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# Induction of Cell Adhesion by Galectin-8 and its Target Molecules in Jurkat T-Cells

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**We previously showed that tandem-repeat type galectin-8, which has two covalently linked carbohydrate recognition domains (CRDs), induces neutrophil-adhesion through binding to integrin αM. Here, we analysed the function of galectin-8 in Jurkat T-cells. Galectin-8, as well as tandem-repeat galectin-9, and several multivalent plant lectins, induced Jurkat T-cell adhesion to a culture plate, whereas single-CRD galectins-1 and -3 did not. Galectin-8 also induced the adhesion of peripheral blood leucocytes to human umbilical vein endothelial cells. These results suggest that the di- or multi-valent structure of galectin-8 is essential for the induction of cell adhesion and that this ability exhibits broad specificity for leucocytes. The galectin-8-induced cell adhesion was accompanied by stress fibre formation, which suggests that intracellular signalling is required. We have identified integrin α4 as one of the candidate target molecules associated with the induction of cell adhesion. Indeed, inhibition of the function of integrin α4 by treating cells with a blocking-antibody reduced the sensitivity to galectin-8. Also, the phosphorylation of Pyk and ERK1/2, indicators of integrin-mediated signalling, was up-regulated on treatment with galectin-8. Thus, a primary target of galectin-8 must be the sugar chains on members of the integrin family, which are abundantly expressed on the surface of leucocytic cells.**

**Key words: adhesion, cytoskeleton arrangement, galectin-8, integrin, leucocyte.**

Abbreviations: ECM, extracellular matrix; EDTA, ethylenediamine tetra-acetic acid; ERK, extracellular signal-regulated kinase; GST, glutathione S-transferase; MAPK, mitogen-activated protein kinase; Pyk2, proline-rich tyrosine kinase-2; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis.

Galectins comprise a family of lectins that are defined by a common carbohydrate recognition domain (CRD), and which exhibit affinity for β-galactosides (*1*, *2*). Previously, 14 mammalian galectins were identified (*3–8*), and they have been shown to have various biological functions including regulation of cell activation, adhesion, growth and apoptosis (*9–13*).

The galectin family members are classified into three sub-groups based on their structures: (i) proto type, *e.g*. galectin-1, which has a single CRD; (ii) chimera type, *e.g*. galectin-3, consisting of a CRD and a non-CRD and (iii) tandem-repeat type, *e.g*. galectins-8 and -9, which have two non-identical CRDs connected by a linker peptide. In particular, tandem-repeat type galectins have received a lot of attention due to their multifunctions mediated by cross-linking of glycoconjugates.

Galectin-8 has been observed in normal tissues including lung, liver, kidney, brain and cardiac muscle (*14*), and alteration of galectin-8 expression may be correlated with the malignancy of tumour cells (*15–17*). In recent work, soluble galectin-8 was found to form complexes with integrins and to negatively regulate cell adhesion, and conversely immobilized galectin-8 functions as a matrix protein promoting cell adhesion (*18*). On the other hand, our previous study showed that soluble galectin-8 modulates neutrophil functions in addition to the induction of cell adhesion via integrin αM (*19*). Therefore, galectin-8 may play critical roles in some distinct aspects.

In this study, we analysed the cell adhesive function of galectin-8 in comparison with those of plant lectins in various leukaemia cell lines, and attempted to identify its target molecules in Jurkat T-cells as model leucocytes. First, we demonstrate that galectin-8 exhibits broad specificity for various tumour and inflammatory cells, and that multivalent plant lectins can also induce cell adhesion. Secondly, a combination of galectin and plant lectin columns was useful for identifying the common targets for both galectins and plant lectins in Jurkat T- and K562 cells. Then, the common target candidates were identified as integrins α4 and α5 in Jurkat T- and K562 cells, respectively, suggesting that primary target molecules of galectin-8 must be integrin family members in leukaemia cell lines.

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## MATERIALS AND METHODS

*Reagents*—Concanavalin A (ConA, jack bean), Con A-agarose, *Lens culinaris* agglutinin (LCA), LCA-agarose, *Macckia amurensis* lectin (MAM), *Arachis hypogaea* agglutinin (PNA), *Phaseolus vulgaris* agglutinin (PHA)-$E_4$, PHA-$E_4$-agarose, PHA-$L_4$, *Ricinus communis* agglutinin (RCA) 120, RCA120-agarose, *Sambucus sieboldiana* agglutinin (SSA), *Ulex europaeus* agglutinin (UEA-I), wheat germ agglutinin (WGA) and WGA-agarose were obtained from Seikagaku (Tokyo, Japan). Mouse anti-human integrin β1 monoclonal antibodies (MAB1987Z) and rat anti-human integrin β2/CD18 monoclonal antibodies (MAB1388Z) were purchased from Chemicon International (Temecula, CA, USA). Mouse anti-human integrin α4/CD49d (HP2/1) was from Serotec (Oxford, UK). PD98059 was obtained from Calbiochem (San Diego, CA, USA). PA-sugar chains, 001 (*N*-acetyllactosamine type, biantennary), 004 (*N*-acetyllactosamine type, tetra-antennary), 026 (lactose), 029 (GM3-Neu5Ac-trisaccharide) and 047 (A-hexasaccharide) were all purchased from Takara Bio Inc. (Shiga, Japan).

*Preparation of Recombinant Proteins*—The details of the construction of expression vectors for tag-free human galectins-1, -3, -8 and -9 using the pET vector (Novagen, Darmstadt, Germany) were given previously (*20*, *21*). The isoform of galectin-8 with a short linker and that of galectin-9 with the shortest linker were used. Site-directed mutagenesis and construction of the expression vector for the galectin-8 mutants are described elsewhere (*19*). The Arg69 or Arg233 residue was substituted with His. The mutated cDNAs were introduced into the pGEX-4T-2 vector (GE Healthcare Bio-Sciences AB, Uppsala, Sweden), which allowed production of the glutathione-S-transferase (GST)-fusion protein.

Expression of tag-free and GST-fusion proteins in *Escherichia coli* BL21 was performed as described previously (*19*, *22*). The extracted recombinant proteins were purified by affinity chromatography on a lactosyl-agarose column (Seikagaku) (galectin-1, -3, -8, -8R233H and -9) or a glutathione-Sepharose column (GE Healthcare UK Ltd., Buckinghamshire, England) (galectin-8R69H). The GST-fusion galectin-8 was digested with thrombin, and the released GST moiety was removed with a glutathione-Sepharose column. Then, the purity of the protein was evaluated by SDS-PAGE and staining with Coomassie brilliant blue R-250 (CBB R-250).

For affinity purification of galectin-8-binding proteins, recombinant galectin-8 was coupled to a HiTrap N-hydroxy succinimide-activated column according to the manufacturer's instructions (GE Healthcare UK Ltd.).

*Cell Culture*—Jurkat E6-1, MOLT-4, NAMALWA, K562 and THP-1 cells were obtained from the American Type Culture Collection (Manassas, VA, USA). These cells were cultured in RPMI1640 medium supplemented with 10% fetal bovine serum (FBS), 100 U/ml penicillin and 100 μg/ml streptomycin at 37°C under a 5% $CO_2$–95% atmosphere. Human umbilical vein endothelial cells (HUVEC) were purchased from Cambrex (Walkersville, MD, USA) and cultured in EGM-2 Bullet Kit medium (CC-3162; Cambrex) according to the manufacturer's recommendation. HUVEC monolayers were used for experiments at passage 2 or 3.

*Preparation of Glutaraldehyde-fixed Cells*—For chemical fixation, cells were treated with 1% glutaraldehyde (Wako, Osaka, Japan) in phosphate-buffered saline (PBS) for 1 h at room temperature. After washing with PBS, containing 0.1 M glycine and Tris-buffered saline (TBS), the fixed cells were suspended in TBS/0.05% $NaN_3$ and stored at 4°C.

*Cell Separation by Magnetic Labelling*—Mononuclear and polymorphonuclear cells were separated from the blood of healthy donors by Polymorphprep™ (Axis-Shield PoC AS, Oslo, Norway) centrifugation. Then, target cells were enriched by magnetic cell sorting using a Mini-MACS Separator (Miltenyi Biotec, Bergisch Gladbach, Germany) according to the manufacturer's protocol. T- and B-cells in the mononuclear cell fraction were isolated using micromagnetic beads to anti-CD3 and anti-CD19 antibodies (Miltenyi Biotec), respectively. Monocytes were purified using a CD16+ Monocyte Isolation Kit (Miltenyi Biotec). Neutrophils and eosinophils were isolated from the polymorphonuclear cell fraction by positive and negative selection, respectively, using anti-CD16 antibodies (Miltenyi Biotec). The labelled cells were placed on a column attached to a MACS magnet, the flow-through fraction being collected as the negative one. Enriched cells were collected from the column after removal from the magnet. The purity of the isolated CD3+ T-cells, CD19+ B-cells, CD16+ neutrophils and CD15-56-16+ monocytes was >99, 87, 96 and 95%, as determined, respectively, by flow cytometry. The purity of CD16− eosinophils was >88%, as determined by staining with the Diff-Quick solution (International Reagents, Kobe) after centrifugation in a cell settling chamber (Neuro Probe, Cabin John, MD, USA) at 50*g* for 30 s.

