Glycobiology vol. 12 no. 2 pp. 111–118, 2002

Regulation of galectin-9 expression and release in Jurkat T cell line cells

Sophie Chabot[2,3], Yumiko Kashio[2], Masako Seki[2], Yukako Shirato[2], Kazuhiro Nakamura[2], Nozomu Nishi[4], Takanori Nakamura[4], Ryoji Matsumoto[5], and Mitsuomi Hirashima[1,2]

[2]Department of Immunology and Immunopathology, Kagawa Medical University, 1750-1 Ikenobe, Miki-Cho, Kita-gun, Kagawa 761-0793, Japan; [3]Department of Neuroscience, University of Calgary, Calgary, Alberta, Canada; [4]Department of Endocrinology, Kagawa Medical University, 1750-1 Ikenobe, Miki-Cho, Kita-gun, Kagawa 761-0793, Japan; and [5]Department of Immunology, Mayo Clinic, Rochester, MN 55905

Received on July 18, 2001; revised on September 13, 2001; accepted on September 14, 2001

Ecalectin/galectin-9 was recently described as a novel eosinophil chemoattractant highly expressed in immune tissues. We investigated the regulation of galectin-9 expression and release in Jurkat (a T cell line) cells. We demonstrated that medium and long-sized galectin-9 isoforms were constitutively expressed, and phorbol 12-myriastate 13-acetate (PMA) upregulated the level of galectin-9 mRNA in Jurkat cells. Western blotting and flow cytometry analyses revealed that PMA stimulation resulted in the upregulation of both intracellular and surface galectin-9 protein. The stimulated Jurkat cells simultaneously released evident eosinophil chemoattractant activity (ECA). Main ECA was adsorbed by both lactose and anti-galectin-9 antibody affinity column, suggesting that the ECA was ascribed to galectin-9. When Jurkat cells were stimulated with PMA in the presence of a BB94, a matrix metalloproteinase (MMP) inhibitor, but not tissue inhibitor of metalloproteinase-1 (TIMP-1), the release of galectin-9 was suppressed in a dose-dependent manner. We further found that calphostin c, a protein kinase c (PKC) inhibitor, weakly but significantly suppressed the release of galectin-9. The present data suggested that galectin-9 production in Jurkat cells is provoked by the stimulation with PMA and that some MMP and PKC is, at least, partly involved in the release of galectin-9 from Jurkat cells.

Key words: eosinophils/galectin-9/human/Jurkat/PMA

Introduction

Galectins are S-type lectins that are defined by two characteristic features: affinity for β-galactoside and a conserved specific sequence motif called lectin domain (Paroutaud *et al.*, 1987). In contrast to C-type lectins, its carbohydrate-binding capacity is calcium independent (Perillo *et al.*, 1998). All galectins have at least one characteristic carbohydrate recognition domain (CRD) that consists of about 135 amino acids (Gitt and Barondes, 1986; Caron *et al.*, 1990). To date, 12 members of galectins (galectin-1 to -12) have been cloned, sequenced, and functionally characterized to a certain extent in mammals. Indeed, galectins appear to play a role in diverse biological processes, including cell adhesion, cell proliferation/cell death, and chemoattraction (Paroutaud *et al.*, 1987; Oda *et al.*, 1991; Barondes *et al.*, 1994; Yang *et al.*, 1996; Matsumoto *et al.*, 1998).

However, the mechanisms by which galectins exert their diverse effects are largely unknown. Galectin members have been identified in a wide variety of cell types, and they are localized in the cytoplasm and in the nucleus of cells, or attached to the extracellular matrix (Moutsatsos *et al.*, 1987; Sato *et al.*, 1993). So far, the secretory pathway of galectins remains unknown. One of the reasons is that all known galectins lack signal peptides necessary for their insertion into the endoplasmic reticulum membrane and its subsequent secretion via the classical pathway (Cleves *et al.*, 1996). Recently, a sequence in the N-terminal domain of galectin-3 has been proposed as a determinant for its secretion (Menon and Hughes, 1999). However, the presence of such secretion signal sequence in other galectins and the precise mechanism of galectin-3 secretion remain unknown.

Galectin-9, first cloned from tissues of Hodgkin's disease (Tureci *et al.*, 1997), has two CRDs connected by a linker peptide. Wada and Kanwar (1997) and Wada *et al.* (1997) demonstrated that it causes the apoptotic death of thymocytes in mouse, suggesting a role in the process of negative selection occurring during T cell development. We have recently described that a T cell line–derived ecalectin/galectin-9 (Hirashima *et al.*, 1991, 1992) has a novel eosinophil chemoattractant activity (ECA) *in vitro* and *in vivo* (Matsumoto *et al.*, 1998). We have also shown that divalent galactoside-binding activity of galectin-9 is required for eosinophil chemoattraction (Matsushita *et al.*, 2000). Galectin-9 is highly expressed in tissues of the immune system, such as bone marrow, spleen, thymus, and lymph nodes (Wada and Kanwar, 1997; Matsumoto *et al.*, 1998).

Eosinophils are one of the major effector cells in allergic diseases, such as asthma and allergic rhinitis. Eosinphil chemoattractants are required for preferential eosinophil accumulation in such allergic inflammation sites. Therefore it is conceivable that galectin-9 is an attractive target for the development of new therapeutic strategies designed to treat eosinophil-dependent pathological conditions due to its ability to selectively attract eosinophils. An understanding of galectin-9 biology is an essential step toward this goal; this obviously includes the characterization of galectin-9 expression at the cellular level. The purpose of this study is to investigate

[1]To whom correspondence should be addressed

© 2002 Oxford University Press

the regulation of galectin-9 expression and its release from Jurkat cells during activation.

Results

Description of galectin-9 isoforms

Using sequencing analysis to detect the presence of galectin-9 cDNA in Jurkat cells, we identified 3 isoforms of galectin-9 that differ only in the length of their linker peptide region (Figure 1). The short-sized isoform of galectin-9 was found to have a linker peptide region of 14 amino acids, whereas the medium and the long-sized isoform of galectin-9 have a linker peptide region of 26 and 58 amino acids, respectively. The calculated molecular weight of each galectin-9 isoform was determined from amino acid sequences by computer analysis and was estimated to be of 34.7, 35.9, and 39.5 kDa. As previously demonstrated, galectin-9 is a variant of the 36-kDa isoform of galectin-9, which is now identified as the medium isoform of galectin-9. The existence of the long-sized isoform of galectin-9 has already been established, and it is highly expressed in the intestine (GenBank accession number AB006782).

