Visualization of Galectin-3 Oligomerization on the Surface of Neutrophils and Endothelial Cells Using Fluorescence Resonance Energy Transfer*

Received for publication, May 10, 2006, and in revised form, October 6, 2006. Published, JBC Papers in Press, November 2, 2006, DOI 10.1074/jbc.M604506200

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Galectin-3, a member of the galectin family of carbohydrate binding proteins, is widely expressed, particularly in cells involved in the immune response. Galectin-3 has also been indicated to play a role in various biological activities ranging from cell repression to cell activation and adhesion and has, thus, been recognized as an immunomodulator. Whereas those activities are likely to be associated with ligand cross-linking by this lectin, galectin-3, unlike other members of the galectin family, exists as a monomer. It has consequently been proposed that oligomerization of the N-terminal domains of galectin-3 molecules, after ligand binding by the C-terminal domain, is responsible for this cross-linking. The oligomerization status of galectin-3 could, thus, control the majority of its extracellular activities. However, little is known about the actual mode of action through which galectin-3 exerts its function. In this report we present data suggesting that oligomerization of galectin-3 molecules occurs on cell surfaces with physiological concentrations of the lectin. Using galectin-3 labeled at the C terminus with Alexa 488 or Alexa 555, the oligomerization between galectin-3 molecules on cell surfaces was detected using fluorescence resonance energy transfer. We observed this fluorescence resonance energy transfer signal in different biological settings representing the different modes of action of galectin-3 that we previously proposed; that is, ligand cross-linking leading to cell activation, cell-cell interaction/adhesion, and lattice formation. Furthermore, our data suggest that galectin-3 lattices are robust and could, thus, be involved, as previously proposed, in the restriction of receptor clustering.

Galectin-3 is a member of the family of soluble host carbohydrate-binding proteins called galectins (1–3). Members of this lectin family are characterized by conserved peptide sequences in their carbohydrate recognition domains (CRDs), which have an affinity for β-galactoside containing glycoconjugates (1–3). Galectin-3 has been implicated in various biological functions such as cell activation and cell adhesion (for a review, see Refs. 4–7). It has been reported that galectin-3 can induce mast cell degranulation (8), oxidative burst and interleukin-1 production in monocytes (9, 10), and migration of monocytes/macrophages (11) as well as L-selectin shedding and interleukin-8 production in neutrophils (12). Galectin-3 is also involved in cell adhesion of different cell types, such as neutrophils, to extracellular matrix proteins and to the endothelium (13, 14).

Most galectins are multivalent, being intrinsically composed of two CRDs or existing in a dimerized form (1–3). This multivalency of galectins enables their involvement in cell-cell and cell-pathogen interactions along with signal transduction events and lattice formation upon ligand cross-linking (for a review, see Refs. 4–7 and 15–19). Interestingly, galectin-3 does not comprise two CRDs nor does it form dimers in solution (20, 21). Rather, galectin-3 is composed of a N-terminal, non-lectin domain consisting of multiple repeats of a peptide sequence rich in proline, glycine, and tyrosine in addition to its C-terminal CRD (22–24). Others and our previous study have underlined the importance of the two domains of galectin-3 for its biological functions through the demonstration that both the C-terminal CRD domain and the N-terminal non-lectin domain are essential for its role in signal transduction, cellular adhesion, and lattice formation (Refs. 11–14 and 25–28; for a review, see Refs. 4–7 and 15–19). It has been reported that proteolytic cleavage of galectin-3, resulting in the removal of the N-terminal domain, prevents galectin-3 from exerting its functions whereas it still binds to its ligands (11–14, 25–28). Since most of its functions are thought to depend on oligomerization, the lack of extracellular function of truncated galectin-3 presumably resides in the impossibility for the truncated form to oligomerize. This implies that the N-terminal domain is involved in oligomerization (for a review, see Refs. 4–7 and 15–19). In fact, it has been proposed and generally accepted that, upon oligosaccharide binding by the CRD, the non-lectin

* This work was supported by grants from the Canadian Institutes of Health Research (to S. S.) and the Mizutani Foundation for Glycoscience (to S. S.), by equipment grants from the Canadian Foundation for Innovation (to S. S. and to the Centre de Recherche en Infectiologie), and by grants from the Program for Promotion of Basic Research Activities for Innovative Bioscience (Japan) (to A. K.) and from New Energy and Industrial Technology Development Organization (Japan) (to J. H.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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‡ The abbreviations used are: CRD, carbohydrate recognition domain; FRET, fluorescence resonance energy transfer; PBS, phosphate-buffered saline; HUVEC, human umbilical vascular endothelial cells; TBS, Tris-buffered saline; FRAP, fluorescence recovery after photobleaching.
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EXPERIMENTAL PROCEDURES

Reagents—Chemicals and other reagents were obtained from Sigma unless specified otherwise. Neutrophil elastase was obtained from Calbiochem.