*Inhibition of N- or O-glycan Synthesis*—After incubation with deoxymannojirimycin (DMNJ, Calbiochem) or benzyl-2-acetamide-2-deoxy-D-galactopyranoside (BG, Calbiochem) for 3 days at 37°C, Jurkat T-cells were harvested and centrifuged at 5,000 r.p.m. for 5 min. Then, the pellet was washed twice with the medium and used.

*Cell Adhesion Assay*—The cells were plated on 48-well tissue culture plates ($5 \times 10^4$ in 200 μl of medium/well) for the cell adhesion assay. After the addition of 50 μl of an assay sample, the cells were allowed to adhere for 30 min at 37°C. At the end of the incubation, the cultured medium was removed from the plates, and the plates were washed with 250 μl of fresh medium to harvest floating or loosely attached cells using a pipette. The total cell suspensions were centrifuged at 5,000 r.p.m. for 5 min. Then, the cell pellets were suspended in 50 μl of a 0.1% trypan-blue solution containing 0.1 M lactose and the cell numbers were determined with a haemocytometre. Adherent cells were quantified as the percentages of total cells added. The adhesion of human leucocytes to HUVEC grown to confluency on 48-well plates was similarly examined as described.

To examine the effect of pyridylaminated (PA)-sugar chains on Jurkat T-cell adhesion, we used 96-well tissue culture plates ($2 \times 10^4$ in 25 μl of medium/well). After the addition of 25 μl aliquots of mixtures of galectin-8 and PA-sugar chains or lactose, the cells were incubated for

30 min at 37°C. After centrifugation, the cell pellets were suspended in 25 μl of a 0.1% trypan-blue solution containing 0.1 M lactose and the cell numbers were determined.

*Confocal Microscopy*—Fluorescence microscopy was performed as described previously (*19*). Cells adhering to a plastic chamber slide (Nalge Nunc International, Naperville, IL, USA) were washed with PBS and then fixed with 4% paraformaldehyde. Then, the cells were permeabilized with 0.1% Triton X-100 in PBS. After blocking with 1% BSA for 1 h, $1.65 \times 10^{-7}$ M FITC-phalloidin (Molecular Probes, Eugene, OR, USA) was added to each slide. Following overnight incubation at 4°C in a moist chamber, the cells were washed with PBS. All samples were covered with 50% glycerol and a coverslip, and then subjected to confocal laser scanning microscopy. The immunofluorescence localization of galectin-8 was carried out using affinity-purified anti-galectin-8 polyclonal antibodies. After blocking with 1% BSA, the cells were incubated with anti-galectin-8 antibodies (10 μg/ml) overnight at 4°C. The cells were then washed with PBS, followed by incubation with anti-rabbit IgG-FITC (10 μg/ml. Kirkegaard & Perry Laboratories, Gaithersburg, MD, USA) for 1 h at room temperature. Then, the cells were subjected to confocal microscopy as described earlier.

*Isolation of Galectin-8-interacting Proteins and Protein Sequencing*—Jurkat T-cells suspended in 20 mM Tris–HCl (pH 7.6), 0.15 M NaCl, 2 mM EDTA, 5 mM benzamidine-HCl, 2 mM diisopropyl phosphofluoridate (DFP) and 0.1 M lactose were subjected to two cycles of freezing–thawing and then centrifuged at 5,000 r.p.m. for 10 min. The cell pellet was washed twice with the above buffer without lactose and then homogenized in a Polytron homogenizer. Then, the homogenate was centrifuged at 15,000 r.p.m. for 30 min to obtain crude membranes and the resulting membranes were solubilized in 4-fold 20 mM Tris–HCl (pH 7.6), 0.15 M NaCl, 5 mM benzamidine-HCl, 2 mM DFP and 2% Triton X-100 by stirring for 30 min at 4°C. After centrifugation at 15,000 r.p.m. for 30 min, the resulting supernatant was adjusted to 0.5 M NaCl and then loaded onto a galectin-8 column, and washed with 20 mM Tris–HCl (pH 7.2), 0.5 M NaCl, 5 mM benzamidine-HCl and 0.1% Triton X-100. After washing, galectin-8-binding proteins were eluted with the above buffer containing 0.2 M lactose. The eluted samples were applied to a RCA column after dialysis (Seikagaku Co., Tokyo) and washing, followed by elution with buffer containing 0.2 M lactose. The eluted proteins were dissolved, and then subjected to 10% SDS–PAGE and stained with CBB R-250. To determine the N-terminal amino acid sequences of the galectin-8-binding proteins, the proteins separated by SDS-PAGE were transferred to a polyvinylidene difluoride (PVDF) membrane (Bio-Rad, Hercules, CA, USA). The blotted proteins were stained with CBB R-250 and cut out, and then the N-terminal amino acid sequences were determined with a gas-phase sequencer.

*Sequential Affinity Chromatography (ConA and Galectin-8 Column)*—The solubilized membrane fraction of Jurkat T-cells was first applied to a ConA column. The bound proteins were eluted with 0.8 M α-methylglucoside. The eluted fraction was directly subjected to second affinity chromatography on a galectin-8 column (ConA→Gal-8; Fig. 5B, left panel). The proteins that bound to the galectin-8 column were eluted with 0.2 M lactose. The same experiment performed under the same conditions except for the order of the columns used (Gal-8→ConA; right panel). The proteins in the pass-through and eluted fraction were analysed by western blotting. The data for an experiment representative of three are shown.

*Western Blot Analysis*—The proteins (20 μg protein equivalent/lane) eluted from affinity-columns of galectins or plant lectins, from DMNJ- or BG-treated whole cell membranes, and from native whole cell membranes were subjected to SDS-PAGE (10% gels), and then transferred to PVDF membranes. After blocking with 5% non-fat milk-TBS at room temperature for 1 h, each membrane was incubated with antibodies against integrin α1 (R-164), α2 (H-293), α3 (H-43), α4 (H-210), α5 (H-104), αL (C-17) or β1 (M-106), all antibodies being purchased from Santa Cruz Biotechnology, CA, USA in 1% non-fat milk-TBS overnight at room temperature. To determine the level of protein phosphorylation in Jurkat T-cells, we prepared lysates of galectin-8-treated cells. The cells were suspended in 2% SDS-TBS and then sonicated. The cell lysates were heated for 5 min at 95°C and then centrifuged, and the supernatants were subjected to SDS-PAGE. Phosphorylated proteins were detected by using the following antibodies: anti-p44/42 MAPK, anti-phospho-p44/42 MAPK (Thr202/Tyr204), anti-Pyk2 and anti-phospho-Pyk2 (Tyr402) (all antibodies were purchased from Cell Signaling Technology, Beverly, MA, USA). Then, each membrane was washed with 5% non-fat milk-TBS for 5 min and then three times with 0.1% Tween 20-TBS for 5 min. After incubation with the second antibody (peroxidase-conjugated anti-rabbit IgG; GE Healthcare UK Ltd.) in 1% non-fat milk-TBS for 1 h, the membrane was washed three times with 1% non-fat milk-TBS for 5 min and then twice with TBS. The secondary antibody was detected by means of chemiluminescence using an ECL kit (GE Healthcare UK Ltd.).

*Small interfering (si)RNA Design and Transfection*—Three siRNAs (nucleotide annotation: 1, sense 5′ gaacaga ucugaugaaugaTT 3′, antisense 5′ ucauucaucagaucuguuc TT 3′; 2, sense 5′ gugaagacauggaugcuuaTT 3′, antisense 5′ uaagcauccaugucuucacTT 3′ and 3, sense 5′ gguagaaag ucgggacaaaTT 3′, antisense 5′ uuugucccgacuuucuaccTT 3′) specific for integrin β1 were designed by B-Bridge international, Inc. (Sunnyvale, CA, USA). For transfection, a 100 nM three siRNA cocktail was added to $2.0 \times 10^6$ cells using Lipofectamine 2000 and Opti-MEM I Reduced Serum Medium (Gibco, Invitrogen, Carlsbad, CA, USA) according to the manufacturer's protocol. Control cells were transfected with a negative control duplex provided by B-Bridge international, Inc. At 48 h transfection, cells were harvested and used for the adhesion assay. Transfection efficiency was examined by means of western blotting using the membrane fraction of Jurkat T-cells described earlier.

*Statistical Analysis*—Data are presented as means ± SD with the number of experiments indicated. Most data were analysed by Student's *t*-test or one-way analysis of

variance (ANOVA) in combination with Dunnett's multiple comparison test. A value of $P < 0.05$ was considered significant.