Lipkowitz *et al.* (2001) recently reported the genomic structure of human galectin-9/urate transporter. Their results indicated that the three isoforms of galectin-9 are generated from a single gene by alternative splicing. In our preliminary experiments, reverse transcriptase polymerase chain reaction (RT-PCR) was used to detect levels of galectin-9 mRNA in Jurkat cells. Using primers that we have generated, two cDNA PCR products of different size (483 bp and 560 bp) were amplified by RT-PCR. Sequencing of the PCR products revealed that those bands correspond to the medium and long-sized isoforms of galectin-9, respectively, as determined by Blast Search. Even though cells may have the cDNA for the short-sized isoform of galectin-9, the presence of its mRNA could not be detected.

Effects of phorbol 12-myriastate 13-acetate (PMA) on galectin-9 mRNA expression

Experiments were done to clarify the mechanisms of galectin-9 release from Jurkat cells. First, time-course assay of galectin-9 mRNA expression was performed. Figure 2 showed that Jurkat cells constitutively expressed galectin-9 mRNA, and that the mRNA levels of both the medium and long-sized galectin-9 were slightly decreased after 6 h of treatment with PMA, but the levels of both galectin-9 mRNA were increased after 12 h of the treatment (Figure 2). The mRNA levels of galectin-9 were still higher than that found in untreated cells even at 24 h after the treatment (Figure 2). The effect on galectin-9 mRNA expression was shown to be much more evident in the long-sized galectin-9 isoform (Figure 2).

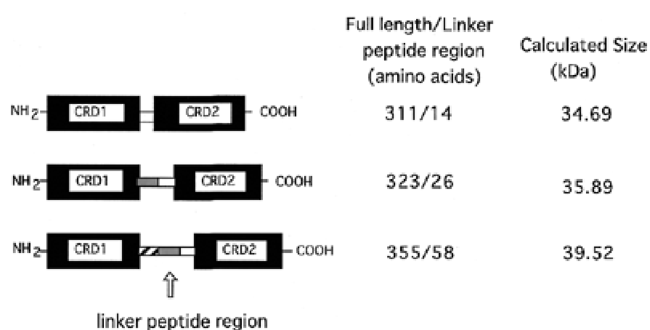

Fig. 1. Schematic representation of galectin-9 isoforms. Sequencing analysis demonstrated that there were three isoforms of galectin-9 that differ in the length of their linker peptide region.

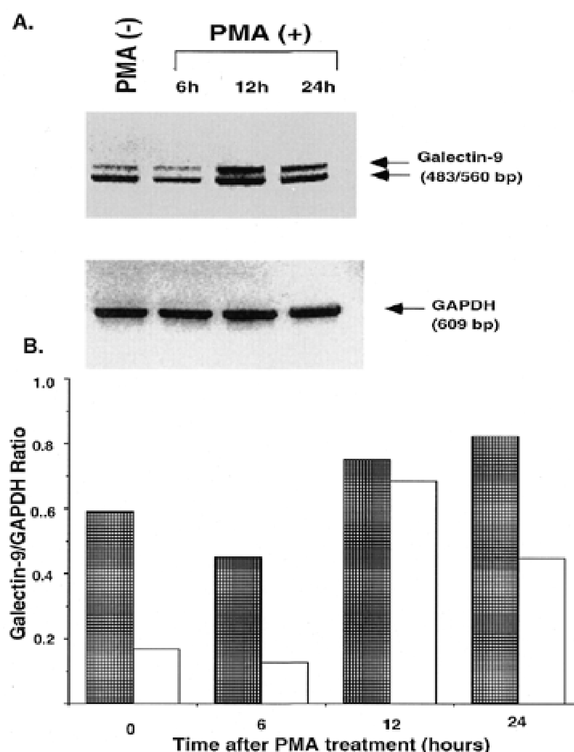

Fig. 2. Time course assay of PMA effect on galectin-9 mRNA in Jurkat cells. PMA treatment induced the increment of galectin-9 mRNA levels in Jurkat cells. (**A**) Quantification of galectin-9 band intensity is shown. (**B**) Band intensity of the medium galectin-9 (shaded bars); and that of the long-sized galectin-9 (clear bars). Representative data from four experiments was shown.

Galectin-9 protein expression in Jurkat cells

Following the generation of a galectin-9 polyclonal antibody that recognizes the C-terminal of galectin-9, we performed western blot analysis to detect the protein levels of galectin-9 in lysates of Jurkat cells. Like the RT-PCR results, immunoblotting revealed that the medium and long-sized galectin-9 were present in Jurkat cells even under resting conditions (Figure 3A). The results obtained from RT-PCR and western blot analysis where galectin-9 mRNA levels correlate with protein levels suggest that there is a constant and functionally active production of galectin-9 in Jurkat cells.

The presence of an additional band of 42 kDa was also observed (Figure 3A). To determine whether this band was of nonspecific nature, we preabsorbed the antibody with glutathione-S-transferase (GST)-galectin-9 fusion protein prior to immunoblotting. As expected, a great reduction in the intensity of specific bands (36 and 39.5 kDa) was observed, but the intensity of the 42-kDa band was also attenuated (data not shown). This data suggested that the 42 kDa protein may

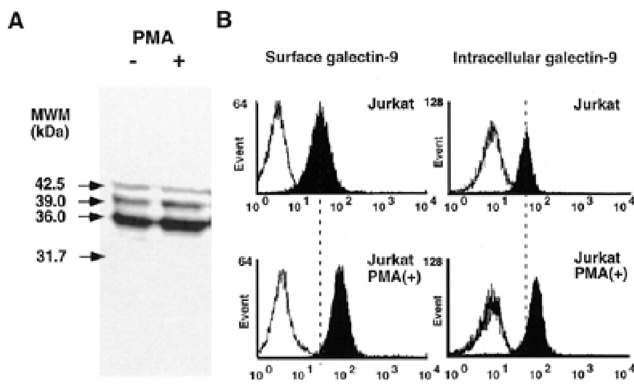

Fig. 3. Galectin-9 protein expression in Jurkat cells. (**A**) Western blot analysis performed on cell lysates of Jurkat cells demonstrates that there is a constitutive protein expression of medium and long-sized galectin-9 isoforms of 36 and 39 kDa, respectively. PMA treatment increased the expression of the long-sized galectin-9 isoform (39 kDa). (**B**) Flow cytometry analysis demonstrates that only Jurkat cells express high levels of surface galectin-9. PMA significantly induced the expression of surface galectin-9 in Jurkat cells. Representative data of four experiments was presented.