Expression and Purification of Human Galectin-3—Recombinant human galectin-3 was purified as described previously and was passed through Detoxi-Gel endotoxin-removing gels (Pierce) (14). Truncated galectin-3 was prepared by incubation with human elastase (70 milliunits/ml) at 37 °C for 1 h followed by the addition of Pefabloc (5 mg/ml) to inhibit enzyme activity.

Nonspecific Labeling of Human Galectin-3—For nonspecific fluorescent labeling, the succimidyl ester-based method was used. Alexa 488- and Alexa 555-labeled galectin-3 (referred here as galectin-3-Alexa 488 or -Alexa 555) were prepared following the manufacturer’s instructions (Molecular Probes) with a slight modification as previously described (28, 37).

C-terminal-specific Fluorescence Labeling of Human Galectin-3 Tagged with Short Transglutaminase Peptide Sequence (TG1)—The expression vector (pET27/galectin-3-TG1) was constructed to produce galectin-3 tagged with the TG1 sequence, HSV®-tag (NOVAGEN), and His6 at the C terminus. Site-directed fluorescence labeling of human galectin-3 at the C terminus was performed as described by Taki et al. (36). Briefly, galectin-3-TG1 was incubated with either Alexa 488-cadaverine or Alexa 555-cadaverine; Molecular Probes (final concentration 1 mM) in the presence of transglutaminase (0.05 unit) at room temperature for 2 h in the dark. Labeled galectin-3-TG1 was purified on a lactose-Sepharose column. Galectin-3-TG1 was eluted with 20 mM lactose in PBS containing EDTA. The purity of galectin-3-TG1-Alexa 488 and galectin-3-TG1-Alexa 555 was analyzed by SDS-PAGE. Fluorescence intensities of galectin-3-TG1-Alexa 488 and galectin-3-TG1-Alexa 555 were measured by Fluoromax-3 (Jovan-Yvon). Fluorescent molecule (Alexa) to protein ratios of galectin-3-TG1 were 0.58 ± 0.10 and 0.40 ± 0.06, respectively. While a report suggests that the N-terminal domain of hamster galectin-3 contains a potential sequence for transglutaminase (38), very little if any fluorescence was associated with human galectin-3 when galectin-3 without TG1 was used as a substrate for labeling, suggesting that in the condition we used the majority of Alexa dyes incorporated to galectin-3-TG1 were attached to the TG1 sequence (data not shown). Before use, galectin-3-TG1-Alexa was extensively dialyzed against PBS to remove lactose and EDTA.

Fluorescence Intensity and Galectin-3 Purity—All galectins-3 molecules were fractionated on a SDS-PAGE gel, and fluorescent galectins-3 were visualized using the ProXpress two-dimensional proteomic imaging system (PerkinElmer Life Sciences) (excitation filter 480 nm, bandpass 30 nm; emission filter 530 nm, bandpass 30 nm) and Typhoon 3200 variable mode imager (GE Healthcare) (excitation 532 nm; emission filter 555 nm, bandpass 20 nm). Galectin-3-TG1-Alexa had a slightly higher molecular mass (32 kDa) than unlabeled galectin-3 or chemically labeled-galectin-3 (~30 kDa) (data not shown). Protein concentrations for the assays were then adjusted to achieve similar fluorescence intensities. The pair Alexa 488 and Alexa 555 was chosen for FRET analysis because the Alexa 488 emission spectra overlaps the Alexa 555 excitation spectra and the domain of galectin-3 molecules could oligomerize, thereby cross-linking ligands of the cell surface (20, 29, 30). Despite of a relatively limited set of available data, the multivalency of galectin-3 is conceptually accepted.

