,

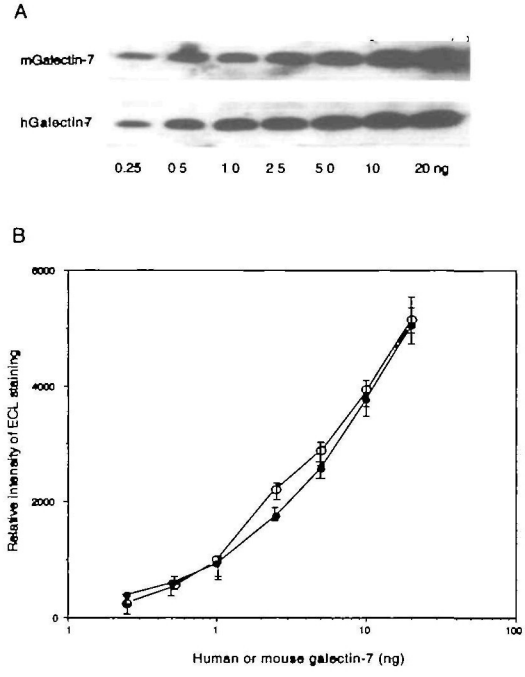

Fig. 2. **Quantitative analyses of mouse and human galectin-7 by the Western blotting method.** A: Native mouse galectin-7 or recombinant human galectin-7 was subjected to SDS-PAGE (12.5% gel) in the range of 0.1 to 20 ng/lane. After blotting onto PVDF membranes, the galectin-7 blots were treated with anti–rhgalectin-7 antiserum, and then visualized with an ECL system. B: The relative intensities of the galectin-7 blots on X ray film were integrated with NIH image. The relationships between the amounts of galectin-7 and its staining intensity are presented as semi-log plots (○: mouse galectin-7, ●: recombinant human galectin-7).

TABLE I. **The amounts of galectin-7 in adult mouse tissues.**

Tissue	Mean value ± SD[a] (ng galectin-7/mg tissue)
Skin	62 ± 3
Esophagus	23 ± 8
Stomach	18 ± 6
Anus	13 ± 1
Tongue	12 ± 2
Trachea	5.9 ± 1.4
Ovary	2.7 ± 0.6
Small intestine	n.d.[b] (<0.5)
Large intestine	n.d.
Brain	n.d.
Heart	n.d.
Lung	n.d.
Spleen	n.d.
Pancreas	n.d.
Kidney	n.d.
Bladder	n.d.
Liver	n.d.
Thymus	n.d.
Testis	n.d.
Uterus	n.d.
Muscle	n.d.
Blood cells	n.d.

[a]SD: standard deviation; [b]n.d.: not detectable (less than 0.5 ng/mg).

