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Review

Intracellular functions of galectins

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Abstract

Many galectin family members are detected primarily intracellularly in most of the systems studied, although certain members can be found both inside and outside of cells. Specific functions that are consistent with their intracellular localization have now been documented for some of the galectins. Galectin-1 and -3 have been identified as redundant pre-mRNA splicing factors. Galectin-3, -7, and -12 have been shown to regulate cell growth and apoptosis, being either anti-apoptotic or pro-apoptotic. Galectin-3 and -12 have been shown to regulate the cell cycle. In some cases, the mechanisms by which galectins exert their functions have been partially delineated in relation to known intracellular pathways associated with these processes. In addition, a number of intracellular proteins involved in these processes have been identified as the interacting ligands of certain galectins. This review summarizes the intracellular activities displayed by several galectins and discusses the possible underlying mechanisms.

Keywords: Galectin; RNA splicing; Cell growth; Apoptosis; Cell cycle; Galectin-interacting protein

1. Introduction

The galectin family of carbohydrate-binding proteins was initially defined on the basis of structural analysis and binding specificity studies. Membership in this family required fulfillment of two key criteria: (a) binding affinity for β-galactosides; and (b) conserved sequence elements in the carbohydrate-binding site [1]. To date, 14 mammalian galectins have been identified and numbered sequentially according to the accepted numbers for their genes in the Genome Data Base. Galectins have also been identified in many nonmammalian species, including birds, amphibians, fish, worms, sponges and fungi [2]. Screening the databases of genomic DNA sequences and expressed sequence tags has revealed additional candidates for membership in the mammalian galectin family, as well as putative galectins in plants and viruses [3]. [See related articles in this issue on Galectinomics by Cooper [4] and on How Galectins Have Evolved Oligosaccharide Specificity by Hirabayashi et al. [5]].

Each member of the galectin family contains at least one domain of about 130 amino acids, which is responsible for the observed carbohydrate-binding activity, and therefore is designated the Carbohydrate Recognition Domain (CRD) [see related article in this issue by Gabius et al. [6]]. Comparison of the amino acid sequences of the galectin polypeptides suggests a classification into subfamilies, based on the content and organization of the domains: (a) the Prototype group (galectin-1, -2, -5, -7, -10, -11, -13, and -14) contains one domain, the CRD; (b) the Tandem Repeat group (galectin-4, -6, -8, -9, and -12) contains two CRDs; and (c) the Chimera group (galectin-3) contains an unusual proline- and glycine-rich domain (also about 130 amino acids) fused onto the CRD [see related articles in this issue by Cooper [4] and Hirabayashi et al. [5]].

The three-dimensional structures of the CRDs derived from galectin-1, -2, -3, -7 and -10 have been elucidated by X-ray crystallography. These studies revealed that the CRD is folded tightly, with two anti-parallel β-pleated sheets forming a sandwich-like structure [7]. Amino acid side chains on one of these sheets form the core carbohydrate-binding site [see related article in this issue on Principle of Structures of Animal and Plant Lectins by Loris [8]]. The interaction between a galectin CRD and the monosaccharide ligand galactose is weak, with a dissociation constant in the micromolar range. For most galectins tested, the disacchar-

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ide lactose binds with about 100-fold higher affinity than galactose alone [9]. Some larger oligosaccharides exhibit even higher affinity than lactose, suggesting that the carbohydrate-binding site extends beyond the core binding site for galactose. The amino acid residues forming this extended binding site are much less conserved among the galectins than those of the core binding site and different galectins show different affinity and specificity for longer oligosaccharides. [See related articles in this issue on Binding and Cross-linking Properties of Galectins by Brewer and Dam [10]].

Studies on the expression of galectins highlight three important facts: (a) any given organism usually expresses multiple members of the galectin family; (b) different cells within an organism usually contain a different complement of galectins; and (c) almost all cells have at least one galectin. Studies on galectin-1 and -3 during mouse embryonic development suggest that each individual galectin is expressed in some tissue-specific or developmentally regulated fashion [11]. In the adult murine system, galectin-1 is abundant in a variety of cells and tissues, particularly those of mesodermal origin. Galectin-3 is found in various epithelial cells and cartilage, as well as inflammatory cells such as macrophages. On the other hand, galectin-4 expression appears to be restricted to epithelial cells of the gastrointestinal tract, while galectin-7 is confined to stratified epithelia such as the epidermis [2].