We previously proposed that galectin-3 oligomerization would lead to different galectin-3 functions through three different cross-linking modes (7, 16). The different activities expected from those three cross-linking modes, cell–cell adhesion, signal transduction, and lattice formation, were previously observed by our group through the interaction of galectin-3 with different stages of neutrophils (12). Furthermore, a recent elegant study by Brewer and co-workers (30) indeed indicated that upon binding to synthetic sugars in solution, high concentrations of galectin-3 precipitate as pentamers, most likely through oligomerization of galectin-3 N-terminal domain. However, to date the molecular mechanism leading to galectin-3 activities has not been elucidated, and the expected galectin-3 oligomerization has not been demonstrated in biological settings with physiological concentrations of galectin-3. We, thus, sought to investigate the oligomerization of galectin-3 at the cellular level through the three different proposed cross-linking modes previously observed in neutrophil adhesion, signal transduction, and lattice formation (12).

Fluorescence resonance energy transfer (FRET) is a technique that allows the visualization of molecular interactions that would otherwise be well beyond the resolution of a fluorescence microscope (31, 32). This technique implies the use of fluorescent molecules with emission (donor) and excitation (acceptor) spectra that overlap. When the energy from the emission of the donor is transferred to the acceptor, the acceptor fluorophore is excited, and its fluorescence (FRET signal) can be detected by fluorescence spectrometry, including fluorescence microscopes. Unlike colocalization, which merely indicates physical proximity, a FRET signal indicates that the two molecules are separated by a very small distance (less than 10 nm) and are presumably interacting (33–35).

In this study, galectin-3 tagged at its C terminus with a short transglutaminase substrate peptide sequence (galectin-3-TG1) was labeled with fluorescent Alexa molecules (Alexa 488 or Alexa 555) using a technique involving an enzymatic reaction (33–35). Galectin-3-TG1-Alexa had a slightly higher molecular mass (32 kDa) than unlabeled galectin-3 or Alexa 488 and Alexa 555 had similar fluorescence intensities. The pair Alexa 488 and Alexa 555 labeled galectin-3 (referred here as galectin-3-Alexa 488 or -Alexa 555) were prepared following the manufacturer’s instructions (Molecular Probes) with a slight modification as previously described (28, 37).

Expression and Purification of Human Galectin-3—Recombinant human galectin-3 was purified as described previously and was passed through Detoxi-Gel endotoxin-removing gels (Pierce) (14). Truncated galectin-3 was prepared by incubation with human elastase (70 milliunits/ml) at 37 °C for 1 h followed by the addition of Pefabloc (5 mg/ml) to inhibit enzyme activity.
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Alexa 555 emission can be detected using Far Red filters (≥660 nm) with minimum cross-talk originating from the Alexa 488 emission (39).

**Microplate FRET Measurement**—96-Well plates (Nunc) were coated with 4 mg/ml asialofetuin overnight. Residual binding sites on the plates were blocked using 5% bovine serum albumin for 4 h. After washing with Tris-buffered saline (TBS; 20 mM Tris-HCl (pH 7.4) and 0.15 M NaCl), different concentrations of galectin-3-TG1-Alexa 488 and/or galectin-3-TG1-Alexa 555 in 0.05% Triton X-100-TBS were added to the plates and incubated for 30 min. Plates were fixed in 2% paraformaldehyde for 30 min after brief washing with 0.05% Triton X-100-TBS. Plates were again washed 3 times with 0.05% Triton X-100-TBS, then PBS was added to the plates. Fluorescence was read using a Wallac Victor fluorescent reader (PerkinElmer Life Sciences). Green fluorescence, red fluorescence, and FRET signal were read alternatively using a 485-nm excitation filter with a 535-nm emission filter, a 545-nm (bandpass 30 nm) excitation filter with a 620-nm (bandpass 30 nm) emission filter, and a 485-nm excitation filter with a 620-nm (bandpass 30 nm) emission filter, respectively. Truncated galectin-3-TG1-Alexa 488 and/or truncated galectin-3-TG1-Alexa 555 were also tested in the same manner. However, the binding of truncated galectin-3 was as low as 4–7% compared with full-length galectin-3 (data not shown). Therefore, the presence or absence of FRET could not be evaluated due to the limited sensitivity of the detector. Furthermore, truncated galectin-3 was not retained in an asialofetuin-agarose column, whereas it was retained in a lactose-Sepharose column. This is consistent with the results obtained during microplate assays. These results suggest that stable association of soluble galectin-3 with asialofetuin requires the N-terminal domain.