## RESULTS

*Induction of Immune Cell Adhesion by Human Galectins and Plant Lectins*—Tandem-repeat galectins, *i.e*. galectin-8 and galectin-9, induced Jurkat T-cell adhesion to culture plates in a dose-dependent manner after 30 min incubation at 37°C (Fig. 1A). In contrast, both proto-type galectin-1 and chimera-type galectin-3 did not induce cell adhesion at any dose. Furthermore, among several plant lectins, RCA120, LCA, WGA, ConA, MAM, PHA-$L_4$ and -$E_4$, and SSA also induced cell adhesion, as shown in Fig. 1B. These effects of galectins on cell adhesion were abrogated on the addition of lactose as a common hapten sugar, but not with sucrose as a control sugar (Fig. 1C), showing that the carbohydrate-binding activity of galectins is essential for their cell adhesive function. A similar inhibitory effect of each hapten sugar was observed on plant lectin-induced cell adhesion (Fig. 1C). These results suggest that the di- or multi-valent structures of the lectins are essential for the adhesion-inducing activity, since all the positive lectins, including the plant ones, are multivalent, while monovalent galectins-1 and -3 did not show this activity. Although each plant lectin exhibits a different sugar-binding specificity, they still mimicked galectins as to adhesion-inducing activity. This suggests that plant lectins act through binding to sugar-chains other than the ones recognized by galectins, but attached to common membrane glycoproteins that are implicated in the cell adhesion.

Galectins-8 and -9 also induced the adhesion of other leucocytic cell lines, *i.e*. MOLT-4, NAMALWA, K562 and THP-1 cells (Fig. 2), and exhibited critical adhesion activity toward T-cells, B-cells, neutrophils, eosinophils and monocytes derived from human peripheral blood on plastic culture plates (data not shown). Thus, tandem-repeat galectins has broad specificity on the cell adhesion in various leucocytic cell lines. However, the ability of galectins on the adhesion varied among the cell lines. MOLT-4 and THP-1 cells showed low reactivity at low concentration (1 μM) of galectin-8. Leucocytic cell lines and peripheral blood cells express common and different kinds of cell adhesion molecule such as integrin family. The different ability of galectins may be due to the different target molecule(s) or the expressing level of common target molecule among the cell lines.

Next, we examined whether or not galectin-8 induced leucocyte attachment to cultured HUVEC grown to confluency on culture plates, in order to deduce its role *in vivo*. Galectins-8 and -9 triggered marked increases in the adhesion of T-cells, B-cells, neutrophils, eosinophils and monocytes to HUVEC. On the other hand, galectins-1 and -3 moderately and weakly induced the selective leucocyte adhesion to HUVEC, as shown in Fig. 3. These results indicate that tandem-repeat galectins can behave as cell adhesion-inducing molecules for functional leucocytes *in vivo*.

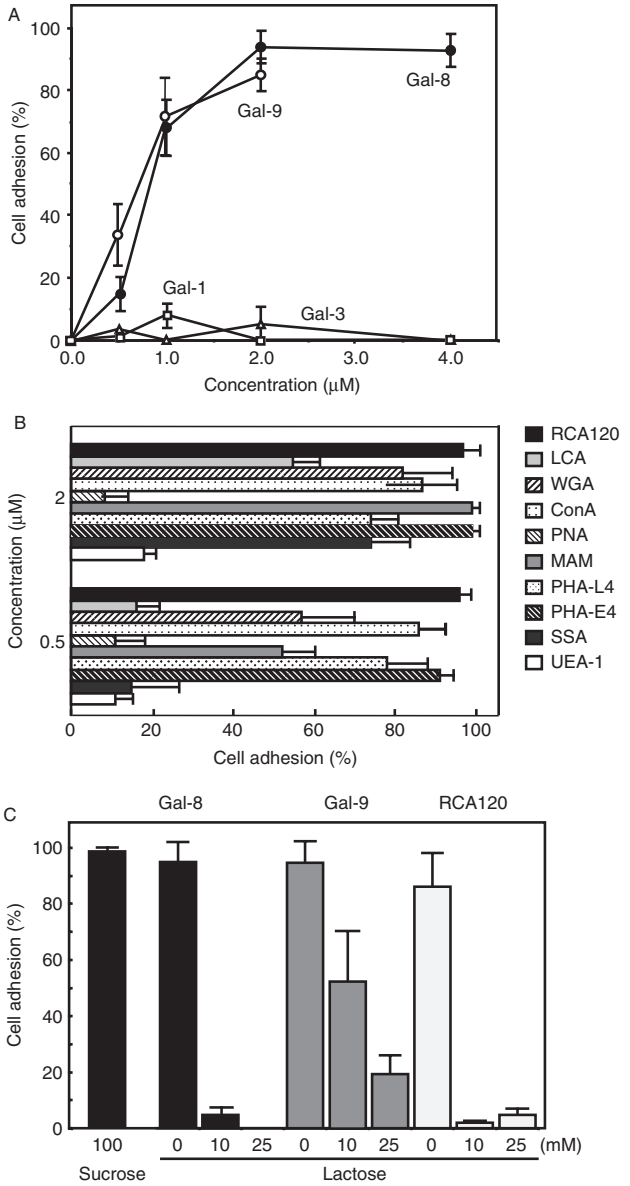


Fig. 1. **Comparison of various types of galectins and plant lectins in Jurkat T-cell adhesion.** (A and B) Jurkat T-cells were cultured with galectins or plant lectins at the indicated concentrations, and allowed to adhere for 30 min at 37°C. Adherent cells were quantified as the percentage of total cells added. Gal, galectin; ConA, concanavalin A; LCA, *Lens culinaris* agglutinin; MAM, *Macckia amurensis* lectin; PNA, *Arachis hypogaea* agglutinin; PHA-$E_4$ and -$L_4$, *Phaseolus vulgaris* agglutinins; RCA 120, *Ricinus communis* agglutinin; SSA, *Sambucus sieboldiana* agglutinin; UEA-1, *Ulex europaeus* agglutinin; WGA, wheat germ agglutinin. (C) Effects of lactose and sucrose on lectin-induced Jurkat T-cell adhesion. Jurkat T-cells were cultured with galectins-8 (2 μM) and -9 (2 μM), and RCA (1 μM) for 30 min at 37°C in the presence of lactose at the indicated concentrations. Data represents means ± SD of triplicate measurements.

*Cytoskeleton Rearrangement by Galectin-8*—We examined the distribution of galectin-8 and stress fibre formation in Jurkat T-cells treated with soluble galectin-8 or plant lectins. As shown in Fig. 4B, galectin-8 was

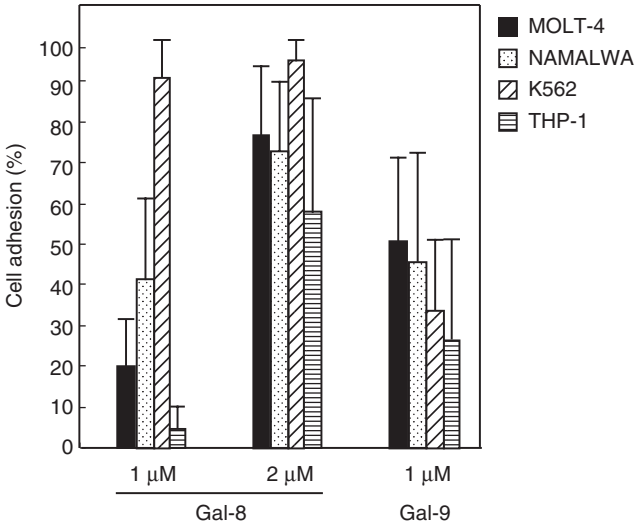


Fig. 2. **Galectins-8 and -9 induce adhesion in various cell lines.** Jurkat T-, MOLT-4, NAMALWA, K562 and THP-1 cells were cultured for 30 min at 37°C in the presence of the indicated lectins. Adherent cells were quantified as the percentage of total cells added. Data represents means ± SD of triplicate measurements.

localized on the surface of adhered T-cells, and the accumulation of stress fibres was observed (Fig. 4C). Similar stress fibre formation was induced by plant lectins, *i.e.* ConA, RCA120 and WGA (Fig. 4D and unpublished data). In contrast, we did not observe any adherence or stress fibre formation in non-treated cells (data not shown).

In order to clarify if the stress fibre formation is required for the cell adhesion induced by galectin-8, we examined whether glutaraldehyde-fixed cells (Jurkat T- and K562) still adhere to a culture plate or not. As a result, we found that fixed cells do not adhere to a culture plate in presence of galectin-8 (Table 1). Fixed cells were aggregated in presence of galectin-8 at a concentration of as low as 100 nM, which is a similar phenomenon to haemagglutination (data not shown). These results show that intracellular signalling from the cell surface is required for the induction of cell adhesion by galectin-8.

