Complement Receptor 3 of Macrophages Is Associated with Galectin-1-Like Protein

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We have previously identified a 16-kDa protein with a pI of 5.1 (P16/5.1) that is associated with macrophage CR3. Microsequencing of P16/5.1 indicated exclusive homology to the β-galactoside-binding lectin, galectin-1. Abs specific to a galectin-1 unique peptide reacted with P16/5.1. The association of P16/5.1 with CR3 was specifically inhibited by lactose, which binds with high affinity to galectin-1. These data together with similarities in molecular mass and pI suggest that P16/5.1 is galectin-1. Two-color immunofluorescence staining revealed the expression of galectin-1 on the macrophage surface and its colocalization with CR3. However, a surplus of CR3 was free of galectin-1, and some galectin-1 molecules were associated with cell surface receptors other than CR3. Based on these results we propose two models depicting the functional significance of CR3-galectin-1 association: 1) homodimeric galectin-1 possessing a divalent sugar binding site may act as an extracellular adapter molecule that cross-links CR3 with other receptors; and 2) association of galectin-1 with β-galactosides on the extracellular domain of CR3 may modify the binding affinity of the receptor to its ligand. These possibilities are not mutually exclusive and can clarify the mode by which CR3 transmits signals in macrophages. The Journal of Immunology, 1998, 160: 6151–6158.

Complement receptor 3 (CR3) is a heterodimer of α (CD11b) and β (CD18) transmembrane glycoproteins. While the α-chain is unique to CR3, the β subunit is shared by LFA1, CR4, and α(v)β3 (1, 2), all belonging to the β2 integrin leukocyte receptor family. CR3 is mainly expressed on macrophages, monocytes, granulocytes, and NK cells, playing a role in cell-cell and cell-matrix interactions. It enables the establishment of leukocyte adherence to the endothelium and facilitates movement of leukocytes through the endothelial intercellular junctions (3, 4; reviewed in Ref. 5). CR3 is also involved in phagocytosis (6), cellular killing by oxidative burst (7), and it regulates homoeostasis of inflammation by mediating apoptosis of extravasated neutrophils (8). The multiple activities of CR3 depend upon its ability to interact with a wide variety of ligands, such as iC3b, fibrinogen, ICAM-1, ICAM-2, zymosan, LPS, factor X, haptoglobin, and oligodeoxynucleotides (9–19).

A dynamic regulation of CR3 function allows rapid on and off switches of receptor adherence to some of its ligands (reviewed in Refs. 20–22). Such modifications in CR3 occur following activation of other cell surface molecules, such as selectins and receptors for chemotaxants and cytokines (23–26). These receptors transmit signals into the cell, leading to conformational changes in CR3 that convert it to an active adhesive form (“inside-out” signaling). In its active form, CR3 can bind specific ligands, which, in turn, induce a cascade of “outside-in” signaling events (27, 28, reviewed in Ref. 29). Recent data suggest that CR3 can also transmit signals emanating at glycosylphosphatidylinositol (GPI)3-linked proteins such as FcγRIIB, CD14 (receptor for LPS), and urokinase plasminogen activator receptor (uPAR) (reviewed in Ref. 30). It was speculated that these GPI-anchored proteins, which are devoid of a transmembrane domain, trap the ligand while floating in the membrane lipid bilayer and transmit inflammation signals via co-associated CR3 molecules. Accordingly, CR3 has been termed a public transducer (30).

Even though CR3 is a bidirectional signaling molecule, its α- and β-chains possess short cytoplasmic domains (22 and 46 amino acids, respectively) devoid of an intrinsic catalytic activity (31; reviewed in Ref. 32). Cumulative evidence suggests that CR3 signaling is mediated either via other cell surface receptors, such as FcγRII (33, 34), or through intracellular molecules, such as cytoskeleton-associated proteins (35, 36), acting as initiators of the signaling cascade. In an attempt to clarify how CR3 functions as a signal transducer, we searched for CR3-associated molecules. In our previous study (37) we reported about the characterization of a 16-kDa protein with an isoelectric point of 5.1 that is specifically coimmunoprecipitated with CR3 of murine macrophages. We designated this protein P16/5.1. In the present study we identify P16/5.1 as galectin-1-like protein and demonstrate a novel mode of CR3 interaction with a secreted lectin.

Materials and Methods

Mice

C57BL/6 female mice were obtained from Harlan Sprague-Dawley (Indianapolis, IN) and maintained at the specific pathogen-free unit of the Hebrew University-Hadassah Medical Center (Jerusalem, Israel).