**Neutrophils and Galectin-3**—Neutrophils were purified from heparinized blood of healthy volunteers, as previously described (12, 14). Freshly isolated neutrophils are referred here as unprimed neutrophils. A portion of the neutrophils was primed before the experiment by a 5-min preincubation with 5 μM cytochalasin B (referred here as primed neutrophils) (12, 26, 40). Primed and unprimed neutrophils were then incubated for the indicated times with Alexa labeled galectin-3 at 4 or 37 °C. Fluorescence intensity and protein content (2 μM) of each preparation was adjusted to similar levels with unlabeled galectin-3. After incubation galectin-3 was removed by washing neutrophils twice with PBS, then cells were washed with PBS. Then the cell layer was incubated with galectin-3-TG1-Alexa 555 for 15 min at 4 °C. After removing galectin-3 was washed by rinsing the cell layers with PBS twice, the cell layer was washed extensively with 5 mM HEPES (pH 7.8)-buffered PBS, then succimidyl ester-Alexa 488 was added, and cells were incubated for 1 h at 4 °C. The reaction was stopped by adding Tris-HCl (pH 7.5; final concentration 20 mM), and cells were washed with PBS. Then the cell layer was incubated with galectin-3-TG1-Alexa 555 for 15 min at 4 °C. After unbound galectin-3 was removed by rinsing the cell layers with PBS twice, the T4 dish was mounted onto the stage of the Olympus IX70 inverted microscope equipped with a Delta T4 dish controller (Bioptechs) and kept at 37 °C in 5 mM HEPES-buffered medium 199 (M199), as previously published (41). Green (for surface proteins) and red (for galectin-3) fluorescence of a small region of an ECV cell layer was photobleached by increasing the intensity of the 488 and 543 nm argon-ion and helium-neon laser lines to 100%. Fluorescence recovery was then monitored using normal confocal settings at 0.5, 15, 30, 45, 60, and 75 min post-photobleaching. When ECV cell layers were incubated with N-terminal-truncated galectin-3-TG1-Alexa, we were unable to detect significant amounts of truncated galectin-3 when cell layers were warmed at 37 °C (before bleaching) (data not shown). These results suggest that, like the interaction between truncated galectin-3 and asialofetuin, the stable retention of soluble galectin-3 on ECV cell layers requires the N-terminal domain, which implies the importance of oligomerization in the avidity of galectin-3. The lipid bilayer of an ECV cell layer was labeled with Dio (Molecular Probes) following the manufacturer’s instructions. Using the same conditions for ECV incubated with galectin-3-Alexa 555, a small region of the ECV layer was then bleached, and

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3 J. Nieminen, A. Kuno, J. Hirabayashi, and S. Sato, unpublished observations.
fluorescence recovery was monitored. As expected, quick fluorescence recovery (less than 95 s) was detected when the lipid layer of the plasma membranes were labeled with DiO (data not shown).

RESULTS

Detection of FRET Signal from Galectin-3-TG1-Alexa Bound to Asialofetuin—It has been suggested that galectin-3, upon binding to its ligands through its CRD, can oligomerize through its N-terminal non-lectin domain (for a review, see Refs. 4–7 and 15–19). However, previous reports have relied on precipitation techniques with galectin-3 in solution (30), whereas most galectin-3 activities are expected to occur at the cellular surface. Furthermore, the doses of galectin-3 required for precipitation assays are 10 to ~100-fold higher than the doses at which galectin-3 induces biological functions (11–14, 25–28).

We, thus, investigated whether oligomerization of galectin-3 indeed takes place when bound on a ligand matrix such as asialofetuin using the FRET technique. FRET occurs when two molecules, in this case galectin-3-TG1-Alexa 488 (donor) and galectin-3-TG1-Alexa 555 (acceptor), are in close proximity (<10 nm) and interact in such a way that the light used to excite the donor fluorochrome will be transferred as internal energy to the acceptor fluorochrome, which will then emit at its fluorescence wave length (42). This emission of fluorescence can then be detected using a fluorescence reader or microscope.