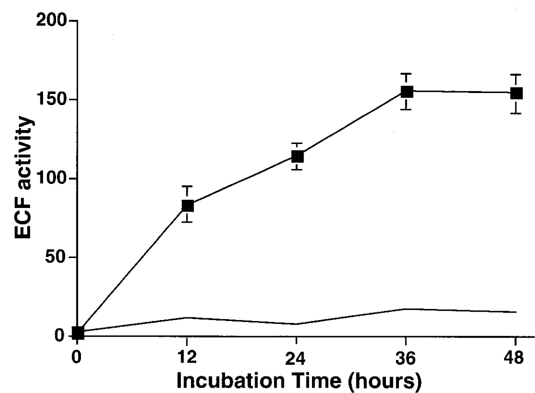

Fig. 4. Time course assay of PMA effect on ECA release in Jurkat cells. ECA release from Jurkat cells was induced by PMA in a time-dependent fashion. (Squares) ECA from PBS-treated cells, and that from PMA-treated cells (line). Data represent the mean ± SEM of three experiments in triplicate.

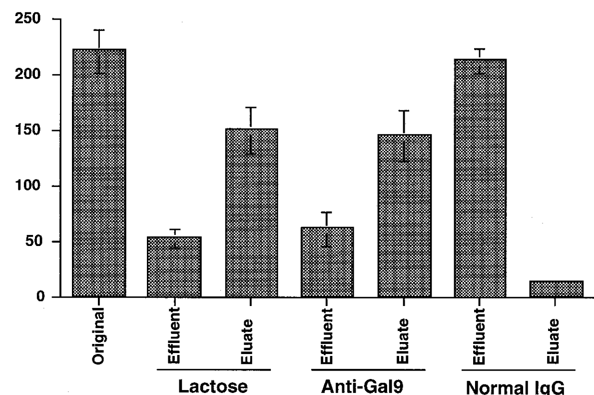

Fig. 5. Affinity chromatography of ECA from PMA-stimulated Jurkat cells. ECA from Jurkat cells was adsorbed by lactose and anti-galectin-9 antibody. Effluent and eluate of respective affinity column were assessed for chemotaxis. Data represent the mean ± SEM of three experiments in triplicate.

correspond to a galectin-9-related protein of unknown nature, or to a modified form of the long-sized isoform of galectin-9. As also shown in Figure 3A, increased levels of galectin-9 protein expression were detected in PMA-treated Jurkat cells.

Flow cytometry analysis was used to assess the levels of galectin-9 intracellular and surface expression. Figure 3B showed that even under resting conditions Jurkat cells expressed high levels of intracellular galectin-9 in the cytoplasm and that intracellular galectin-9 level was expectedly upregulated in Jurkat cells after PMA stimulation (Figure 3B). Figure 3B showed that Jurkat cells also expressed high levels of galectin-9 on their surface even under resting conditions and that this level was enhanced by PMA.

ECA release from Jurkat cells

Experiments were done to clarify whether galectin-9 exhibiting ECA was released by PMA stimulation from Jurkat cells. Figure 4 showed that the supernatants of untreated Jurkat cells exhibit negligible ECA. Evident ECA was released from Jurkat cells by PMA stimulation in a time-dependent manner (Figure 4). When the culture supernatants were applied on a lactose affinity column, we failed to detect evident ECA in the effluent fraction (Figure 5). After elution with 0.2 M lactose, the eluate fraction was dialyzed against phosphate buffered saline (PBS). We thus found that evident ECA was recovered in the eluate fraction but not in the effluent fraction (Figure 5). The results suggested that main ECA in the culture supernatants of PMA-stimulated Jurkat cells has β-galactoside-binding capacity.

The culture supernatants were also applied on an anti-galectin-9 antibody-conjugated column, and eluted with 0.1 M glycine-HCl (pH 2.5) and 10% ethylene glycol. After dialysis against PBS, ECA of the effluent and the eluate fractions were assessed. Figure 5 shows that ECA was adsorbed by anti-galectin-9 antibody but not by normal IgG, suggesting that main ECA from PMA-stimulated Jurkat cells is ascribed to galectin-9.

Adsorbed fractions on anti-galectin-9 column were further assessed for western blot analysis and ECA activity. Figure 6A shows that galectin-9 is eluted in two different fractions: one was recovered in the fraction 3 to 5, and another in the fraction 7 to 9. Main molecular weight of the galectin-9 in the first fraction was about 36 kDa and that in the second fraction was about 39 kDa, corresponding to the medium and the long-sized isoforms of galectin-9, respectively. ECA was also assessed using those fractions after dialysis against PBS. Figure 6B showed that comparable ECA was detected in the 10-times diluted first and second fractions. However, when those fractions were 50-times diluted, the ECA in the first fraction decreased significantly though that of the 50-times diluted second fractions exhibited comparable ECA to 10-times diluted second fraction (Figure 7B). These results suggest that the long-sized isoform of galectin-9 exhibits more evident ECA than the medium isoform of galectin-9, and avidity of the long-sized isoform of galectin-9 is higher than the medium isoform of galectin-9.