Cell lines

CCL1 (L-929) is a CSF-1-secreting fibroblast line. RAW-309-Cr.1, J774A.1, and P388D1 are macrophage cell lines obtained from the American Type Culture Collection (Manassas, VA). The cells were grown in DMEM supplemented with 10% FCS, 5 mM HEPES, 1 mM glutamine, and antibiotics.

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Abbreviations used in this paper: GPI, glycosylphosphatidylinositol; uPAR, urokinase plasminogen activator receptor; BMIMβ, bone marrow macrophages; 2D, two-dimensional; IEF, isoelectric focussing; PVDF, polyvinylidene difluoride; KLH, keyhole limpet hemocyanin; LAMP, lysosome-associated membrane protein.
Bone marrow macrophages (BMMφ)

BMMφ were prepared as previously described (38). Briefly, bone marrow cells were removed from C57BL/6 mouse femurs and cultured in miniplast bacteriologic grade culture dishes (BCD, Ein-Shemer, Israel) in DMEM supplemented with 15% FCS, 5% horse serum (Bio-Lab, Jerusalem, Israel), 30% L-929 conditioned medium, 1 mM glutamine, 5 mM HEPES, and antibiotics (BMMφ medium). The medium was replaced twice a week, and the cells were harvested 1 to 2 wk after plating.

Monoclonal Abs

Hybridomas secreting anti-CD11b (M1/70-TiB128), anti-CD11b (5C6-CRL 1969), and anti-CD18 (M18/2.a.8-T1B 218) Abs were obtained from American Type Culture Collection. Anti-F4/80 Abs were obtained from Serotec (Oxford, U.K.). Hybridoma secreting anti-Thy-1.2 Abs (G7) was provided by Dr. Ethan Shevach, National Institutes of Health (Bethesda, MD) (39). mAbs were affinity purified on protein G-Sepharose beads (Pharmacia, Uppsala, Sweden). For staining, rhodamine (tetramethylrhodamine B isothiocyanate (TRITC))-conjugated F(ab')2 donkey anti-rabbit IgG (H+L) Abs and FITC-conjugated F(ab')2 donkey anti-rat IgG (H+L) Abs were used. Mouse Fc fragments (Jackson ImmunoResearch, West Grove, PA) were used for blocking of FcγR.

Metabolic labeling

Macrophages (3 × 10⁶ cells/ml) were plated in methionine/cysteine-free DMEM and pulsed with a 0.1 mCi/ml [³⁵S]methionine/cysteine labeling mixture (DuPont-New England Nuclear, Boston, MA) for 16 h in the absence or the presence of the indicated sugar (0.15 M). Labeled cells were harvested, washed, and processed for immunoprecipitation.

Immunoprecipitation

For immunoprecipitation of CR3 and co-associated molecules, protein G-Sepharose beads (Pharmacia, Piscataway, NJ) were preincubated for 2 h at 4°C with 5 mg of rabbit Abs directed against the Fc region of rat IgG. The beads were washed and incubated for an additional 2 h at 4°C with a combination of Abs as specified. Ab-coated beads were washed with PBS and kept at 4°C. In parallel, [³⁵S]-labeled macrophages (1.6 × 10⁷/ml) were lysed for 30 min at 4°C with lysis buffer comprised of 1% Triton X-100, 2 mM PMSF, 20 mM iodoacetamide, 0.4 U/ml aprotinin, 20 mM leupeptin, 1 mM pepstatin, 10 mM NaF, 5 mM sodium pyrophosphate, 0.5 mM sodium orthovanadate, and 50 mM HEPES at pH 7.4. In some experiments, the lysis buffer contained 0.2 M of the indicated sugar. The soluble cell fraction was sequentially immunoprecipitated with beads precoated with control Abs followed by specific Abs. For purification of P16/5.1, the immunoprecipitation procedure was 10-fold up-scaled.