*Analysis of the Membrane Glycoproteins Implicated in Cell Adhesion*—To identify the target glycoproteins for galectins and plant lectins derived from a solubilized membrane fraction of Jurkat T-cells, we first tried single affinity chromatography on galectin- or plant lectin-immobilized columns (data not shown). On SDS-PAGE analysis of each eluted fraction, we detected several protein bands on every chromatography, some proteins seeming to be common for the lectin-columns and some not. However, most of the protein bands were very faint, and the only abundant and commonly detected protein was CD45, which is a dominant glycoprotein in leucocytes and is not very interesting from our point of view. In the end, it was difficult to identify the main target molecule(s) associated with cell adhesion.

In order to overcome this problem, we performed several series of sequential affinity chromatography, for which we combined the galectin-8 column and a plant lectin column. After the first chromatography on the galectin-8 column, the eluted proteins were applied to a plant lectin column, and the finally eluted proteins were analysed by SDS-PAGE followed by CBB staining. The fractions obtained were expected to include highly concentrated glycoproteins that are implicated in the cell adhesion. We tried several plant lectins for the second chromatography and a main protein band corresponding to 160 kDa, other than the 200 kDa band (CD45), appeared when we used a column of RCA, which exhibits similar sugar binding specificity to galectins (RCA prefers *N*-acetyllactosamine). The N-terminal amino acid sequence of the 160 kDa protein completely matched the sequence of integrin α4 (Asn-Val-Asp-Thr-Asp-Ser-Ala-Leu-Leu; Fig. 5A). Integrin αL was also detected as a minor protein band (140 kDa, Fig. 5A).

During this protein purification study, we found that integrin α4 has the potential to be activated by a variety of lectins with different sugar-binding specificities. For example, *N*-glycans of integrin α4 seems to consist of a more high-mannose type than a complex type. As shown in Fig. 5B, when we performed sequential affinity chromatography on ConA and galectin-8 columns, in that order, integrin α4 molecules recognized by ConA appeared in the pass-through fraction and were eluted from the secondary galectin-8 column. With the reverse combination, all the integrin α4 molecules recognized by galectin-8 bound to the ConA column. In fact, both galectin-8 and ConA are able to induce Jurkat T-cell adhesion through binding to integrin α4.

*Target Integrin Complex Recognized by Galectin-8*—Since integrins are αβ heterodimeric transmembrane receptors inducing cell adhesion, we then investigated the subunit structures of target molecules of galectin-8. After affinity chromatography on galectin or plant lectin columns, the eluted proteins were analysed by SDS-PAGE and western blotting with anti-integrin antibodies in Jurkat T- and K562 cells. As a result, we could identify the subunits of integrin α4, α5, αL and β1 in Jurkat T-cells, and those of integrin α5 and β1 in K562 cells as common integrin complexes for galectins-8 and -9, RCA120, WGA and ConA (Fig. 5C and D). We did not detect either integrin α1, α2 or α3 in the eluted fraction on any lectin-affinity chromatography using an extract of Jurkat T-cells (data not shown). In order to clarify whether the cell adhesion by galectin-8 depends on the interaction with common or specific integrin subunit(s), we compared the expression level of integrin α4, α5, αL and β1 in the membrane extracts of Jurkat T-, MOLT-4, NAMALWA, K562 and THP-1 cells. As shown in Fig. 5E, we observed common expression of integrin α5 and β1 in all cell lines. Although integrin α4 and αL were detected in Jurkat T-, MOLT-4 and THP-1 cells, the expression of both subunits was low or not detectable in NAMALWA and K562 cells. Thus, integrin α4, α5, αL may be common targets for T-cell lines, Jurkat T- and MOLT-4. Integrin α5 may be a main target for B-cell line, NAMALWA, monocytic cell line, THP-1 and erythroleukaemia cell line, K562. However, the expression level of integrin subunits varied in the cell lines, and dual protein bands of integrin α4, αL and β1 consisting of

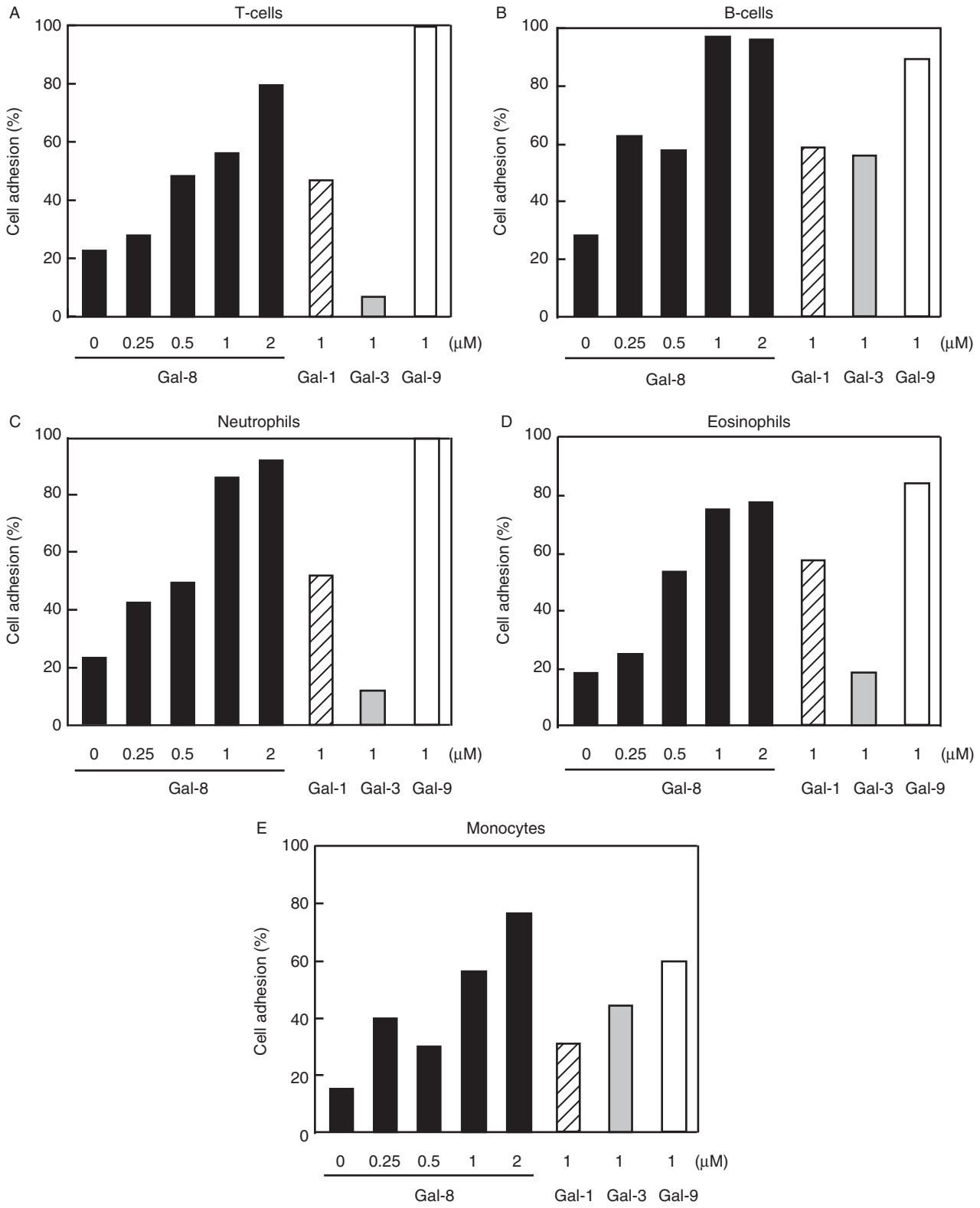


Fig. 3. **Galectin-8 induces adhesion of peripheral blood leucocytes to HUVEC.** We isolated human peripheral blood leucocytes using a MiniMACS Separator, and then assayed the adhesion to cultured HUVEC grown to confluence on plates. Isolated T-cells (A), B-cells (B), neutrophils (C), eosinophils (D) and monocytes (E) were incubated with various concentrations of galectins-1, -3, -8 or -9 for 30 min at 37°C. Adherent cells were quantified as the percentage of total cells added. Only the mean values for three independent experiments are shown.