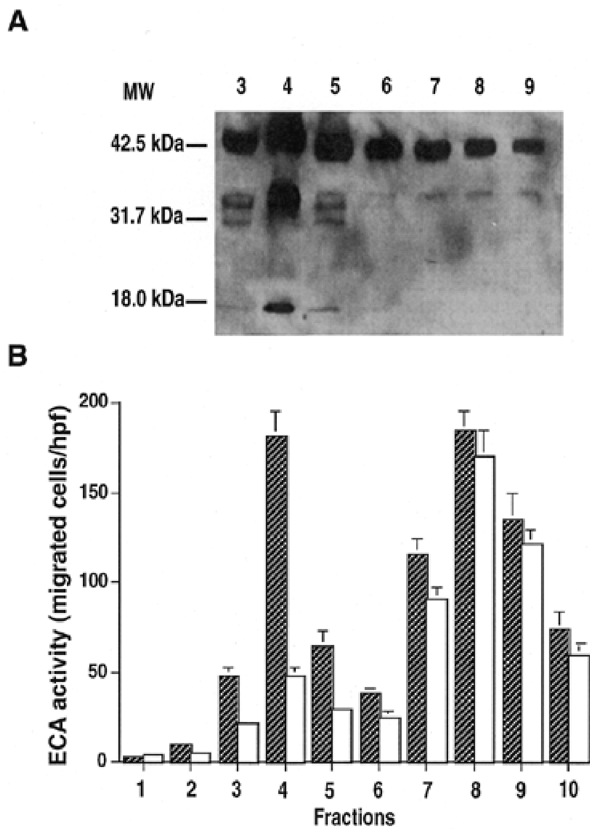

Fig. 6. Immunoaffinity chromatography. (**A**) Western blot analysis revealed that galectin-9 is eluted in two different fractions. In the first fraction, the medium galectin-9 is eluted, and the long-sized galectin-9 is eluted in the second fraction. (**B**) ECA activity of the eluted fractions after immunoaffinity column chromatography. ECA activity of 10-times diluted ECA (shaded bars); that of 50-times diluted one (clear bars). Data represent the mean ± SEM of three experiments in triplicate.

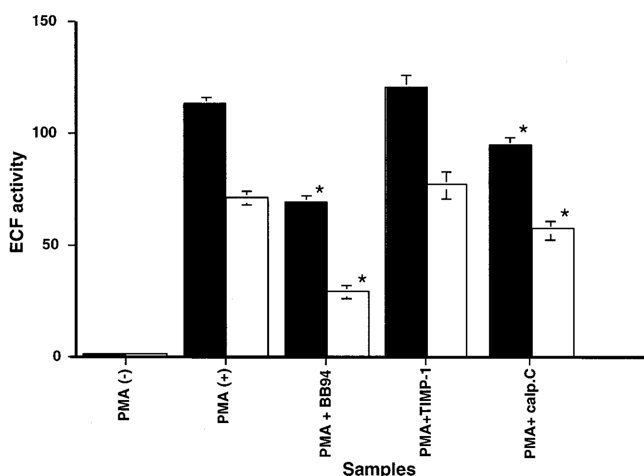

Fig. 7. Suppression of ECA release from Jurkat cells by BB-94 and calphostin c. Treatment of PMA-stimulated Jurkat cells with BB-94 and calphostin c suppressed the release of ECA activity. ECA activity before lactose agarose affinity chromatography, (black bars); that of lactose agarose-adsorbed fraction after elution with 0.2 M lactose, (clear bars). Data represent the mean ± SEM of three experiments in triplicate. Asterisk indicates $P < 0.05$.

Suppression of galectin-9 release by BB-94

Next, we examined the effects of matrix metalloproteinase (MMP) and protein kinase c (PKC) inhibitors on galectin-9 (ECA) release. We used tissue inhibitor of metalloproteinase-1 (TIMP-1), and BB-94 as MMP inhibitors; we used calphostin c as a PKC inhibitor. Figure 7 showed that ECA release was significantly suppressed by BB-94, whereas TIMP-1 failed. Calphostin c also could suppress ECA release weakly but significantly (Figure 7). ECA activity of the adsorbed fractions to lactose beads was also assessed. We thus found that BB-94 strongly suppressed the release of the ECA that binds to lactose beads and that calphostin c suppressed weakly but significantly. Furthermore, we found that the suppression by BB-94 was induced in a dose-dependent manner: BB-94 could suppress even at concentrations about 0.01 to 0.1 μM (Figure 8).

The effects of BB-94 on the levels of galectin-9 protein were also assessed using western blot analysis. We thus found that only BB-94 evidently suppressed the release of the medium-sized isoform of galectin-9 and 42 kDa protein, though we failed to detect the long-sized isoform of galectin-9 in Western blot (Figure 9).

Discussion

In our previous articles, we have demonstrated that ecalectin is a potent and selective ECA (Matsumoto *et al.*, 1998), and that divalent galactoside-binding activity is essentially required for its ECA (Matsushita *et al.*, 2000). We first noted that galectin-9 represents a variant form of human galectin-9, because there are as many as five amino acids that are different between the two proteins (Tureci *et al.*, 1997; Matsumoto *et al.*, 1998). Subsequently, by RT-PCR using oligonucleotide primers that correspond to internal sequences of galectin-9, we generated galectin-9 cDNA from a human T cell line, Jurkat, which express galectin-9; the cell supernatant contains ECA that is absorbable by lactose agarose (Figure 5) and by anti-galectin-9 antibodies (Figures 5 and 6), suggesting that galectin-9 produced from PMA-stimulated Jurkat cells exhibiting potent ECA. DNA sequencing revealed that all of these 12 independent

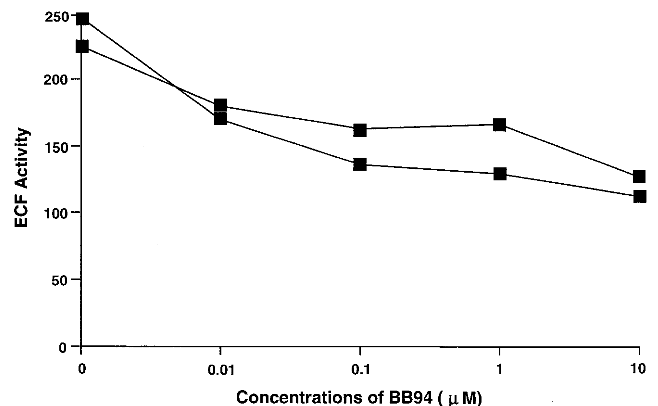

Fig. 8. Dose dependency of BB-94. ECA release from PMA-stimulated Jurkat cells was suppressed by BB-94 in a dose-dependent manner. Data of two separated experiments in triplicate were represented.

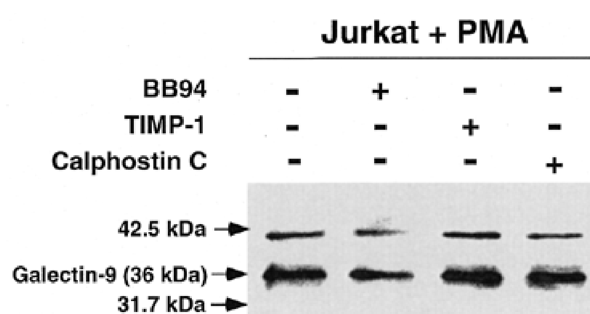

Fig. 9. Suppression of galectin-9 from Jurkat cells by BB-94. Western blot analysis revealed that the release of the medium galectin-9 was suppressed by BB-94 treatment. Representative data of three experiments was presented.

clones share a common sequence, and the deduced protein sequence differs from our previously published galectin-9 sequence (Matsumoto *et al.*, 1998) by one amino acid and from the published galectin-9 sequence (Tureci *et al.*, 1997) only by four amino acids. Therefore, it is now apparent that galectin-9 is identical to ecalectin, and the sequence differences noted before are likely due to allelic variations and/or cloning/sequencing artifacts (Matsushita *et al.*, 2000).