In addition to binding galactose-containing glycoconjugates, some members of the galectin family share another property in terms of their cell biology. They exhibit dual localization, being found in both the intracellular (cytoplasm and, in some cases, the nucleus) as well as the extracellular (cell surface and medium) compartments [12]. The mechanism of externalization appears to be unusual because none of the galectins contains an obvious signal sequence for directing the polypeptide into the classical endomembrane pathway for secretion. On the other hand, many of the galectins are predominantly intracellular proteins. It is the purpose of the present article to review their intracellular activities (see Table 1).

Table 1
Intracellular activities documented for some of the galectins

Galectin	Activity
1	Regulation of cell transformation: promotes membrane anchorage of Ras Nuclear splicing of pre-mRNA
3	Regulation of the cell cycle through G_1 or G_2/M arrest Regulation of cell growth through an anti-apoptotic effect: protects cells from a variety of death signals, including Fas receptor cross-linking and loss of cell anchorage Nuclear splicing of pre-mRNA
7	Regulation of cell growth through a pro-apoptotic effect: programmed cell death of epithelial cells in response to UV irradiation and p53 expression
12	Regulation of the cell cycle through G_1 arrest Regulation of cell growth through a pro-apoptotic effect: programmed cell death in adipocytes in response to ligands of the peroxisome proliferator-activated receptor

2. Intracellular localization of galectins

It is generally assumed that all known galectins are synthesized on cytoplasmic ribosomes. Following synthesis, there appears to be selective intracellular targeting of specific galectins to subcompartments of the cytosol, to distinct subcellular organelles, and even to membranes and membrane-bounded vesicles.

2.1. Galectin-1

Galectin-1 was shown to be synthesized on free ribosomes [13]. In addition, amino acid sequence analysis showed that the polypeptide, purified from tissue extracts, was acetylated at the N terminus [14]. Both of these observations are more typical for cytosolic proteins than secreted proteins. Indeed, comparisons of the immunofluorescence staining of live cells versus fixed and permeabilized cells showed that galectin-1 was found predominantly in the intracellular compartment. Within cells, galectin-1 was distributed diffusely in the cytoplasm.

Observations and interpretations pertaining to the nuclear localization of galectin-1 were somewhat more difficult to establish. For example, there are reports that explicitly state that anti-galectin-1 antibodies failed to label the cell nucleus. Two separate studies on mouse C2 myoblasts showed that galectin-1 was found in a diffuse distribution in the cytoplasm but no nuclear localization could be detected [15,16]. More recently, immunofluorescence analysis of Chinese hamster ovary cells showed that galectin-1 was exclusively cytoplasmic [17].

On the other hand, there are also studies that show galectin-1 localization in the nucleus as well as in the cytoplasm. Cryostat sections of tissues subjected to immunofluorescence showed labeling of both nuclei and cytoplasm in anti-galectin-1 staining of adult chicken kidney [18] and calf pancreas [19]. In studies on chick embryonic skin, Akimoto et al. [20] reported a chicken homolog of galectin-1 in both the cytosol and nuclei of cells in the intermediate layer of the epidermis and in some dermal fibroblasts. Similar results were also reported for normal human skin [21]. More recent studies using confocal fluorescence microscopy showed galectin-1 of HeLa cells throughout most of the sections, from the top of the cell to the bottom. As the plane of focus cuts through the nucleus in the middle sections, there was definite fluorescence staining in the nuclear compartment [22]. Finally, Choi et al. [23] have identified galectin-1 as a component of the nuclear matrix in rat osteoblasts. Galectin-1 has also been

recently identified as a component of the Survival of Motor Neuron (SMN) complex in HeLa cells [24]. Both of these latter observations are consistent with a role of galectin-1 in RNA processing inasmuch as such nuclear events have been ascribed to the nuclear matrix in general and to the SMN complex in particular.