Two-dimensional (2D) gel electrophoresis

Immunoprecipitated proteins were eluted from the beads and subjected to 2D isoelectric focusing (IEF)-SDS. In the first dimension, proteins were separated on a 12% gel, and in the second dimension, separation was based on molecular mass. Briefly, IEF tube gels (5% acrylamide) containing two ampholites of pI 5 to 7 (1.6%) and pI 3.5 to 10 (0.4%); Sigma, St. Louis, MO) were prerun, after which samples were applied and resolved for 19 h at 400 V and for 1 h at 1000 V. The gel tubes were reduced with 30 mM dithiothreitol, laid on top of a discontinuous (10–20%) gradient of SDS-polyacrylamide gel, and resolved at 30 V. The separated, radiolabeled proteins were transferred onto a nitrocellulose membrane and exposed to Kodak XAR-8 films (Eastman Kodak, Rochester, NY).
**Microsequencing analysis**

For purification of P16/5.1, a large scale immunoprecipitation was performed, proteins were resolved on 2D IEF-SDS gels and transferred onto a polyvinylidene difluoride (PVDF) membrane (Bio-Rad, Richmond, CA). Visualization and quantification of the protein that corresponded to P16/5.1 were performed using Ponceau-s staining of the membrane. Subsequently, 1 μg of P16/5.1 protein bound to the PVDF membrane was subjected to microsequencing analysis performed at the Protein Research Center at the Technion (Haifa, Israel).

**Preparation of anti-galectin-1 Abs**

Anti-galectin-1 Abs were raised in rabbits by an initial s.c. injection of 1 mg of galectin-1 peptide (CLKVGRVEVASDAKS) coupled to KLH, in CFA. During the following 2 mo, the rabbits were boosted twice with 1 mg of peptide-KLH in IFA. Antiserum was collected 2 wk after the final injection.

**Western blot analysis**

Following 2D IEF-SDS gel electrophoresis, separated proteins were transferred onto PVDF membrane. The latter was incubated with anti-galectin-1 antisera (1/150 dilution) or normal rabbit serum. Proteins were detected by using protein A-horseradish peroxidase and enhanced chemiluminescence (Pierce, Rockford, IL).

**Preparation of F(ab’)_2**

F(ab’)_2 were prepared from rabbit antisera after ammonium sulfate precipitation and digestion with pepsin (Sigma; 5 mg enzyme/mg protein) for 16 h at 37°C. Uncleaved Abs were removed by affinity chromatography on protein A-Sepharose beads. Purity of the F(ab’)_2 was confirmed by analysis in nonreducing and reducing SDS-PAGE followed by staining with Coomassie brilliant blue.

**Immunofluorescence**

Macrophages (2 × 10^5) were plated on coverslips for 16 h. Adherent cells were rinsed with washing buffer (PBS containing 2% BSA and 0.05% NaN₃) and incubated with 20 μg of murine Fc fragments in 50 μl of washing buffer for 60 min at 4°C to block FcRs. Subsequently, cells were incubated at 4°C with 5 μg of the indicated first Ab, followed by incubation with fluoresceinated or rhodaminated second Ab. In the co-capping experiments, double staining was performed simultaneously at 4°C, and the washing buffer was devoid of NaN₃. Following incubation with the corresponding labeled second Abs at 4°C, the cells were transferred to 37°C for 10 min. The cells were fixed with 1% formaldehyde in PBS for 20 min at room temperature, and the coverslips were mounted on slides. Immunofluorescent cells were analyzed and photographed using a UV microscope attached to a camera (Zeiss, Jena, Germany) or a confocal fluorescence microscope (LSM-410 Zeiss).

**Results**

**Microsequencing analysis of P16/5.1**

We have previously demonstrated that P16/5.1 is a novel protein associated with CR3 (CD11b/CD18), but not with CR4 (CD11c/CD18) (37). It is specifically coimmunoprecipitated from BMMΦ and J774A.1 cells using a combination of three anti-CR3 mAbs: M1/70 and 5C6 (anti-CD11b) and M18/2.9.8 (anti-CD18; Fig. 1). In an attempt to characterize P16/5.1, we performed a large scale immunoprecipitation of CR3 from BMMΦ using the same combination of anti-CR3 Abs. Proteins recovered from the immunoprecipitate were resolved on a 2D IEF-SDS gel and transferred onto a PVDF membrane. The P16/5.1 was located by staining the membrane with Ponceau-s (data not shown). The PVDF membrane piece containing the P16/5.1 protein was excised and subjected to microsequencing analysis. Two P16/5.1 peptides were obtained after enzymatic cleavage, one of seven amino acids and the other of nine amino acids. The amino acid sequence of these peptides displayed exclusive homology to the murine lectin, galectin-1 (Fig. 2).