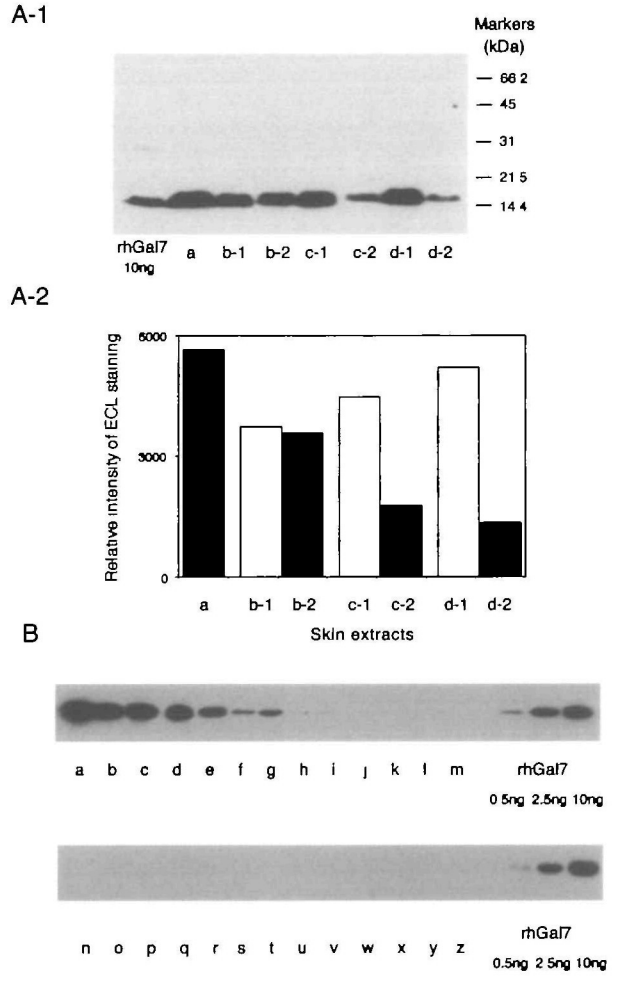

Fig. 3. **Expression of galectin-7 in adult mouse tissues.** A-1: Mouse skin extracts, obtained as described under "MATERIALS AND METHODS," were subjected to 12.5% SDS-PAGE, and galectin-7 was detected with rhgalectin-7 as an internal standard by Western blotting. rhGal7, 10 ng of rhgalectin-7; a, total skin extract prepared with 80% methanol–20% TBS; b-1, c-1, and d-1, supernatants of skin extracts prepared with TBS, TBS/Lactose, and TBS/Lactose/Triton, respectively; b-2, c-2 and d-2, pellets of skin extracts prepared with TBS, TBS/Lactose, and TBS/Lactose/Triton, respectively. A-2: The relative intensities of galectin-7 blots visualized on X-ray film were taken from the data shown in Fig. 2A-1. B: Total proteins extracted from 500 μg of adult mouse tissues were subjected to 12.5% SDS-PAGE and Western blotting. a, skin; b, esophagus; c, stomach; d, anus; e, tongue; f, trachea; g, ovary; h, brain; i, duodenum; j, jejunum; k, ileum; l, cecum; m, colon; n, rectum; o, heart; p, lung; q, spleen; r, pancreas; s, kidney; t, bladder; u, liver; v, thymus; w, testis; x, uterus; y, muscle; z, blood cells; and rhGal7, 0.5–10 ng of rhgalectin-7.

brain, heart, lung, spleen, pancreas, kidney, bladder, liver, thymus, testis, uterus, muscle, and blood cells.

Next, we determined galectin-7 produced in each tissue by quantitative Western blot analysis using serially diluted extracts for skin and esophagus, extracts of 500 μg tissue for other tissues, and 0.25, 1, 5, and 20 ng of rhgalectin-7 as an internal standard. The amounts of mouse galectin-7 were calculated in comparison with rhgalectin-7 using tissue extracts from two mice in triplicate. Table I shows that the mean values for galectin-7 were as follows: skin, 62 ± 3 ng/mg of tissue; esophagus, 23 ± 8 ng/mg; stomach, 18 ± 6 ng/mg; anus, 13 ± 1 ng/mg; tongue, 12 ± 2 ng/mg; trachea, 5.9 ± 1.4 ng/mg; and ovary, 2.7 ± 0.6 ng/mg.

Localization of Galectin-7 in Mouse Ovaries and Trachea—As the trachea and ovaries seem not to be organs that include typical stratified epithelia, we examined the localization of galectin-7 in comparison with that in skin as a positive control by immunohistochemistry. In the skin, strong staining of galectin-7 was observed in epithelial cells (keratinocytes), which comprise a typical stratified epithelium, but not in dermal fibroblasts (Fig. 4B). The outer root sheath (keratinocytes) of hair follicles was also labeled in the skin. Galectin-7 was moderately stained in ovarian stromal epithelium (Fig. 4D), but not in follicular granulosa or theca cells. Similarly, tracheal epithelial cells were labeled moderately while chondrocytes were labeled weakly (Fig. 4F).