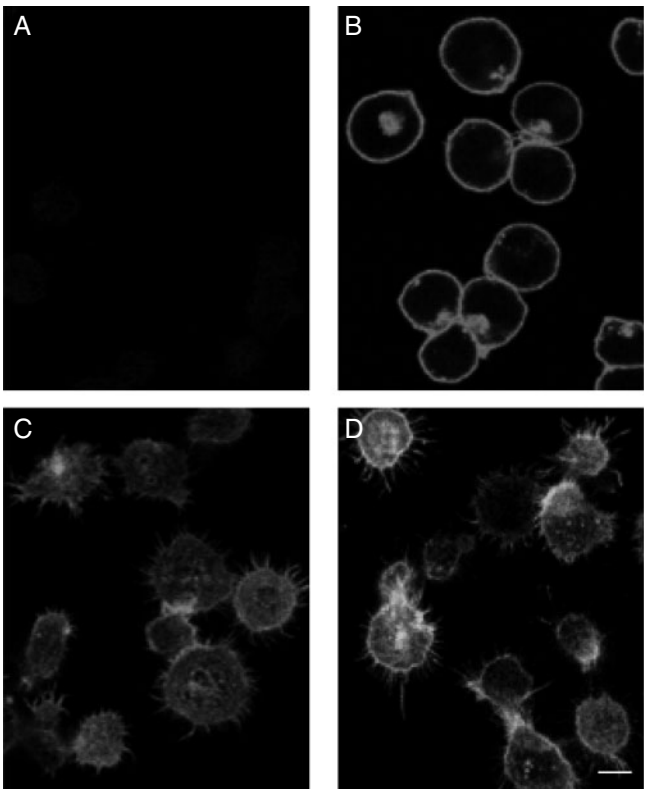


Fig. 4. **Immunofluorescence localization of galectin-8 and F-actin in Jurkat T-cells treated with galectin-8 or ConA.** After incubation with galectin-8 (2 μM) (A–C) or ConA (2 μM) (D) for 30 min at 37°C, adherent Jurkat T-cells were fixed and permeabilized. The immunofluorescense localization of galectin-8 was examined with control IgG (A) or anti-galectin-8 polyclonal antibodies (B), followed by incubation with anti-rabbit IgG-FITC. F-actin was stained with FITC-phalloidin (C and D). Then, the cells were subjected to confocal microscopy. The sections obtained in an experiment representative of four are shown. Scale bar, 10 μm.

Table 1. **Effect of galectin-8 on glutaraldehyde-fixed cell adhesion to a culture plate.**

| | | Living cells | | Fixed cells | |
|---|---|---|---|---|---|
| | | Jurkat T-cells | K562 cells | Jurkat T-cells | K562 cells |
| Gal-8 | 0 μM | <5% | <5 | <5 | <5 |
| | 2 μM | 93 ± 15 | 96 ± 8 | <5 | 17 ± 6 |

different molecular size were detected. These results indicate that the number and/or structure of the sugar chains attached to integrins are various among leucocytic cell lines. Furthermore, it seems that expression level of integrin α4, α5, αL and β1 does not correlate with the lower ability of galectin-8 towards MOLT-4 and THP-1 cells (Fig. 2). Other integrin subunits or adhesion molecules might contribute the adhesive response by galectin-8 in MOLT-4 and THP-1 cells.

Next, we examined the effects of anti-integrin antibodies on galectin-8-induced adhesion of Jurkat T-cells. Anti-integrin α4 and β1 antibodies, but not anti-integrin β2 ones, suppressed galectin-8-induced cell adhesion in dose-dependent manner (Fig. 6A and B). The cell adhesion induced by ConA was also moderately reduced by treatment with anti-integrin β1 antibodies (data not shown). Furthermore, the reduction in integrin β1 expression caused by transfection of siRNA resulted in a lower sensitivity to galectin-8 (Fig. 6D and E). In addition, treatment with anti-integrin β1 antibodies completely inhibited galectin-8-stimulated adhesion of K562 cells (Fig. 6C), demonstrating that the common target molecules of galectin-8 concerning cell adhesion are members of the integrin family in leucocytic cell lines.

*Integrin-mediated Signalling by Galectin-8*—It is well known that Pyk2 and ERK1/2 (p44/42 MAPK) phosphorylation are stimulated on integrin-mediated cell adhesion (*23*, *24*), so we examined the activation of these molecules in galectin-8-treated Jurkat T-cells. As shown in Fig. 7A, galectin-8 enhanced both Pyk2 and ERK1/2 phosphorylation within 15 min. PD98059, a MAPK kinase (MEK)-specific inhibitor, moderately suppressed both the activation of ERK1/2 and cell adhesion (Fig. 7B). These results indicate that activation of the MAPK pathway is required for the cell adhesion induced by the galectin family (*25*).

*Sugar-recognition by Galectin-8 in the Induction of Cell Adhesion*—In order to clarify whether *N*- and/or *O*-glycans play a critical role in galectin-induced cell adhesion, the effects of galectin-8, ConA and RCA were analysed in the presence of inhibitors for *N*- (DMNJ) or *O*- (BG) glycan synthesis. As shown in Fig. 8A, DMNJ significantly inhibited the cell adhesion induced by galectin-8 and RCA, whereas BG had no effect (right panel). No effect of DMNJ on the ConA treatment was observed either. Furthermore, treatment with DMNJ reduced the molecular weights of both integrins α4 and β1, which was confirmed by western blotting analysis using cell lysates of inhibitor-treated cells (left panel). These results indicate that the *N*-glycans attached to integrins α4 and β1 are the major target sugar chains recognized by galectin-8 in the induction of cell adhesion.

Next, we screened several commercially available sugar chains to find an effective inhibitor of galectin-8 in cell adhesion. Among them, A-hexasaccaride strongly inhibited the activity of galectin-8 (Fig. 8B). It was effective at the concentration of 10 μM, whereas a concentration of more than millimolar order was required for sufficient inhibition by lactose. Our previous study showed that A-hexasaccharide is recognized by the C-terminal CRD of galectin-8 with high affinity ($K_d$ = 3.3 μM as analysed by frontal affinity chromatography as in ref. 26). In contrast, GM3, which has been shown to be recognized by the N-terminal CRD of galectin-8, had much less effect. Consistent with these results, it was revealed that the C-terminal CRD of galectin-8 plays a critical role in the induction of cell adhesion. When we introduced a point mutation at amino acid 233 (R233H), which abolishes the lectin activity of the C-terminal CRD, the cell adhesion-inducing activity of galectin-8 was completely lost, whereas a mutation in the N-terminal CRD had no or much less effect (Fig. 8C).