The intracellular localization of galectin-1 is consistent with several interacting ligands identified for the protein. These include the oncogene H-Ras [25] and Gemin4 [24]. Galectin-1 was identified to be a binding partner of Ras on the basis of chemical cross-linking of the two polypeptides when a membrane particulate fraction of H-Ras(12V)-transformed Rat-1 (EJ) cells was treated with dithiobis(succinimidyl propionate). The association between galectin-1 and Ras was also demonstrated by reciprocal co-immunoprecipitation experiments using antibodies directed against the respective proteins. In this study, galectin-1 was shown to be essential for membrane localization of Ras: overexpression of galectin-1 results in increased membrane-associated Ras, while galectin-1 antisense RNA inhibits such membrane anchorage. Consistent with the known significance of membrane anchorage of Ras to malignant transformation, galectin-1 overexpression results in cell transformation, while antisense RNA inhibits such transformation [25]. The significance of galectin-1-Gemin4 interaction is discussed in a later section in relationship to the role of galectin-1 in pre-mRNA splicing.

2.2. Galectin-3

Like galectin-1, the N terminus of the galectin-3 polypeptide, isolated from cell extracts, was blocked by acetylation [26]. There are a number of studies, at both the light microscopy and ultrastructure levels, documenting the localization of galectin-3 in both the nucleus and the cytoplasm of various cell types [27–30]. In fibroblasts, the nuclear versus cytoplasmic distribution of the protein was dependent on the proliferation state of the cells under analysis. In quiescent cultures of fibroblasts, galectin-3 was predominantly cytoplasmic; proliferating cultures of the same cells, on the other hand, showed intense nuclear staining for the protein [31]. These conclusions were substantiated by quantitative immunoblotting of subcellular fractions derived from quiescent and proliferating cultures.

Galectin-3 expression and its intracellular distribution have also been shown to vary along the crypt-to-surface axis of human colonic epithelia [32]. The protein is concentrated in the nuclei of differentiated colonic epithelial cells. The progression from normal mucosa to adenoma to carcinoma is characterized by a striking absence of galectin-3 in the nuclei of adenoma and carcinoma cells.

Biochemical characterization revealed that, in 3T3 fibroblasts, galectin-3 exists in two isoelectric variants: (a) a nonphosphorylated native polypeptide (pI ~ 8.7); and (b) a phosphorylated derivative (pI ~ 8.2). The former is found exclusively in the nucleus, while the latter is found both in the nucleus and in the cytoplasm [33]. In digitonin-permeabilized cells, in which the integrity of nucleo-cytoplasmic transport is preserved, galectin-3 is rapidly and selectively exported from the nucleus [34]. Although both phosphorylated and nonphosphorylated forms of galectin-3 are found in the nuclear fraction, only the phosphorylated form is identified in the exported fraction, implying that phosphorylation may be important for nuclear export of the protein. Studies on the canine homolog of galectin-3 identified serine-6 as the major site of phosphorylation in vivo, with a minor site at serine-12 [35]. These sites were phosphorylated by casein kinase I in vitro.

The application of proteomics in two recent studies has identified galectin-3 as a component of intracellular vesicles: phagosomes in macrophages and exosomes in dendritic cells. The J774 mouse macrophage-like cell line was allowed to ingest latex beads and the phagosomes so formed were isolated. More than 140 proteins were identified to be associated with the latex bead-containing phagosomes [36]. Many of these were hydrolases, proton pump ATPase subunits, etc., expected for the function of the phagolysosome. Surprisingly, a number of proteins not previously described along the endocytic/phagocytic pathway were also identified, including proteins related to apoptosis such as protein 14-3-3, Alix/AIP-1, and galectin-3. This same set of proteins related to apoptosis was also identified in a systematic analysis of proteins of the exosome [37]. Exosomes are small (50–90-nm diameter) antigen-presenting and immunostimulating vesicles secreted by a number of cell types, including dendritic cells. The identification of galectin-3 with these intracellular vesicles has important implications on the role of the protein in apoptosis (see below for discussion of galectin-3 and apoptosis). It will be important to rigorously determine whether galectin-3 is on the lumenal or cytoplasmic side of such vesicles, which in turn may shed light on a mechanism for its secretion [12].

The intracellular localization of galectin-3 is consistent with several interacting ligands identified for the protein. These include: (a) Bcl-2 [38,39]; (b) Alix/AIP-1 (Chang, E.Y. and Liu, F.T., unpublished results); (c) Gemin4 [24]; (d) CBP70 [40]; (e) Chrp [41]; and (f) cytokeratin [42]. The first three of these will be discussed in later sections of this review, under the context of apoptosis and pre-mRNA splicing.