The characterization of galectin-9 cellular expression has never been established. We demonstrate herein that galectin-9 is constitutively expressed in Jurkat cells. We also found that other immune cell line cells, such as THP-1 (a macrophage line), HL-60 (a promyelocyte line), RPMI-8866 (a B cell line), and HMC-1 (a mast cell line), expressed galectin-9 (data not shown). Two isoforms of galectin-9, varying in the length of their linker peptide region (the medium and long-sized isoforms of galectin-9) are expressed, and PMA appears to enhance the expression of galectin-9 mRNA in Jurkat cells (Figure 4).

Galectins are most commonly described as cytoplasmic proteins lacking a transmembrane segment (Ohyama *et al.*, 1986; Cleves *et al.*, 1996). However, here we could provide evidence by flow cytometry analysis that galectin-9 has membrane localization (Figures 3 and 6). The surface expression of other galectins has also been documented. For example, galectin-3 surface expression has been shown in normal human monocytes, and its level increases as monocytes differentiate into macrophages (Liu *et al.*, 1995). The fact that galectin-3 and -9 have membrane localization without having a trans-membrane segment suggests that they interact with other proteins to facilitate their membrane localization. In the case of galectin-3, there is evidence to suggest that the lysosomal-membrane-associated glycoproteins (LAMPs) 1 and 2 are potential interacting-proteins candidates because an enhanced binding of galectin-3 correlated with an increased in LAMPs surface expression in the A2058, HT1080, and CaCO-2 human tumor cells (Sarafian *et al.*, 1998).

We have described that the level of intracellular galectin-9 staining well correlates with the results of western blot analysis and the mRNA levels of galectin-9 in Jurkat cells. The level of surface galectin-9 in Jurkat cell is upregulated by PMA stimulation (Figure 3). We have further shown that avidity of the long-sized galectin-9 to anti-galectin-9 antibody is higher than the medium galectin-9, and that the former may exhibits more evident ECA than the latter (Figure 6A, B). However, further information is required to ascertain it because it cannot be excluded the possibility that some co-factor for enhancement or suppression of ECA activity is contaminated in the fractions.

The observation that there are several isoforms of galectin-9 is not surprising because isoforms of other galectins have been shown to exist, and their profile appears to be tissue-specific. For instance, six isoforms of bovine galectin-1 have been identified in the spleen, whereas in the heart, only four isoforms can be detected based on their isoelectric point (Ahmed *et al.*, 1996).

This study also addressed the mechanisms by which PMA affects expression and release of galectin-9. Stimulation of Jurkat cells with PMA resulted in the release of galectin-9 exhibiting ECA (Figures 4 and 5). PMA is a strong activator of PKC that is known to be involved in the regulation of numerous signaling pathways. Thus it is possible PMA regulates the production of galectin-9 through PKC activation. A PKC inhibitor, calphostin c, weakly but significantly suppressed PMA-induced ECA release from Jurkat cells (Figure 7), but we failed to detect obvious change in PMA-induced release of galectin-9 in calphostin c–treated cells (Figure 9).

Because galectins lack a signal sequence in the structure, the release may not be induced by classical pathway (Cleves *et al.*, 1996). It is well known that tumor necrosis factor (TNF)α and IL-1β also lack signal peptide like galectins. Recent studies have revealed that IL-1β converting enzyme and TNFα-converting enzyme is involved in the release of IL-1β (Howard *et al.*, 1991; Kronheim *et al.*, 1992; Nett *et al.*, 1992) and TNFα (Black *et al.*, 1997; Moss *et al.*, 1997), respectively. Lunn *et al.* (1997) have shown that ADAM 10 play a role as a TNFα convertase, though Black *et al.* (1996) suggested that major TNFα converting enzyme is not an MMP. Thus, we cannot exclude the hypothesis that some enzyme action is involved in PMA-induced release of galectin-9. Recently, it has been found that galectin-3 is a substrate of MMP-2 and -9 (Ochieng *et al.*, 1994) and its biological functions are modulated by those MMP (Ochieng *et al.*, 1998). In the present article, we have shown that BB-94, an MMP inhibitor with broad spectrum, suppresses PMA-induced both ECA and galectin-9 release (Figures 7 to 9) though TIMP-1 fails, suggesting that some MMP differing from MMP-2 and -9 is involved in the release of galectin-9 from Jurkat cells.

The present studies suggest that there are, at least, two isoforms of galectin-9 constitutively expressed in immune cells, and that PMA treatment enhances the expression level and the release of ecalectin/galectin-9 from Jurkat cells.

Materials and methods

Cell culture and stimulation with PMA

Jurkat cells were obtained from American Type culture collection and maintained in RPMI 1640 medium supplemented with 10% fetal calf serum and 2×10^{-5} M 2-mercaptoethanol at 37°C with 7 % CO_2. PMA was used for Jurkat cells as a stimulant.

In some experiments, Jurkat cells were cultured in the presence of an MMP inhibitor or a PKC inhibitor. As MMP inhibitors, BB-94 (0.01–10 μM) and TIMP-1 (0.25 μM) were kindly donated by Dr. Yong at University of Calgary and used in the present experiments. Calphostin c (100 nM, Sigma) was used as a PKC inhibitor.

RT-PCR of galectin-9 and sequencing of galectin-9 PCR products

Total RNA from Jurkat cells was isolated using TRIZOL reagent (Gibco BRL, Rockville, MD). Using a Gene Amp RNA PCR kit (Perkin-Elmer, Branchburg, NJ), 0.5 μg of total RNA was reversed transcribed to DNA in one-step reaction followed by PCR to amplify transcripts of human galectin-9 and GAPDH. RT reaction and PCR steps were performed following detailed instructions from the manufacturer. The following primer sequences synthesized at Amersham Pharmacia Biotech (Buckingshire, England) were used:

- galectin-9 sense 5′-GAGAGGAAGACACACATGCCTTTC-3′,
- galectin-9 anti-sense 5′-GACCACAGCATTCTCATCAAA-ACG-3′,
- GAPDH sense 5′-GCCATCAATGACCCCTTCATTGAC-3′, and
- GAPDH anti-sense 5′ ACGGAAGGCCATGCCAGTGAG-CTT-3′.