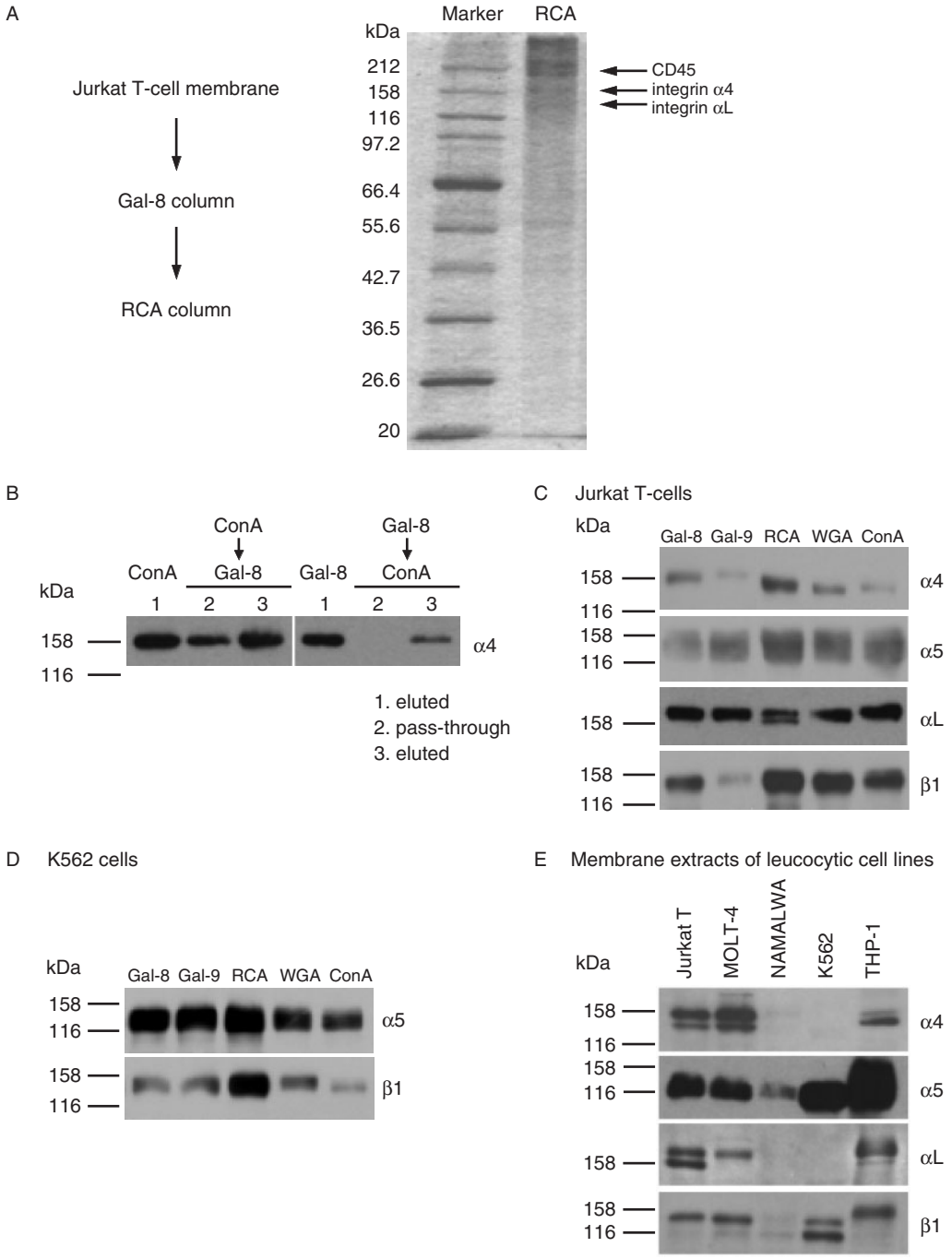


Fig. 5. **Identification of target proteins for galectin-8 in Jurkat T-cells.** (A) Solubilized membranes of Jurkat T-cells were applied to a galectin-8 affinity column. Then, the bound proteins were eluted with 0.2 M lactose-containing buffer. The eluted protein sample was dialysed to remove lactose and then applied to a RCA column, followed by elution with buffer containing 0.2 M lactose. The eluted proteins were analyzed by SDS-PAGE. The proteins eluted from the RCA column were transferred to a polyvinylidene difluoride (PVDF) membrane, and then main protein bands were excised, and the N-terminal amino acid sequences were determined with a gas-phase sequencer. (B) An example of the behavior of integrin α4 after sequential affinity chromatography (combination of ConA and galectin-8 columns) detected on western blotting. The solubilized membrane fraction of Jurkat T-cells was first applied to the ConA column, and then the bound proteins were eluted and fractionated on the second affinity chromatography on a galectin-8 column (ConA→Gal-8; left panel), the experiment also being performed in the reverse order (Gal-8→ConA; right panel). The data for an experiment representative of three are shown. (C–E). Western blot analysis of integrins in the eluted fraction on lectin-affinity chromatography (C and D) and the membrane extracts of leucocytic cell lines (E). Proteins of Jurkat T- or K562 cells eluted from the indicated affinity columns and the extracts of the indicated cell lines were analysed by western blotting using anti-integrin α4 (H-210), α5 (H-104), αL (C-17) or β1 (M-106) antibodies. The data for an experiment representative of three are shown.

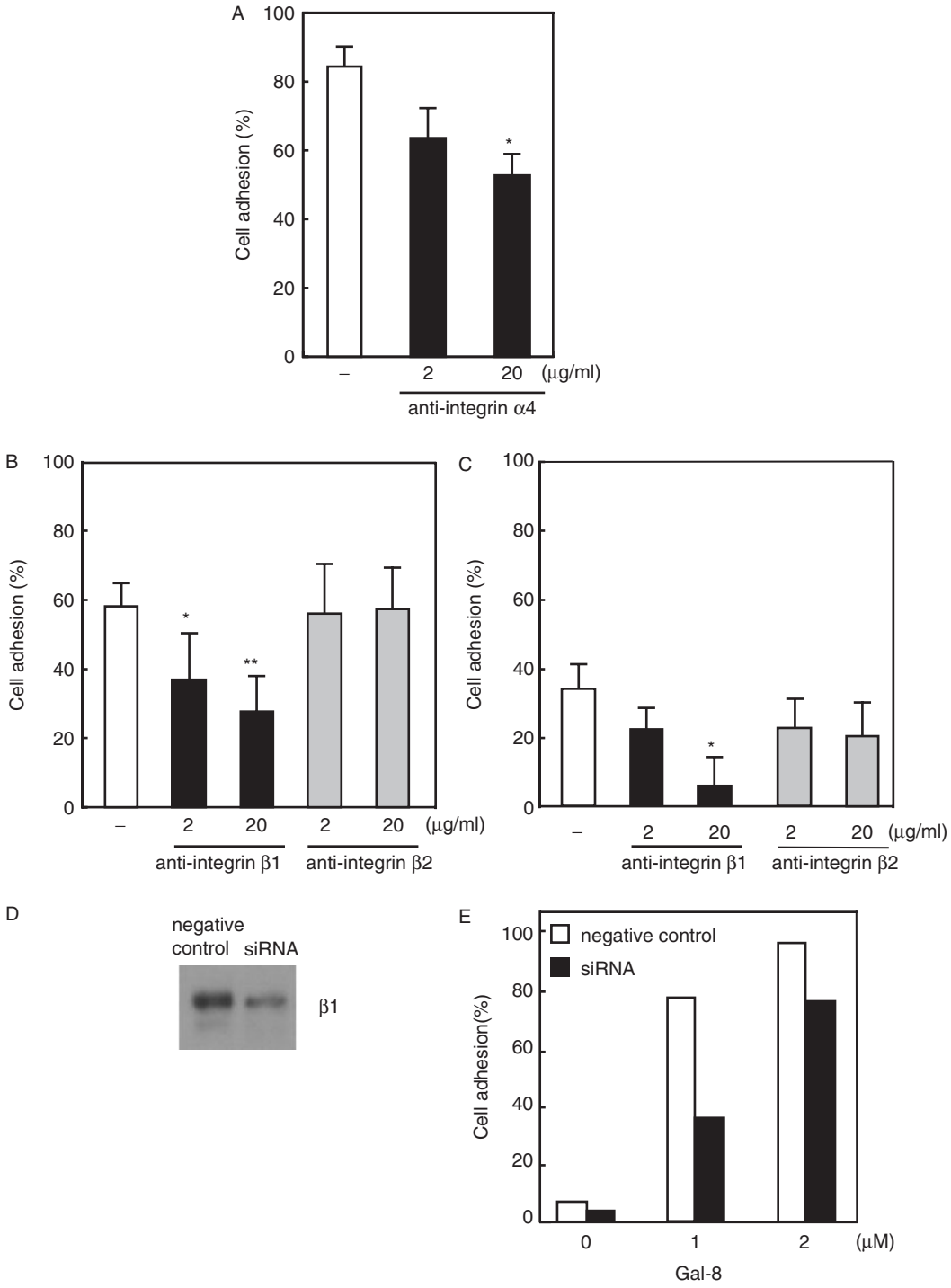


Fig. 6. **Effects of anti-integrin α4 or β1 antibodies and integrin β1-specific small interfering (si) RNA on galectin-8-induced Jurkat T-cell adhesion.** (A–C) Jurkat T- (A and B) and K562 cells (C) were pre-cultured with inhibitory antibodies against integrin α4 (HP2/1), β1 (MAB1987Z) or β2 (MAB1388Z) at the indicated concentrations for 15 min, and then exposed to galectin-8 with concentrations of 1 μM in (B) and 0.25 μM in (C) for 30 min at 37°C. The data in A, B and C represent the means ± SD of triplicate measurements. $^{*}P < 0.05$; $^{**}P < 0.01$ versus galectin-8-treated cells in the absence of antibodies. (D) The efficacy of integrin β1-specific siRNA as to integrin β1 protein expression. After 100 nM siRNA or negative control treatment, an equal amount of the membrane fraction was lysed and then analysed by western blotting using integrin β1 antibodies. (E) Following 48 h post-treatment with 100 nM integrin β1-specific siRNA or negative control, cells were incubated with galectin-8 (1 or 2 μM) for 30 min at 37°C. The data for an experiment representative of three are shown.