**Reactivity of P16/5.1 with anti-galectin-1 Abs**

To confirm the homology between P16/5.1 and galectin-1, anti-galectin-1 Abs were prepared. To this end, we synthesized a 14-amino acid galectin-1 peptide (Fig. 2) corresponding to an immunodominant region predicted by the crystallographic model of bovine galectin-1 (Protein Data Bank (PDB), Brookhaven, NY) (41). The peptide was conjugated to a carrier protein (KLH) and injected into rabbits for Ab generation. Western blot analysis revealed that antisera of the immunized rabbits contained anti-galectin-1 Abs, which exclusively recognized the P16/5.1 protein (Fig. 3). Preimmune rabbit serum did not react with P16/5.1 (data not shown). This finding indicates that P16/5.1 is galectin-1. The possibility that P16/5.1 is a novel protein that shares a high degree of homology with galectin-1 is unlikely, but cannot be excluded.

**Galectin-1 binds to CR3 through lectin-carbohydrate interactions**

We analyzed whether galectin-1 associates with CR3 via its carbohydrate binding domain. To this end we used lactose, which binds with high affinity to galectins, and tested its effect on CR3-galectin-1 interaction. In these experiments, BMMΦ were metabolically labeled in the presence or the absence of lactose or sucrose (for control). Treated cells were lysed with buffer containing the corresponding sugar. CR3 was immunoprecipitated and resolved on 2D IEF-SDS gels. As shown in Figure 4A, when BMMΦ were grown in medium with no addition of sugars, and galectin-1 was specifically immunoprecipitated with anti-CR3 Abs, but not with nonspecific Abs (Fig. 4B). This association was specifically interrupted by lactose (Fig. 4D), but not by sucrose (Fig. 4C).
CR3 and galectin-1 colocalize on the macrophage cell surface

Galectin-1, like other members of the galectin family, lacks a signal peptide and is found in the cytosol. It can, however, be exported to the cell surface and extracellular matrix via a nonclassical secretion pathway (42–44). To determine whether galectin-1 is expressed on the macrophage cell surfaces, we used F(ab’)2 of anti-galectin-1 Abs and the macrophage line RAW-309-Cr.1, from which CR3 and P16/5.1 are coimmunoprecipitated (37). As shown in Figure 5A, galectin-1 is expressed on the macrophage cell surface, and both galectin-1 and CR3 (Fig. 5B) staining displayed a dispersed membrane distribution. We next assessed whether cell surface-expressed galectin-1 is associated with CR3 by double staining and co-capping. Cells were incubated simultaneously with anti-CR3 and anti-galectin-1 Abs, followed by secondary Abs conjugated with FITC and TRITC, respectively (Fig. 6, upper panel).

The dominant yellow patches that appeared on the merged images (Fig. 6C) indicated colocalization of CR3 (Fig. 6B, green) and galectin-1 (Fig. 6A, red). Control, double staining of F4/80 (Fig. 6E) and galectin-1 (Fig. 6D) demonstrated no colocalization of these two membrane proteins (Fig. 6F). A similar pattern of double staining was recapitulated in BMMφ (Fig. 6, lower panel).

Control staining with nonspecific Abs (rat anti-mouse Thy.1 or normal rabbit serum) and analysis of cross-reactivity among the various Abs used in the experiments yielded negative results. Likewise, staining of the P338D1 macrophage cell line, in which CR3 is not associated with galectin-1 (37), yielded negative results.

To determine whether all the cell surface CR3 molecules are associated with galectin-1, high resolution confocal fluorescence microscope was employed. We first stained the macrophage line J774A.1 for galectin-1 by red fluorescence (Fig. 7C) and incubated the cells at 37°C to allow redistribution. The cells were then fixed and stained for CR3 expression by green fluorescence (Fig. 7D).

Superimposed yellow patches represent colocalization of CR3 and galectin-1, whereas green patches indicate an excess of CR3 that does not colocalize with galectin-1 (Fig. 7, A and B). We then asked whether all galectin-1 molecules expressed on the cell surface are associated with CR3. To answer this question, we reversed the order of staining, with CR3 first (Fig. 7G) followed by galectin-1 (Fig. 7H). The red staining pattern shown in the merged images (Fig. 7, E and F) demonstrates a surplus of galectin-1 molecules not associated with CR3. An identical pattern of staining was observed with BMMφ (data not shown). This finding suggests that galectin-1 associates with other cell surface molecules in addition to CR3.