Galectin-9 transcripts of two different sizes (483 and 560 bp) were obtained, and G3PDH transcripts were of 609 bp. Thirty PCR cycles were used for amplification of both transcripts, and all reactions were performed in a GeneAmp PCR System 9600 (Perkin Elmer) unless otherwise specified. PCR products were run on a 1.5% agarose gel containing ethidium bromide (1 μg/ml) for visualization under UV. After purification of galectin-9 PCR products, sequencing of galectin-9 PCR products was performed using a ABI PRISM BigDye Terminator Cycle Sequencing Ready Reaction Kit (Perkin Elmer). For each reaction, the following reagents were added to a tube: 8 μl of Terminator Reaction Mix, 500 ng of PCR product, 3.2 pmol of galectin-9 primers, and deionized water. Sequencing of DNA was performing on a GeneAmp PCR System 2400. Sequences obtained from both lower and higher molecular band PCR products corresponded to galectin-9 sequence with a difference in the length of the linker peptide region. Finally, band intensity was measured using the NIH image 1.61 program

Generation of GST-galectin-9 fusion proteins

The following forward (S) and reverse (AS) primers were used to amplify the cDNA of galectin-9-WT (full-length) and galectin-9-CT (C-terminal only):

- galectin-9-WT-S (5′-CCCGAATTCCCATGGCCTTCAGCA-GTTCC-3′),
- galectin-9 WT-AS (5′-CCCCTCGAGCTATGTCTGCACAT-GGGT-3′),
- galectin-9-CT-S (5′-CCCGAATTCCCATTACCCAGAGAGT-CATC-3′), and
- galectin-9-CT-AS (5′-CCCCTCGAGCTATGTCTGCACCTG-GGT-3′).

Amplified cDNA was inserted into the GST fusion plasmid vector pGEX (Pharmacia Biotech, Uppsala, Sweden) into the BamHI/XhoI or EcoRI/XhoI sites. Competent BL21 *Escherichia coli* were transfected by electroporation with pGEX-galectin-9 vectors, and were grown in 2×YT medium supplemented with 10% (w/v) glucose and 100 μg/ml ampicillin to an optical density of 0.7 at 600 nm. To induce the expression of the GST-galectin-9 fusion proteins, transfected *E. coli* were cultured in the presence of the lactose analog isopropyl β-D-thiogalactoside. Induced cultures were allowed to express GST-fusion protein for several h and were then lysed by mild sonication. After clearing the bacterial lysate of cellular debris, it was applied directly to glutathione Sepharose 4B. Fusion proteins were then eluted with an elution buffer containing 50 mM Tris (pH.8.0) and 10 mM glutathione. The presence of GST-galectin-9 fusion proteins with a molecular weight of 62 kDa was verified by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE), and its concentration was determined by spectrophotometry (optical density at 280nm).

Generation and purification of a polyclonal antibody for human galectin-9

Every 2 weeks, rabbits were immunized subcutaneously with a mixture of GST-galectin-9-CT and complete Freund's adjuvant. Animals received a total of four injections, after which they were bled for serum collection. The anti-serum was loaded onto a CNBr-activated Sepharose 4B column coupled with GST-galectin-9-CT fusion protein. After four subsequent washes using 25 mM Tris–HCl (pH.7.5) + 0.15 NaCl, 1 M NaCl, and 1% Triton-X-100, 25 mM Tris–HCl (pH.7.5) + 0.15 NaCl, acidic elution was achieved with a buffer solution containing 0.1 M glycine-HCl (pH 2.5) and 10% ethylene glycol. The pH was immediately neutralized by adding 1 M Tris–HCl (pH.8.0). To ensure the presence of galectin-9 antibody, SDS–PAGE was performed and the antibody solution was dialyzed overnight for functional assays. Finally, the specificity of rabbit-anti-human galectin-9 was confirmed by performing western blot analysis of GST-galectin-9 (CT) fusion proteins. Moreover, using the same approach, no or little cross-reactivity with galectin-1 and galectin-3 fusion proteins was detected.

Western blotting of galectin-9

Before and after PMA treatment of the cell line cells, cell lysates were obtained by adding a small volume of lysis buffer (10 mM Tris–HCl, 0.15 M NaCl, 2 mM ethylenediamine tetra-acetic acid, 2 mM ethylene glycol bis(2-aminoethyl ether)-tetra acetic acid, and freshly added 0.5 mM phenylmethylsulfonyl fluoride, 10 μg/ml of leupeptin, antipain, pepstatin A, and 1 mM dithiothreitol) to cells pellets. SDS sample buffer was then added to cell lysates, and samples were incubated for 5 min at 100°C and then placed on ice. Samples were run on a 12% acrylamide-SDS gel, and were transferred to a polyvinylidene difluoride membrane (BioRad Laboratories, Hercules, CA). Nonspecific binding was blocked using 5% skim milk in PBS containing 0.1% Tween-20 (PBS-T). After several washing with PBS-T, membranes were incubated for 1 h with 0.5 μg/ml of purified rabbit anti-human galectin-9 diluted in PBS-T. Membranes were then washed, followed by incubation with PBS-T containing anti-rabbit peroxidase-linked (Amersham Pharmacia Biotech) for a period of 45 min. Finally, membranes were immersed into electrochemiluminescence–horseradish peroxdiase substrate solution obtained from the ECL kit (Amersham Pharmacia Biotech), and immunoblotting could be visualized by exposing the membrane to an XJB-1 X-ray film (Kodak, Rochester, NY).

Flow cytometry analysis of galectin-9

To assess surface galectin-9 expression, cells were collected by centrifugation, and were washed with PBS containing 0.05% NaN_3 and 2% fetal calf serum (PBS+) followed by an incubation of 1 h on ice with the primary antibody solution containing 50 μg/ml of rabbit-anti-human galectin-9 and 10% goat serum

(Santa Cruz Biotechnology, Santa Cruz, CA). After several washes with PBS+, cells were then incubated on ice with fluorescein isothiocyanate–conjugated goat-anti-rabbit antibody (Santa Cruz Biotechnology) for a period of 45 min.