Different concentrations of galectin-3-TG1-Alexa 488 and/or galectin-3-TG1-Alexa 555 were added to asialofetuin-coated microtiter plates. Asialofetuin is commonly used as a galectin-3 ligand since this glycoprotein contains several N-acetyl lactosamine epitopes to which galectin-3 can bind (43). After removal of unbound galectin-3 molecules, fluorescence was measured using a Victor3 microplate reader. If galectin-3 molecules oligomerize on the plate surface, FRET originating from the interaction of Alexa 488 and Alexa 555 results in (a) increased emission from the Alexa 555 fluorochrome upon excitation of the Alexa 488 fluorochrome (Fig. 1A) and (b) quenching of the Alexa 488 fluorochrome (Fig. 1B) due to internal transfer of energy from Alexa 488 to Alexa 555 (45). The increase emission from Alexa 555 was monitored after excitation of Alexa 488 using filters that extract the spectra between 605 and 635 nm. The intensity of fluorescence from the wells containing galectin-3-TG1-Alexa 488 and Alexa 555 alone (Fig. 1A), suggesting that internal fluorescence transfer from Alexa 488 to Alexa 555 occurred. Furthermore, significant quenching of the Alexa 488 fluorochrome was also observed in wells containing higher amounts of galectin-3-TG1-Alexa 488 and galectin-3-TG1-Alexa 555 but not in wells containing only galectin-3-TG1-Alexa 488 molecules. Together, these results suggest that galectin-3 molecules interact when they bind to the ligand.
trophils (Fig. 2I), suggesting that the galectin-3 molecules are oligomerized on the surface of unprimed naïve neutrophils. Neither galectin-3-TG1-Alexa (A–I) or G3-Alexa (J–L) or truncated G3TG1-Alexa, which lacks the N-terminal non-lectin domain (M–P) at 37 °C for 10 min. Oligomerization of galectin-3 was evaluated by detecting far-red fluorescence emission (≥660 nm) resulting from the transfer of energy from G3TG1-Alexa 488 to G3TG1-Alexa 555 interacted. Representative data from four separate experiments are shown.

The majority of galectin-3 activities rely on the presence of the C-terminal, CRD domain and the N-terminal, non-lectin domain. Our previous results indicate that upon interaction with primed neutrophils, galectin-3 is deactivated through cleavage by elastase, a neutrophil enzyme. Galectin-3-TG1-Alexa 488 and/or galectin-3-TG1-Alexa were, thus, incubated with 70 milliunits of purified neutrophil elastase, and truncated galectin-3, consisting of the C-terminal CRD but lacking the majority of the N-terminal domain, was incubated with unprimed neutrophils. Although truncated galectin-3 retained the ability to bind to neutrophils, its binding level was lower than of full-length galectin-3. The affinity of truncated galectin-3 for lactosamine-containing glycans is similar to that of full-length galectin-3 when galectin-3 is immobilized to agarose (49). Thus, the lower binding of truncated galectin-3 to neutrophils is likely due to lower avidity rather than an alteration of its binding affinity. Nonetheless, we were able to observe that a significant amount of truncated galectin-3-TG1-Alexa 488 and G3-Alexa was colocalized (Fig. 2M–O) but very little if any FRET signal could be detected by confocal

FIGURE 2. Detection of FRET signal from galectin-3-TG1-Alexa interacting with unprimed neutrophils. Unprimed neutrophils were incubated with G3TG1-Alexa (A–I) or G3-Alexa (J–L) or truncated G3TG1-Alexa, which lacks the N-terminal non-lectin domain (M–P) at 37 °C for 10 min. Oligomerization of galectin-3 was evaluated by detecting far-red fluorescence emission (≥660 nm) resulting from the transfer of energy from G3TG1-Alexa 488 to G3TG1-Alexa 555 interacted. Representative data from four separate experiments are shown.

FIGURE 3. Analysis of fluorescence signal intensity in FRET positive and FRET negative regions of cells incubated with galectin-3-TG1-Alexa or galectin-3-Alexa. Green (y axis) and red (x axis) fluorescence intensity/pixel of regions in the images of G3TG1-Alexa (Fig. 2, G–I) and of G3-Alexa (Fig. 2, J–L) were plotted in A and B, respectively. Pixels showing a far-red fluorescence intensity (≥660 nm) superior to 64 were considered positive for FRET (closed triangles), whereas others were considered negative (open triangles).
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FIGURE 4. Detection of FRET signal from galectin-3-TG1-Alexa interacting with primed neutrophils. Primed neutrophils were incubated with G3TG1-Alexa for 10 min at 37 °C. Green (A, E, and I), red (B, F, and J), merged (C, G, and K), and FRET signals (D, H, and L) are shown here. Representative data from four separate experiments are shown.

microscope (Fig. 2P), confirming the importance of the N-terminal domain of this lectin in oligomerization.

On the surface of primed neutrophils, galectin-3 forms patches instead of the uniform lattices observed on unprimed neutrophils (data not shown and Ref. 12) and is eventually internalized when incubated for 10 min (Fig. 4). Again, FRET signals were visualized only when galectin-3-TG1-Alexa 488 and galectin-3-TG1-Alexa 555 colocalized (Fig. 4L). When compared with unprimed neutrophils (Fig. 2), relatively weaker FRET signals were detected in primed neutrophils (Fig. 4). We previously observed that primed neutrophils have the ability to cleave the N-terminal domain of galectin-3, which can be detectable as early as after 5 min of incubation (12). Thus, the weaker FRET signals of primed neutrophils are likely due to the lower amount of bound full-length galectin-3 in this condition.