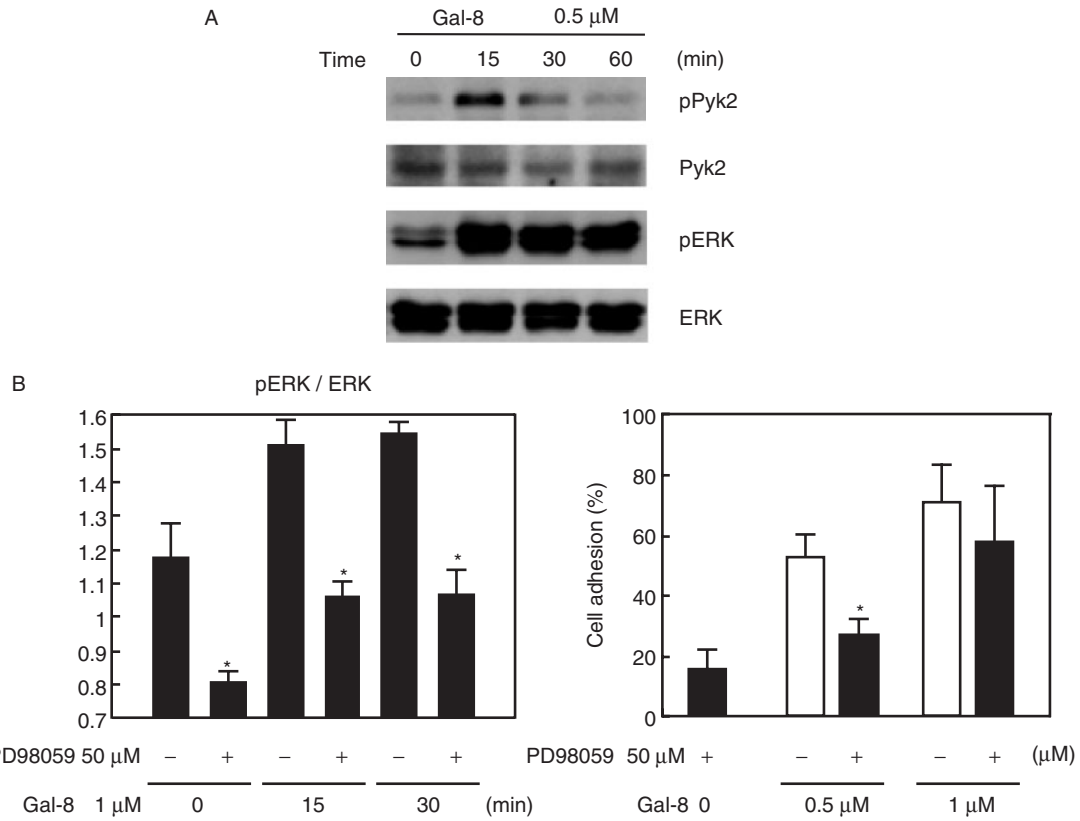


Fig. 7. **Activation of ERK1/2 (p44/42 MAPK) on galectin-8 mediated adhesion.** (A) We prepared lysates of galectin-8 (1 μM)-treated Jurkat T-cells at the indicated times after incubation at 37°C. Phosphorylated proteins were detected by Western blot analysis with the following antibodies: anti-p44/42 MAPK, anti-phospho-p44/42 MAPK (Thr202/Tyr204), anti-Pyk2 and anti-phospho-Pyk2 (Tyr402). (B) Effects of MEK inhibitor PD98059 (50 μM) on galectin-8-mediated phosphorylation (*left*) and adhesion (*right*) in Jurkat T-cells. *Left*, cells were pre-cultured with or without PD98059 for 15 min, and then exposed to galectin-8 (1 μM) for the indicated times at 37°C. The level of phosphorylation of ERK was analysed with NIH image software. The data represent the means ± SD for three experiments. $^{*}P < 0.05$ versus galectin-8-treated cells in the absence of PD98059. *Right*, after incubation with or without PD98059 for 15 min, the cells were cultured with galectin-8 (0.5 or 1 μM) for 30 min at 37°C. The data represents the means ± SD of triplicate measurements. $^{*}P < 0.05$ versus galectin-8-treated cells in the absence of PD98059.

## DISCUSSION

We previously showed that galectin-8 is a potent inducer of neutrophil adhesion to plastic culture plates with the GST-fusion protein, and that its target molecules are integrin αM and proMMP-9 on the cell surface (*19*). Here, we investigated the effects of the galectin family on tumour cells derived from various immune cells *in vitro*. To exclude the effect of the GST–GST interaction that results in artificial dimerization or multimerization of fusion proteins, we used tag-free galectins in this study. Both galectins-8 and -9, as well as several multivalent plant lectins, but not single CRD galectins-1 and -3, markedly induced cell adhesion to culture plates in a sugar-recognition manner. These results indicate that the multivalent structure of the tandem-repeat galectin family, which endows strong ability to cross-link glyco-conjugate ligands, is required to induce cell adhesion. Galectin-8 also induced the adhesion of several other blood cell lines to culture plates. Furthermore, human peripheral blood leucocytes were induced to adhere on HUVEC by galectin-8. Thus, the present results including those of our previous study show that the adhesion-inducing activity of galectin-8 exhibits broad specificity for various leucocytes. Also, the results suggest that galectin-8 could be an *in vivo* factor that plays a role in regulation of the interaction between leucocytes and endothelial cells.

As shown in Table 1, galectin-8 permits cell adhesion only of living cells, *i.e.* not of chemical fixed cells. This finding implies that galectin-8 causes cell adhesion by binding to a cell surface receptor followed by induction of actin cytoskeleton rearrangement, which is a different phenomenon from physical cross-linking between the cell surface receptor and a plastic surface. On the other hand, single CRD galectins-1 and -3 exhibited moderate activity as to peripheral leucocyte adhesion to HUVEC. This effect may be due to the physical cross-linking between the cell surface receptors which allow cell–cell interactions, namely the direct cross-linking of leucocytes to HUVEC. In fact, galectins-1 and -3 have been shown to regulate cell–cell interactions through cross-linking of the cell surface glycoproteins (*27–31*). Therefore, we propose that there are two different interaction systems

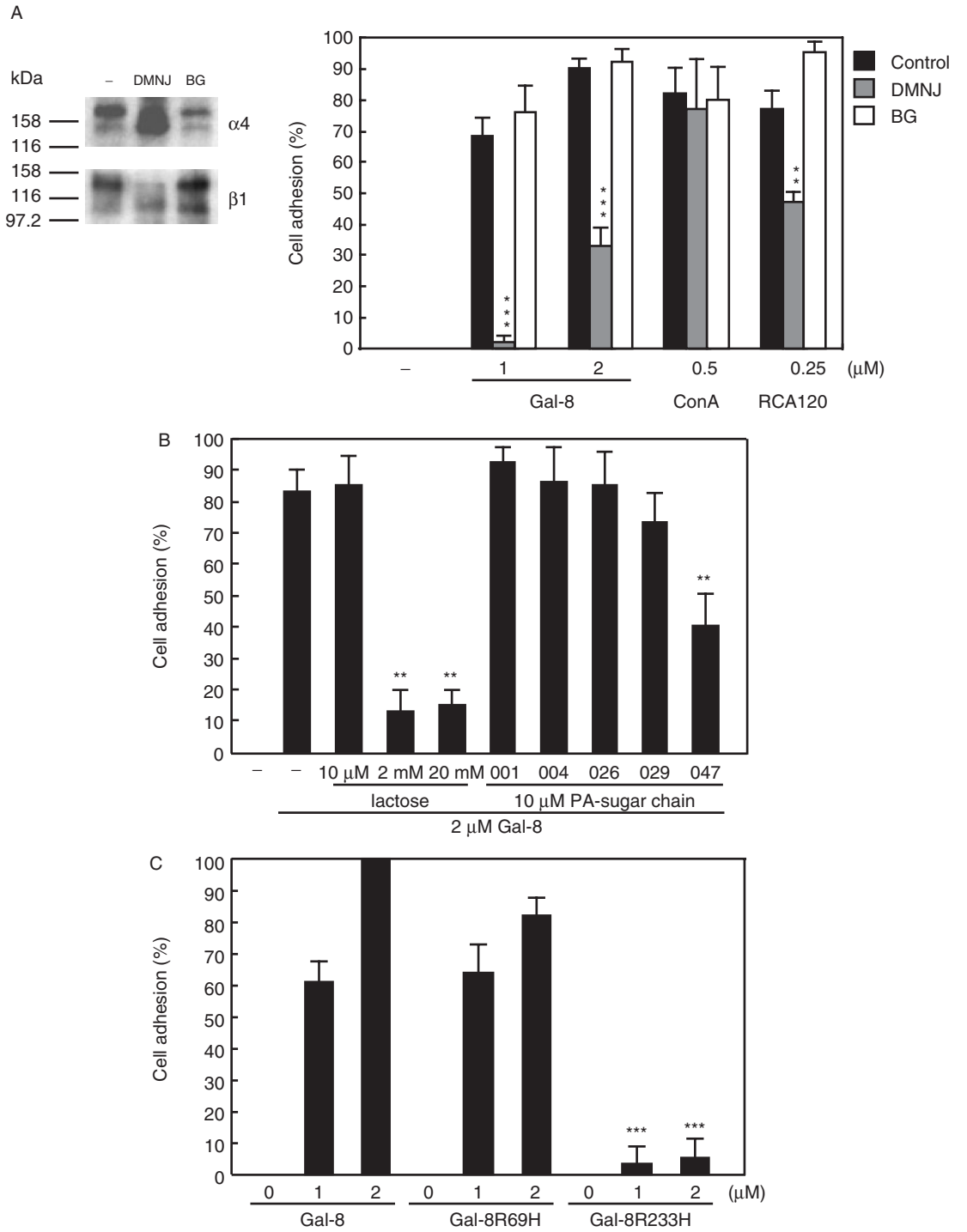


Fig. 8. **Sugar-recognition by galectin-8 in the induction of Jurkat T-cell adhesion.** (A) The effects of *N*- (DMNJ) or *O*- (BG) glycosylation inhibitors of the glycosylation of integrins α4 and β1 (left panel). Cell lysates of Jurkat T-cells, which were treated with the inhibitors, were analysed by western blotting. The data for an experiment representative of three are shown. The right panel shows the effects of the glycosylation inhibitors on the cell adhesion induced by galectin-8, ConA and RCA120. The data represents the means ± SD of triplicate measurements. $^{**}P < 0.01$; $^{***}P < 0.001$ versus lectin-treated cells in the absence of an inhibitor. (B) The effects of commercially available sugar-chains, which had been shown to have affinity with galectin-8, on galectin-8-induced Jurkat T-cell adhesion. 001, *N*-acetyllactosamine type, biantennary; 004, *N*-acetyllactosamine type, tetraantennary; 026, lactose; 029, GM3-Neu5Ac-trisaccharide and 047, A-hexasaccharide. The data represents the means ± SD of triplicate measurements. $^{**}P < 0.01$ versus galectin-8-treated cells in the absence of PA-sugar chains. (C) Effects of site-directed mutagenesis of individual CRDs on galectin-8-induced Jurkat T-cell adhesion. The N-terminal CRD of Gal-8R69H was inactivated, while its C-terminal CRD was active. The situation for Gal-8R233H was the opposite, *i.e.* the N-terminal CRD was active and the C-terminal CRD was inactive. Cells were allowed to adhere for 30 min at 37°C. The data represents the means ± SD of triplicate measurements. $^{***}P < 0.001$ versus galectin-8 wild type-treated cells.

for the cell–cell interactions regulated by the galectin family. One comprises an adhesive interaction with cellular signaling and the other physical cross-linking between the surfaces of the cells without signaling. These functions of galectin in cell–cell interactions might be associated with the cell recruiting system *in vivo*.