Intracellular expression of galectin-9 was assessed by a modification of the methods by Krug *et al.* (1996). In brief, the cells were incubated in 30 mM lactose-containing PBS+ on ice for 30 min to eliminate surface galectin-9 and fixed with ice-cold PBS containing 4% paraformaldehyde for 10 min. After washing with PBS+, the cells were resuspended in 25 μl saponin buffer (PBS containing 0.1% saponin and 0.01 M HEPES buffer). After addition of 50 μl of rabbit-anti-human galectin-9 antibody in saponin buffer, the cells were incubated for 30 min at room temperature, followed by an incubation of 45 min on ice with 50 μl fluorescein isothiocyanate–conjugated goat-anti-rabbit antibody (Santa Cruz Biotechnology).

Galectin-9 staining of total cells defined by scatter gates (15,000 events) was analyzed with a flow cytometer using SYSTEM II™ Software version 1.0 (Coulter, Miami, FL). For the verification of flow cytometer's optical alignment and fluidics system, Flow-check™ fluorospheres were used (Coulter).

ECA

This was performed by the same methods described previously (Hirashima *et al.*, 1991). In brief, normodense CD16-negative eosinophils were isolated by subjecting peripheral blood leukocytes to a discontinuous density gradient of Percoll (Pharmacia), followed by immunomagnetic treatment of the obtained cells with anti-CD 16 immunoglobulin (DAKO A.S., Glostrup, Denmark). The purity and viability of the obtained eosinophils were >98% and 95%, respectively. ECA was evaluated with a 48-well chamber (Neuro Probe, Cabin John, MD) containing a polyvinylpyrolidone-free membrane with 5-μm standard pore sizes. Human eosinophils (1×10^6/ml) and a test sample were placed in the top and bottom chambers, respectively. Each assay was performed in triplicate. After a 1.5-h incubation at 37°C in a humidified atmosphere of 5% CO, the filters were stained with Diff-Quik™ (Baxter Healthcare, McGaw Park, IL), and migrated eosinophils were counted. Human C5a (Sigma) and eotaxin-l (Seikagaku, Tokyo) were used as positive controls.

Affinity column chromatograph

To clarify whether ECA has affinity to lactose, the culture supernatants were applied on a lactose-agarose affinity column (Seikagaku). Adsorbed protein was eluted with 0.2 M lactose and assessed for ECA activity after dialysis against PBS.

The culture supernatant of PMA-stimulated Jurkat cells was applied on an Affigel-HZ immunoaffinity column conjugated with antibodies against C-terminal of galectin-9 according to the protocol of the manufacturer. After washing with PBS, adsorbed galectin-9 was eluted with 0.1 M glycine-HCl–10% ethylene glycol (pH 2.5). After immediate neutralization, each fraction (0.5 ml) was dialyzed against PBS and used for western blot and chemotaxis.

Abbreviations

CRD, carbohydrate recognition domain; ECA, esoniophil chemoattractant activity; GST, glutathione-S-transferase; LAMP, lysosome-associated membrane protein; MMP, matrix metalloproteinase; PBS, phosphate buffered saline; PKC, protein kinase c; PMA, phorbol 12-myristate13-acetate; RT-PCR, reverse transcriptase polymerase chain reaction; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; TIMP-1, tissue inhibitor of metalloproteinase-1; TNF, tumor necrosis factor.

References

Ahmed, H., Fink, N.E., Pohl, J., and Vasta, G.R. (1996) Galectin-1 from bovine spleen: biochemical characterization, carbohydrate specificity and tissue-specific isoforms profiles. *J. Biochem.*, **120**, 1007–1019.

Barondes, S.H., Castronovo, V., Cooper, D.N.W., Cummings, R.D., Hirabayashi, J., Hughes, C., Kasai, K.-I., Leffler, H., and Liu, F.-T. (1994) Galectins, a family of animal β-galactoside-binding lectins. *Cell*, **76**, 597–598.

Black, R.A., Durie, F.H., Otten-Evans, C., Miller, R., Slack, J.L., Lynch, D.H., Castner, B., Mohler, K.M., Gerhart, M., Johnson, R.S., and others (1996) Relaxed specificity of matrix metalloproteinases (MMPS) and TIMP insensitivity of tumor necrosis factor-alpha (TNF-alpha) production suggest the major TNF-alpha converting enzyme is not an MMP. *Biochem. Biophys. Res. Commun.*, **225**, 400–405.

Black, R.A., Rauch, C.T., Kozlosky, C.J., Peschon, J.J., Slack, J.L., Wolfson, M.F., Castner, B.J., Stocking, K.L., Reddy, P., Srinivasan, S., and others (1997) A metalloproteinase disintegrin that releases tumour-necrosis factor-alpha from cells. *Nature*, **385**, 729–733.

Caron, M., Bladier, D., and Joubert, R. (1990) Soluble galactoside-binding vertebrate lectins: a protein family with common properties. *Int. J. Biochem.*, **22**, 1379–1385.

Cleves, A.E., Cooper, D.N., Barondes, S.H., and Kelly, R.B. (1996) A new pathway for protein export in *Saccharomyces cerevisiae*. *J. Cell Biol.*, **133**, 1017–1026.

Gitt, M.A. and Barondes, S.H. (1986) Evidence that a human soluble β-galactoside-binding lectins is encoded by a family of genes. *Proc. Natl Acad. Sci. USA*, **83**, 7603–7607.

Hirashima, M., Ueno, M., Higuchi, S., Matsumoto, T., Sakata, K., Matsumoto, R., and Adachi, M. (1992) Establishment of a human T-cell line constitutively producing chemotactic lymphokines and their heterogeneity on eosinophils. *Lymphokine Cytokine Res.*, **11**, 331–338.

Hirashima, M., Ueno, M., Kamiya, K., Higuchi, S., and Matsumoto, R. (1991) Functional heterogeneity of human eosinophil chemotactic lymphokines. *Lymphokine Cytokine Res.*, **10**, 481–486.

Howard, A.D., Chartrain, N., Ding, G.F., Kostura, M.J., Limjuco, G., Schmidt, J.A., and Tocci, M.J. (1991) Probing the role of interleukin-1 beta convertase in interleukin-1 beta secretion. *Agents Actions Suppl.*, **35**, 77–83.

Kronheim, S.R., Mumma, A., Greenstreet, T., Glackin, P.J., Van Ness, K., March, C.J., and Black, R.A. (1992) Purification of interleukin-1 beta converting enzyme, the protease that cleaves the interleukin-1 beta precursor. *Arch. Biochem. Biophys.*, **296**, 698–703.