Detection of FRET Signal at the Adherent Junction between Neutrophils and Endothelial Cells—We and others have reported a role for galectin-3 as a neutrophil adhesion molecule in the firm adhesion of neutrophils to the endothelial cell layer during the first step of extravasation across blood endothelial vessel (14) as well as interaction between neutrophils and a cell matrix component, laminin (13). This activity is assumed to be dependent on galectin-3 oligomerization, since not only the C-terminal CRD domain but also the N-terminal non-lectin domain is required for adhesion (14). However, it remains to be determined if galectin-3 molecules oligomerize and are concentrated at junction sites where neutrophils adhere to an endothelial cell layer. First, an endothelial-like cell line, ECV304, was used to visualize the localization of galectin-3 while it mediates neutrophil adhesion to an endothelial cell layer (Fig. 5). Unlike primary endothelial cells, HUVEC that form thin cell layers (~2 μm), immortalized ECV cells form relatively thick cell layers (~10–15 μm), enabling a better visualization of the interactions between neutrophils and endothelial cells. For this reason, ECV304 cells were first used. Neutrophils and ECV cell layers were both labeled with calcein and incubated at 37 °C for up to 30 min with galectin-3 labeled with succimidyl Alexa 546. Fig. 5 shows confocal images that were reconstructed to reveal the images of the xz and yz planes. Even though galectin-3 was localized on the surface of neutrophils and the endothelial cell layer, concentration of galectin-3 at the junctions between neutrophils and endothelial cells was significant, supporting our previous findings that galectin-3 acts as an adhesion molecule (13, 14). Next, we studied whether FRET signals can be detected in this setting. When galectin-3-TG1-Alexa 488 and -Alexa 555 were both incubated with non-labeled neutrophils and ECV cells, significant FRET signals were observed where galectin-3 molecules were concentrated, such as neutrophil-endothelial cell (white stars in Fig. 6) and endothelial-endothelial junctions (Fig. 6). In Figs. 6–8, as represented in Fig. 6B (yellow highlight), one optical xy cross-section (1-μm thick), where neutrophils adhere to endothelium, was selected for each data set, to pinpoint the interface between interacting cells.

Concentration of galectin-3 at the junctions between neutrophils and the endothelial cell layer and at the borders of endothelial cells was also observed when galectin-3 labeled with succimidyl-Alexa 546 was added to calcein-labeled neutrophils and a HUVEC layer (Fig. 7). The majority of adherent neutrophils and a significant concentration of galectin-3 were located at tricellular corners, where the borders of

FIGURE 5. Distribution of galectin-3 when incubated with neutrophils adherent to an ECV cell layer. Calcein-labeled neutrophils (PMN) (either unprimed (A–F) or primed (G–M)) and ECV cell layers were incubated with Alexa 546-labeled galectin-3 (2 μM) for the indicated times at 37 °C. After removal of unbound galectin-3, cells were fixed and mounted onto slides for observation by confocal microscopy. Fluorescence images were captured at serial optical sections (~30 sections at 1.0-μm intervals) by the FLUOWVIEW 300 confocal scanning unit, and reconstituted yz images are shown here. Representative data from three separate experiments are shown.
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Three endothelial cells intersect (Fig. 7), supporting previous observations that neutrophil transendothelial migration preferentially occurs at those corners (50, 51). Galectin-3 binding on the surface of HUVEC was also observed but was less significant compared with ECV cell layers possibly due to the thinness of the HUVEC layers. When galectin-3-TG1-Alexa 488 and -Alexa 555 were both added to HUVEC layers, FRET signals were again detected at the junctions of neutrophils and ECV cells are shown here (as illustrated by the yellow section in B). White stars indicate neutrophils bound to the endothelium. Representative data from three separate experiments are shown.

Stability of the Galectin-3 Lattice—While observing the oligomerization of galectin-3 at the surface endothelial cells and neutrophils adherent to this endothelial cell layer, we noticed the rather high stability of the lattices at the surface of endothelial cells. In fact, in live cell experiment those lattices were present on the surface of endothelial cells even after 30 min of incubation. White stars indicate neutrophils bound to endothelium. Representative data from two separate experiments are shown.