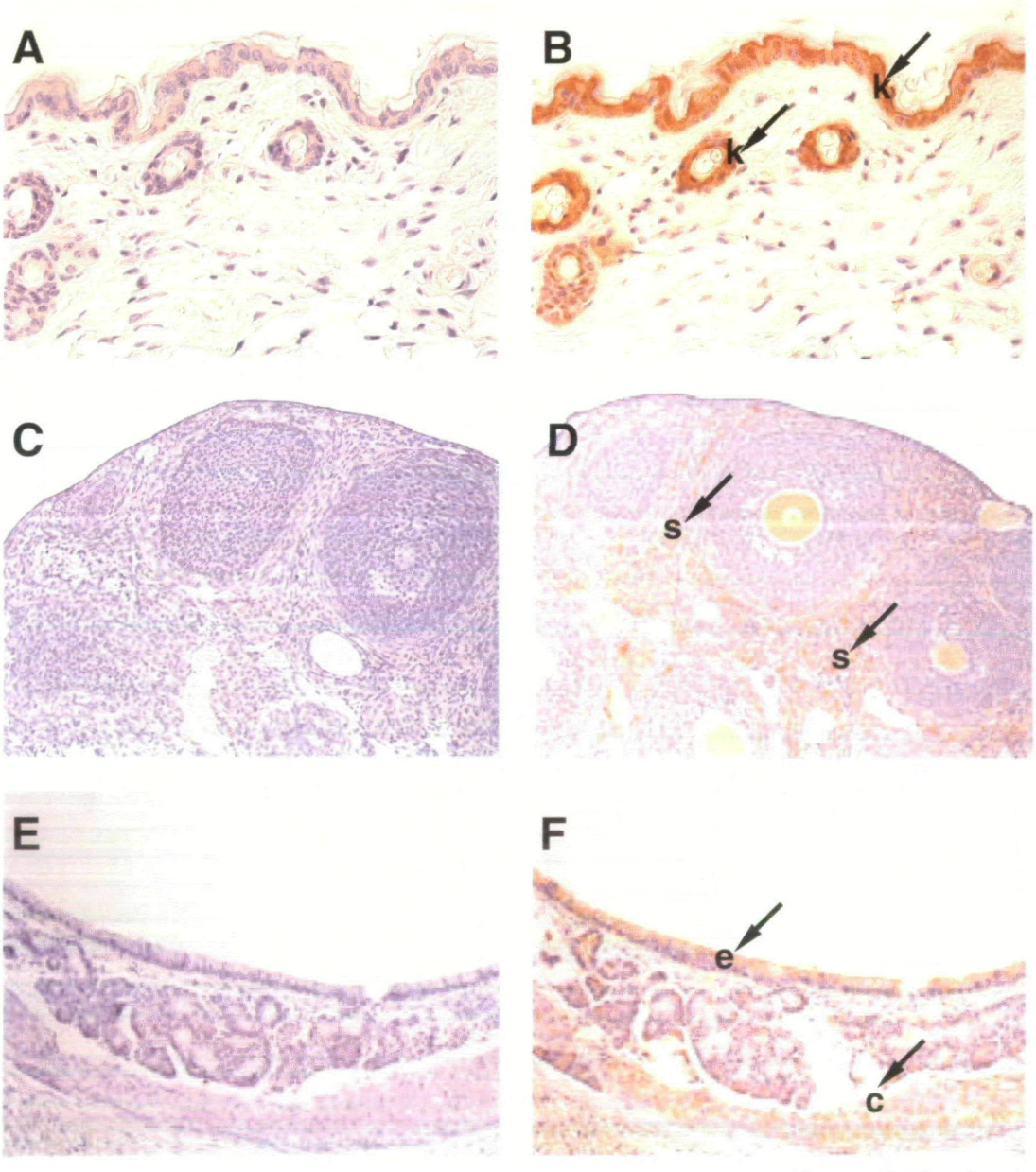

Fig. 4. **Localization of galectin-7 in adult mouse skin, ovary, and trachea.** Sections of skin (A, B), ovary (C, D), and trachea (E, F) were treated with preimmune serum (A, C and E) or anti–rhgalectin-7 antiserum (B, D, and F). The magnification in A–F was ×100. k, keratinocyte; s, stromal cell; e, epithelial cell; c, chondrocyte.

DISCUSSION

Galectin-7 is a member of the 14 kDa galectin subfamily. It is possible to discriminate at least between galectin-7 and ubiquitously expressed galectin-1 by Western blotting, because the apparent molecular weight of galectin-7 is about 15–16 kDa, *i.e.*, slightly larger than that of galectin-1 (14–15 kDa), on SDS-PAGE. Also, it seems not to be difficult to identify galectin-7 on Western blotting, because other members of the 14 kDa galectin subfamily, *e.g.* galectins-2, -5, and -10, are restricted to the digestive organs, red blood cells and peripheral white blood cells, respectively.