Discussion

In the present study we demonstrate that the CR3 coassociated molecule, P16/5.1, is homologous to galectin-1. This finding
FIGURE 6. Galectin-1 and CR3 are colocalized on the cell surface of macrophages. RAW-309-CR.1 cells and BMMφ were saturated with mouse Fc fragments (20 mg/ml). The cells were then double stained at 4°C for detection of galectin-1 (red, A) and CR3 (green, B). Control staining was performed for galectin-1 (red, D) and F4/80 (green, E). The cells were then incubated for 10 min at 37°C, to allow redistribution of receptors. The superimposed yellow patches indicate colocalization of galectin-1 and CR3 (C). No colocalization of galectin-1 and F4/80 was observed (F).
emerged from our previous experiments demonstrating coimmunoprecipitation of a novel protein, P16/5.1, with macrophage CR3 (37) (Fig. 1). The identification of P16/5.1 as galectin-1 is based on the following data: 1) microsequencing of two P16/5.1-derived peptides revealed exclusive homology to galectin-1; 2) polyclonal anti-galectin-1 Abs specifically recognized P16/5.1; and 3) the reported molecular mass (14.7 kDa) and pl (5.3) of galectin-1 are similar to those of P16/5.1. This experimental evidence indicates identity between P16/5.1 and galectin-1. It does not, however, rule out the possibility that P16/5.1 is a novel protein sharing a high degree of homology with galectin-1.

Galectins are β-galactoside binding proteins. There are eight known members in this family of lectins that appear in low (e.g., galectin-1) and high (e.g., galectin-3) molecular masses (reviewed in Refs. 45–48), of which galectin-1 and galectin-3 are the most studied proteins. Galectin-1 appears as a monomer or as a non-covalently associated homodimer. Each of its subunits folds into a compact globular structure, forming a carbohydrate binding site. Galectin-3 is a monomer composed of several domains, one of which is homologous to the galectin-1 carbohydrate binding site (reviewed in Ref. 49). Although the functions of galectins are not fully understood, they have been found involved in growth regulation, cell adhesion, and cell migration. Galectin-1 is abundant in muscle, neurons, thymus, kidney, and placenta. Of the galectin family, only galectin-3 and a 16-kDa galectin-like protein (50) were found in macrophages.

In our current study we demonstrate that galectin-1 is expressed on the cell surface of macrophages in association with CR3. This association is mediated via the galectin-1 carbohydrate binding site. These findings raise the question of the functional significance of galectin-1-CR3 association in macrophages. We discuss herein some possible models based on our own results and on data reported by others.

CR3 is involved in multiple activities that are imperative for the development of innate immunity. The dynamic communication of CR3 with other receptors that are involved in acute inflammation is mandatory for some of its functions. Such CR3-associated receptors are FcγR (51), CD14 (52), uPAR (53, 54), and CD63 (one of the major lysosome-associated membrane proteins (LAMPs)) (55). Based on the versatility of CR3 interactions, we propose a model depicting galectin-1 as an extracellular adapter molecule (Fig. 8A). Homodimeric galectin-1 possesses a divalent carbohy-
drate binding site that cross-links polylectosamines and can potentially bridge between CR3 and other cell surface expressed receptors. This hypothesis is corroborated by the observations that galectin-1 and CR3 are associated on the macrophage cell surface, and that additional receptors are probably involved in this type of interaction.

Previous studies demonstrated that galectin-1 interacts with glycoconjugates on the cell surface and extracellular matrix, including the integrin α5β1, LAMP-1, LAMP-2, fibronectin, and laminin (56–60). The present study is the first to identify CR3 as a galectin-1 glycoconjugate. However, additional macrophage galectin-1 glycoconjugates presumably exist, but have not as yet been identified. Evidence in the literature supports the idea that receptors associated with CR3 are possible galectin-1 glycoconjugates. Some examples are as follows: 1) CD63, a CR3-associated molecule, belongs to the LAMP family, of which some members are galectin-1 glycoconjugates. Hence, an interaction between CD63 and galectin-1 can be envisaged. 2) Galectin-3 interacts with FcγR, some examples are as follows: 1) CD63, a CR3-associated molecule, belongs to the LAMP family, of which some members are galectin-1 glycoconjugates.

We thank Drs. Hanah Margalit and Arie Admon for their assistance and activities. The present study is the first to identify CR3 as a galectin-1 glycoconjugate. However, additional macrophage galectin-1 glycoconjugates presumably exist, but have not as yet been identified. Evidence in the literature supports the idea that receptors associated with CR3 are possible galectin-1 glycoconjugates. Some examples are as follows: 1) CD63, a CR3-associated molecule, belongs to the LAMP family, of which some members are galectin-1 glycoconjugates.

Acknowledgments

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References

ASSOCIATION OF GALECTIN-1-LIKE PROTEIN WITH CR3