Stability of the galectin-3 lattice, FRAP experiments were undertaken. The FRAP technique enables the visualization of protein movement using fluorescence labeling (52). A small region of interest of live cells was first photobleached using high

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intensity argon-ion and helium-neon laser lines, and then the recovery of fluorescence from galectin-3-TG1-Alexa 555 and Alexa 488 non-specifically labeled surface proteins was monitored over time to estimate lateral diffusion or movement of the molecule. For practical reasons detailed before, an endothelial cell line (ECV 304) was used for FRAP experiments. As observed in adhesion experiment, galectin-3 formed lattices on the surface of ECV 304 cells with concentration of the protein between adjacent cells (Fig. 9). Fluorescence recovery from DiO-labeled membranes was as early as 95 s post-bleaching (data not shown). Only a small amount of galectin-3-TG1-Alexa 555 fluorescence, if any, was recovered 45 min post-photobleaching. In contrast, fluorescence recovery of non-specifically labeled surface proteins became evident 30 min post-photobleaching (Fig. 9). Thus, the slow fluorescence recovery of galectin-3-Alexa 555 suggests that galectin-3 lattices are rigid and that their lateral movement is slow on the surface of cells once lattices are formed.

**DISCUSSION**

Despite a lack of evidences suggesting the oligomerization of galectin-3 in biological settings, the importance of oligomerization for the various biological activities of galectin-3 is generally accepted. Thus, in this study we proceeded to show, using site-directed fluorescence-labeling of galectin-3 and FRET detection, that at physiological concentrations of proteins that galectin-3 molecules oligomerize on the surfaces of neutrophils after binding to their ligands. Galectin-3 oligomerization occurred in immunologically different conditions where galectin-3 is reported to exert its functions in neutrophils, including activation, and adhesion to the endothelium (12, 14, 27). These data, thus, demonstrate that ligand cross-linking through oligomerization on the cell surfaces induces the extracellular activities of galectin-3 in physiological settings. We also found that galectin-3 lattices are robust and rather resistant to lateral movement of cell surface once galectin-3 oligomers are formed. These findings also reinforce original works by Dennis and co-workers (47, 48) which indicate that galectin-3 lattices regulate the lateral movement and/or recycling of cell surface receptors.

To visualize the interaction between galectin-3 molecules when they bind to their oligosaccharide ligands at the cell surface, we adapted the FRET technique to our biological assays. The FRET technique enables direct visualization of protein interaction through the exchange of energy between two closely apposed proteins that presumably interact (33). FRET studies are generally conducted using proteins that are fused to green fluorescent protein variants, such as a pair of cyan and yellow fluorescent protein (53). However, the cross-talk between the excitation and emission spectra from this conventional combination has made it difficult to observe FRET signals devoid of background fluorescence (31, 54). The size of fluorescent proteins (~27 kDa) is also considerable and could interfere with assays investigating molecular interactions. Thus, when studying the extracellular functions of proteins, fluorescent dye-based labeling of proteins can replace fluorescent protein fusion proteins since proteins are exogenously added to the assays and can, thus, be manipulated before the experiment (55). Although modification of the lysine and cysteine residues of the molecules with the desired probes can affect the conformation and function of the galectin-3 molecule, these effects can be expected to be small (55).
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teine residues of the target proteins with fluorescent dyes can be carried out using conjugates of succinimidyl ester and C5-maleimide, respectively, multiple modifications of several amino groups may increase the complexity of the FRET analysis. In fact, insertion of two identical fluorescent molecules in close proximity could result in quenching of fluorescence emission due intermolecular interactions (56). Furthermore, orientation of the molecules will affect the efficacy of the energy transfer. Fluorophores need to be aligned with each other for the energy transfer to occur. Thus, we use a technique involving transglutaminase and TG1-tagged galectin-3 to site-specifically label two different sets of galectin-3-TG1 using Alexa 488-cadaverin and Alexa 555-cadaverin as substrates (36). This C-terminal labeling of galectin-3 has enabled the visualization of the interaction between galectin-3 molecules, while it mediated functions such as cellular adhesion and cellular activation. Interaction of galectin-3 with the different stages of neutrophils, as previously observed (12), led to different distribution in neutrophils. Furthermore, a FRET signal could be detected at the surface of neutrophils incubated with galectin-3, suggesting that oligomerization of galectin-3 is indeed responsible for galectin-3 roles in lattice formation and neutrophil activation. FRET signals were significantly low when truncated Alexa-labeled galectin-3 molecules, which lack the N-terminal non-lectin domain, were incubated with neutrophils even though those molecules were colocalized. These data suggest that the N-terminal domain of galectin-3 is responsible for oligomerization. A FRET signal could also be detected when galectin-3-TG1-Alexa 488 and galectin-3-TG1-Alexa 555 were incubated with neutrophils in the presence of an endothelial cell layer, again reinforcing the importance of oligomerization in galectin-3-mediated adhesion of neutrophils to endothelial cells.