In this study, we prepared rabbit antiserum against rhgalectin-7, and first showed that this antiserum is equally useful for the quantitative evaluation of both the pure human and mouse galectin-7 proteins by the Western blot method. We also examined the extraction conditions for galectin-7 using skin, because the galectin family was not effectively extracted with regular buffer for examination of localization in the cytoplasm or cell adhesion apparatus, or attachment to insoluble cellular glycoconjugates (*16–18*). Further, some galectins, such as galectin-4, are susceptible to proteolytic cleavage during extraction (*19*). Skin galectin-7 was efficiently extracted with SDS-containing buffer after homogenization of the tissue in 80% methanol–20% TBS. Therefore, we examined the tissue distribution of galectin-7 in tissue extracts prepared with SDS-buffer, and then determined the amount of galectin-7 in each tissue by the quantitative Western blot method.

As a result, we confirmed the expression of galectin-7 in skin, esophagus, tongue, stomach, and anus, tissues consisting of stratified epithelia, as well as other tissues, including trachea and ovary. The expression profile of galectin-7 was almost identical to that previously reported (*13*). This also indicates that the antiserum does not cross-react with other members of the mouse 14 kDa galectin subfamily. The highest amount of galectin-7 detected was 62 ng/mg tissue in skin. Also, moderate amounts of galectin-7 (12–23 ng/mg) were found in esophagus, stomach, anus and tongue. These findings indicate that galectin-7 production coincides with the degree of stratification of the epithelia. Interestingly, we also detected significant amounts of galectin-7 in trachea and ovary. These tissues seem not to contain typical stratified epithelia. Therefore, we examined the localization of galectin-7 in both trachea and ovary by immunohistochemistry.

Galectin-7 protein was found to be expressed in pseudostratified epithelial cells of the trachea, and in stromal cells derived from embryonic epithelia and/or mesenchyme compartmentalizing each follicle of an ovary. Galectin-7 was previously detected in ovarian epithelia in mouse embryos (*14*). However, it was not labeled in follicular granulosa or theca cells in mature ovaries, which are believed to be developmentally derived from embryonic epithelial cells. Thus, galectin-7 might be expressed primarily in stratified epithelia, but also in some pseudostratified and wet epithelia.

Human galectin-7 exsists as a dimer form in the crystal, but does not possess multivalency. The packing arrangement of galectin-7 differs considerably from that of galectins-1 and -2, and galectin-7 recognizes carbohydrate in its monomeric form (*20*). This monomeric structural property of galectin-7 might be important to exhibit its specific roles *in vivo*. In fact, we could not detect multivalency of galectin-7, namely, neither native mouse nor recombinant human galectin-7 were observed to hemagglutinate rabbit red blood cells (data not shown). Galectins-1 and -2 form a dimer structure in solution and seem to be associated with cell–cell or cell–matrix contact. Moreover, galectin-1 can induce T-cell apoptosis, but not cell attachment activity (*7, 21*). Judging from the present results, galectin-7 may play a unique role in cell-mucus contact in wet epithelia, or in the growth of ovarian follicles besides cell–cell contact.

The sugar-binding ability of rhgalectin-7 was examined by frontal affinity chromatography (unpublised observation). The rhgalectin-7 exhibits a higher affinity for tri- and tetra-antennary glycans derived from glycoproteins than glycolipid glycans. Further, we tried to purify the receptors/binding proteins for galectin-7 in mouse skin membrane fractions. A preliminary result of that trial revealed that the α6 subunit, a member of the integrin family, is a possible galectin-7 receptor (data not shown). Thus, the primary target(s) of galectin-7 may be cell surface glycoproteins in the epidermis, and galectin-7 may regulate epidermal cell polarization, attachment or migration.

The present study represents the first attempt to quantify a member of the galectin family to our knowledge. This quantification method for galectin-7 is conventional, and should be useful for clarifying the functions of both mouse and human galectin-7 *in vivo* and *in vitro*. The galectin-7 gene is one of the targets of p53 in the apoptosis of human cancer cells (*22*). It is also associated with epidermal cell apoptosis induced by UV irradiation (*23*). In chemically-induced mouse breast cancer, galectin-7 is specifically induced in mammary cells (*24*). Thus, it may be valuable to apply this quantitative method to analyses of human diseases.

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