In healthy donors, circulating CD14+CD16+ monocytes comprise a minor subset accounting for ~10% of total monocytes (*32*). This subset is markedly increased in virus infections (*33*) or malignancy (*34*), and contributes to alteration of the proinflammatory cytokine level (*35*). Since galectin-8 exhibits adhesion activity as to CD14+CD16+ monocytes with HUVEC, this function might be relevant to the infiltration of blood monocytes into an organ, making it possible for a disease to progress. This idea might be supported by the finding that galectin-8 has various tumour-modulating properties (*17*).

In the present study, we identified integrin α4 as the major target molecule of galectin-8 in cell adhesion of Jurkat T- cells, and integrin αL as a minor binding protein. Also, integrin α5 is the major target molecule of galectin-8 in K562 cells. The experiments involving antibodies against integrins or involving siRNA confirmed that integrin signalling plays the major role in the cell adhesion induced by galectin-8. In our previous study, we showed that galectin-8 induces the adhesion of peripheral neutrophils through binding to integrin αM (*19*). Moreover, there have been several other manuscripts reporting that galectin-8 induces cell adhesion by activating integrins through direct interaction. Carcamo *et al*. (36) reported that α1, α3 and α5 are important for the cell adhesion of Jurkat T-cells induced by galectin-8, and Hadari *et al*. (*37*) has described the role of α3 in the adhesion of cells such as HeLa ones regulated by galectin-8. Taking all the information together, it is very likely that common target molecules for galectin-8 are the integrin family in leucocytic cells and that the binding of galectin-8 with sugar chains of the integrin family primes cytoskeletal rearrangement. As is well known, there are more than 10 members of the integrin α family and each member exhibits a specific distribution, and it is believed that each member has a specific biological function. However, regarding the function in cell adhesion of leucocytes induced by galectin-8, several members, such as integrin α1, α3, α4, α5 and αM, could act as mediators of the signalling. Therefore, it is very likely that which integrin mediates the signalling depends on the amount of the integrin molecule expressed on the surface of each cell. Hence, we propose that, in most cases, the dominant integrin in each cell could act as the major mediator of the signalling. This idea is supported by the results of our signal-blocking experiments involving anti-integrin antibodies. The antibody against integrin β1 was more effective than that against α4, suggesting that some minor α subunits, such as αL, might still be mediating the signaling by forming complexes with the β1 subunit.

Our hypothesis could be one of the possible explanations for the discrepancy between our study and the recent report by Carcamo *et al*. regarding the target molecules of galectin-8. They identified integrins α1, α3, α5 and β1, but not α4, as the galectin-8 binding proteins in Jurkat T-cells, whereas we identified α4, α5, αL and β1. It is possible to speculate that the amount of the integrin α subunit differs between our Jurkat T-cell subline and the subline Carcamo *et al*. used. Another explanation could be as follows. Carcamo *et al*. also showed that immobilized galectin-8 induces strong adhesion and rapid spreading in 5–7 min, thereby structuring asymmetric lamellipodial protrusions. These intensive reactions must be caused by immobilized not soluble galectin-8, with mediation via an integrin complex other than α4β1.

The advantage of our experiment is that we analysed the galectin-8 binding proteins not only by detection with antibodies but also by CBB staining of the SDS-PAGE gel followed by amino acid sequence analysis. Thus, we directly compared the amounts of the proteins adsorbed to the lectin columns, whereas they detected the proteins only by using antibodies. In that case, it will never be possible to determine which integrin bound the most to the galectin-8, because each antibody may have a different sensitivity, as they mentioned in their manuscript.

Another finding regarding the activation of the integrin complex is that we have observed activation of the integrin complex not only by galectins but also by several plant lectins, which exhibit different sugar-binding specificities. The latter are such as ConA, WGA and MAM, which recognize mannose, *N*-acetylglucosamine and sialic acid, respectively. This finding suggests that integrin molecules are guaranteed to respond to various lectin ligands, which is due to that they are decorated with a variety of sugar-chains. For example, integrin α4 has 12 potential *N*-glycosylation sites and various sugar-chains are attached to the sites. In fact, the *N*-glycans of integrin α4 seems to consist of a more high-mannose type than a complex type (Fig. 5B). And, both galectin-8 and ConA are able to induce Jurkat T-cell adhesion through binding to integrin α4. The contribution of the high mannose type *N*-glycan to ConA-induced cell adhesion was also confirmed by the experiment involving the inhibitor of *N*-glycan synthesis, DMNJ (Fig. 8A). Since DMNJ prevents mannose trimming and subsequent lactosamine elongation on *N*-glycans, high monnose type *N*-glycans were accumulated in the cells treated with DMNJ. This is the reason why the ConA-induced cell adhesion is not prevented by DMNJ treatment (Fig. 8A), and moreover, we have observed that treatment with DMNJ increases the sensitivity of the cells to ConA stimulation (data not shown). Therefore, the most critical event in the integrin activation by lectins seems to be whether the clustering of integrin complexes occurs or not. Also, integrin complexes have the potential to respond to a variety of lectins because they are decorated with various *N*-glycans. This could be an explanation for the mechanism underlying the biological actions of exogenous plant lectins on animal cells, which have been historically utilized as research tools. Furthermore, our hypothesis could be one of the explanations for the mechanism underlying the 'broad specificity' or 'fuzziness' of general lectin activity. We propose that members of the galectin family are some of the major *in vivo* factors for cell adhesion of leucocytes with broad specificity, and whose activity can be mimicked by plant lectins.

Analysis of galectin-8-binding proteins revealed that CD45 is another target candidate. However, anti-CD45 antibodies exhibited no inhibitory or stimulatory effect on galectin-8-induced cell adhesion (data not shown). Previous reports have shown that galectin-1 induces T-cell death and that CD45 is its target receptor for this reaction (*38*, *39*). Hence, we examined whether or not galectin-8 has apoptotic activity, but galectin-8 showed no or only weak apoptotic activity in Jurkat T-cells (*20*). Thus, CD45 may not participate in either cell adhesion or apoptosis in Jurkat T-cells.

Integrin-mediated adhesion causes the recruitment and activation of focal adhesion kinases (FAK), such as Pyk2 (*23*, *40*), leading to the activation of MAPKs (*23*). In this work, we found that galectin-8 augmented the phosphorylation of Pyk2 and ERK1/2 in Jurkat T-cells corresponding to the promotion of adhesion. These events exactly reflect the integrin recognition and activation by galectin-8. A recent report showed that Pyk2 and FAK are required for *trans*-regulation between integrins, which results in cell migration (*41*). We have observed galectin-8 induced chemotaxis of Jurkat T-cells *in vitro* (unpublished data). Hence, this observation may be explained by the *trans*-regulation mechanism after galectin-8-stimulated adhesion.

We have demonstrated that the recognition of *N*-glycans on the integrin family members by the C-terminal CRD of galectin-8 is indispensable for the induction of cell adhesion. This is because (i) A-hexasaccharide, which exhibits high affinity with the C-terminal CRD of galectin-8, inhibits the cell adhesion at low concentration (10 μM; Fig. 8B), while GM3, which exhibits high affinity with the N-terminal CRD, is much less effective. (ii) The introduction of a point mutation, which abolishes the lectin activity of the C-terminal CRD, abrogates the cell adhesion-inducing activity, while disruption of the N-terminal CRD has much less effect. On the other hand, we have examined other mutant galectin-8 proteins, which consist of two tandem repeated N-terminal CRDs (galectin-8NN) or two tandem repeated C-terminal CRDs (galectin-8CC). Neither of these two types of recombinant galectin-8 induced cell adhesion. Thus, taken together the fact that the multivalent structure is one of the indispensable factors, it is suggested that the structures of covalently linked N- and C- terminal CRDs are essential for the cell adhesion-inducing activity of galectin-8, although further analysis is required to reveal the role of the N-terminal CRD.

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