Krug, N., Maddn, J., Redington, A.E., Lackie, P., Djukanovic, R., Schauer, U., Holgate, S.T., Frew, A.J., and Howarth, P.H. (1996) T-cell cytokine profile evaluated at the single cell level in BAL and blood in allergic asthma. *Am. J. Respir. Cell Mol. Biol.*, **14**, 319–326.

Lipkowitz, M.S., Leal-Pinto, E., Rappoport, J.Z., Najfeld, V., and Abramson, R.G. (2001) Functional reconstitution, membrane targeting, genomic structure, and chromosomal localization of a human urate transporter. *J. Clin. Invest.*, **107**, 1103–1115.

Liu, F-T., Hsu, D.K. Zuberi, R.I., Kuwabara, I., Chi, E.Y., and Henderson, W.R. Jr. (1995) Expression and function of galectin-3, a beta-galactoside binding lectin, in human monocytes and macrophages. *Am. J. Pathol.*, **147**, 1016–1028.

Lunn, C.A., Fan, X., Dalie, B., Miller, K., Zavodny, P.J., Narula, S.K., and Lundell, D. (1997) Purification of ADAM 10 from bovine spleen as a TNFalpha convertase. *FEBS Lett.*, **400**, 333–335.

Matsumoto, R., Matsumoto, H., Seki, M. Hata, M., Y. Asano, Y.S. Kanegasaki, S., R.L. Stevens, R.L., and Hirashima, M. (1998) Human galectin-9, a variant of human galectin-9, is a novel eosinophil chemoattractant produced by T lymphocytes. *J. Biol. Chem.*, **273**, 16976–16984.

Matsushita, N., Nishi, N., Seki, M., Matsumoto, R., Kuwabara, I., Liu, F-T., Hata, Y., Nakamura, T., and Hirashima, M. (2000) Requirement of

divalent galactoside-binding activity of ecalectin/galectin-9 for eosinophil chemoattraction. *J. Biol. Chem.*, **275**, 8355–8360.

Menon, R.P. and Hughes, R.C. (1999) Determinants in the N-terminal domains of galectin-3 for secretion by a novel pathway circumventing the endoplasmic reticulum-Golgi complex. *Eur. J. Biochem.*, **264**, 569–576.

Moss, M.L., Jin, S.L., Becherer, J.D., Bickett, D.M., Burkhart, W., Chen, W.J., Hassler, D., Leesnitzer, M.T., McGeehan, G., Milla, M., and others (1997) Cloning of a disintegrin metalloproteinase that processes precursor tumour-necrosis factor-alpha. *Nature*, **385**, 733–736.

Moutsatsos, I.K., Wade, M., Schindler, M., and Wang, J.L. (1987) Endogeneous lectins from cultured cells: nuclear localization of carbohydrate-binding protein 35 in proliferating 3T3 fibroblasts. *Proc. Natl Acad. Sci. USA*, **84**, 6452–6456.

Nett, M.A., Cerretti, D.P., Berson, D.R., Seavitt, J., Gilbert, D.J., Jenkins, N.A., Copeland, N.G., Black, R.A., and Chaplin, D.D. (1992) Molecular cloning of the murine IL-1 beta converting enzyme cDNA. *J. Immunol.*, **149**, 3254–3259.

Ochieng, J., Fridman, R., Nangia-Makker, P., Kleiner, D.E., Liotta, L.A., Stetler-Stevenson, W.G., and Raz, A. (1994) Galectin-3 is a novel substrate for human matrix metalloproteinases-2 and -9. *Biochemistry*, **33**, 14109–14114.

Ochieng, J., Green, B., Evans, S., James, O., and Warfield, P. (1998) Modulation of the biological functions of galectin-3 by matrix metalloproteinases. *Biochim. Biophys. Acta*, **1379**, 97–106.

Oda, Y., Leffler, H., Sakakura, Y., Kasai, K.-I., and Barondes, S.H. (1991) Human breast carcinoma cDNA encoding a galactoside-binding lectin homologous to mouse Mac-2 antigen. *Gene*, **99**, 279–283.

Ohyama, Y., Hirabayashi, J., Oda, Y., Ohno, S., Kawasaki, H., Suzuki, K., and Kasai, K. (1986) Nucleotide sequence of chick 14K beta-galactoside-binding lectin mRNA. *Biochem. Biophys. Res. Commun.*, **134**, 51–56.

Paroutaud, P., Levi G., Teichberg V.I., and Strosberg, A.D. (1987) Extensive amino acid sequence homologies between animal lectins. *Proc. Natl Acad. Sci. USA*, **84**, 6345–6348.

Perillo, N.L., Marcus M.E., and Baum, L.G. (1998) Galectins: versatile modulators of cell adhesion, cell proliferation, and cell death. *J. Mol. Med.*, **76**, 402–412.

Sarafian, V., Jadot, M., Foisdart, J.M., Letesson, J.J., Van den Brule, F., Castronovo, V., Wattiaux, R., and Coninck, S.W. (1998) Expression of Lamp-1 and Lamp-2 and their interaction with galectin-3 in human tumor cells. *Int. J. Cancer*, **75**, 105–111.

Sato, S., Burdett, I., and Hughes, R.C. (1993) Secretion of the baby hamster kidney 30-kDa galactose-binding lectin from polarized and nonpolarized cells: a pathway independent of the endoplasmic reticulum-Golgi complex. *Exp. Cell Res.*, **207**, 8–18.

Tureci, O., Schmitt, H., Fadle, N., Pfreundschuh, M., and Sahin, U. (1997) Molecular definition of a novel human galectin which is immunogenic in patients with Hodgkin's disease. *J. Biol. Chem.*, **272**, 6416–6422.

Wada, J. and Kanwar, Y.S. (1997) Identification and characterization of galectin-9, a novel β-galactoside-binding mammalian lectin. *J. Biol. Chem.*, **272**, 6078–6086.

Wada, J., Ota, K., Kumar, A., Wallner, E.I., and Kanwar, Y.S. (1997) Developmental regulation, expression, and apoptotic potential of galectin-9, a β-galactoside binding protein. *J. Biol. Chem.*, **99**, 2452–2461.

Yang, R.-Y., Hsu, D.K., and Liu, F.-T. (1996) Expression of galectin-3 modulates T cell growth and apoptosis. *Proc. Natl Acad. Sci. USA*, **93**, 6737–6742.