Galectin lattices have been recently proposed to be involved in the molecular dynamism of receptor trafficking and receptor clustering (17, 18, 47, 48, 57). It has been reported that galectin-9 lattices are involved in the metabolism of glucose through their ability to retain glucose receptors on the cell surface (57). In fact, mice that do not express galectin-9 ligands develop type 2 diabetes as a result of a lower expression of glucose transporters on their cell surfaces. It has also been shown that galectin-3 lattices can retain cytokine receptors, such as tumor growth factor-β receptor, on the surfaces of invasive tumor cells (48) by interfering with its endocytosis (58). On the surface of T lymphocytes, galectin-3 lattices are suggested to restrict T cell receptor recruitment to the site of antigen presentation, thereby increasing the threshold for T lymphocyte activation (47). We recently found that galectin-3 interferes with the galectin-1-mediated promotion of human immunodeficiency virus attachment and infectivity in CD4 T lymphocytes even though galectin-3 alone does not have any of those activities (59). Thus, the robustness of galectin-3 lattices revealed by the FRAP technique might contribute to the various regulatory functions of galectin-3 lattices.

Galectin-3 is expressed in cells involved in the immune response (for a review, see Refs. 4–7) and is found to be largely expressed in pathogenic infections such as streptococcal pneumonia (14), which led us and others to suggest its role as a mediator of inflammation. Neutrophils, the dominant leukocyte population in the blood, play a critical role in the initial innate immune response and in inflammation. Neutrophils are also among the first leukocytes to be recruited to an affected site. The present data show that depending on the immune status and on the environment surrounding neutrophils, galectin-3 oligomerization occurs in different modes. For example, oligomerized galectin-3 molecules formed lattices on native neutrophils but not on primed neutrophils. In contrast, in the presence of vascular endothelium, oligomerized galectin-3 molecules were concentrated in the neutrophil-endothelium junction, and this concentration was independent of neutrophils status. Thus, our previous and present results suggest that galectin-3 oligomerization, upon ligand binding, is indeed involved in a wide range of its functions such as cell activation/repression and cell adhesion through three different modes of action: receptor clustering, lattice formation, and cell-cell interactions (Fig. 10). The use of these different modes of action by galectin-3 could represent its involvement in different stages and with different types of cells during the immune response. In this regard we could expect that galectin-3, in the presence of resting inflammatory cells in a healthy individual, would lead to lattice formation, as observed in unprimed neutrophils. Formation of lattices could, thus, prevent unnecessary cell activation that would be damageable to host cells. On the other hand, in the case of pathogenic infections such as lung infection by Streptococcus pneumoniae, the presence of galectin-3 could facilitate neutro-

FIGURE 10. Oligomerization of galectin-3 through three different cross-linking modes. Upon oligosaccharide binding by the CRD, the non-lectin domains of galectin-3 molecules oligomerize, thereby cross-linking ligands of the cell surface. This oligomerization of galectin-3 molecules could stand, as we previously proposed, for most of the extracellular galectin-3 functions, such as cellular adhesion (A), signal transduction through receptor clustering (B), and lattice formation (C) that we observed.
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phil transmigration through its role as an adhesion molecule. Then, when migrated neutrophils are required to clear pathogens through phagocytosis, reactive oxygen species production, and protease release through degranulation, galectin-mediated ligand clustering could trigger cell activation. Lines of evidence for galectin-3 roles in these different settings, namely cell restriction, cell adhesion, and cell activation, have all been described previously, whereas their specific mode of action had remained unknown. In this report we present evidence of such modes of action involving the oligomerization of galectin-3, which leads to lattice formation, cell adhesion, and cell activation (Fig. 10).

Acknowledgments—We acknowledge Dr. Paul De Koninck (Centre de recherche Université Laval Robert-Giffard) for advice in FRAP technique, Julie-Christine Levesque (Bio-imaging platform, Research Centre for Infectious Diseases) for advice in imaging, Dr. Masumi Taki (Okayama University) for advice in fluorescence labeling with trans-gluataminase, and Kyouko Okuyama (Yamagata University) for technical assistance.

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