CBP70 was isolated from HL60 cell nuclei on the basis of its binding to glucose/*N*-acetylglucosamine affinity beads [40]. In this isolation, galectin-3 was co-purified with CBP70. The interaction between CBP70 and galectin-3 was disrupted by lactose binding to the latter protein. Chrp was identified in a yeast two-hybrid screen of a murine 3T3 cell cDNA library using galectin-3 as the bait [41]. Direct interaction between galectin-3 and Chrp was confirmed by immunoprecipitation and in vitro binding assays. The amino acid sequence of this protein showed an unusually high content of cysteine and histidine residues (17 and 11

residues, respectively, in a total of 311 residues) and therefore, it was designated as cysteine- and histidine-rich protein (Chrp). Immunofluorescence microscopy revealed that, in mouse 3T3 fibroblasts, both galectin-3 and Chrp could be found in the cytoplasm. On the other hand, while galectin-3 was also found in the nucleus, Chrp was strikingly excluded from the nucleus. Instead, Chrp appeared to be concentrated in a concentric ring at the nuclear envelope. The functional significance of the Chrp–galectin-3 interaction remains to be elucidated.

It seems important to note that all of the above listed ligands interact with galectin-3 via protein–protein, rather than lectin–glycoconjugate, interactions. The lone exception is the last, the cytokeratins. Goletz et al. [42] have shown that cytokeratins are bound in vitro by mammalian galectin-3 and a galectin homolog from the sponge *Geodia cydonium*, recognizing terminal α-*N*-acetyl-galactosamine (GalNAc)-bearing glycans. The presence of GalNAc residues on highly purified cytokeratins was confirmed by sugar composition analyses using gas chromatography and mass spectrometry. It was proposed that this novel posttranslational modification of the cytokeratin polypeptides might represent a natural cytoplasmic ligand for endogenous galectin-3.

2.3. Other galectins

In addition to galectin-1 and -3, four other galectins have been documented to be in the nucleus and cytoplasm: (a) galectin-7; (b) galectin-10; (c) galectin-11 and (d) galectin-12. Galectin-7 will be discussed below under the context of a pro-apoptotic protein. Confocal fluorescence microscopy on the transformed keratinocyte line HaCaT showed endogenous galectin-7 in cell nuclei [43]. In addition, HeLa cells transfected with galectin-7 yielded nuclear localization of the ectopically expressed protein. Galectin-10, the Charcot–Leyden Crystal protein, can be found in nuclei and cytoplasm of eosinophils and basophils [44]. When basophils are stimulated with either phorbol esters or the chemotactic peptide f-Met, galectin-10 appeared to be targeted into intracellular vesicles [45]. Galectin-11 has been localized in the cytoplasm and nucleus of the upper epithelial cells of the gastrointestinal tract [46]. Finally, galectin-12 will be discussed in the context of apoptosis and cell cycle regulation. Immunocytochemical analysis revealed that in adipocytes, galectin-12 was localized in the nucleus [47]. Immunoblotting of subcellular fractions indicated that the protein was associated with the nuclear and the mitochondrial fractions. The latter is consistent with the dotted staining pattern in the cytoplasmic region in the immunocytochemical studies.

Three other aspects of the intracellular localization of galectins deserve mention. First, Oka et al. [48] have recently cloned and identified rat galectin-2 and reported that it is predominantly expressed in the epithelial cells of the stomach. Under immunohistochemical staining, galectin-2 was found inside cells. In contrast, the protein was barely detectable at the surface of these epithelial cells. Second, galectin-4, like galectin-1 and -3, has been documented to be externalized [49,50]. It is particularly interesting that, in cells where galectin-3 and -4 coexist (e.g., T84 colon adenocarcinoma cells), the two proteins are selectively targeted to distinct regions of the cytosol (galectin-3 to the apical membrane and galectin-4 to the basolateral membrane). Finally, galectin-9 has been studied under various contexts [51–53], but the most interesting is the report that it can be inserted into lipid bilayers as a transmembrane protein that functions as a uric acid transporter in renal epithelial cells [54]. All of these observations highlight the theme that even within the cytosolic compartment, there appears to be selective targeting of individual galectins to subcompartments of the cytoplasm, to subcellular organelles, and to subregions of membranes.

3. Role in pre-mRNA splicing

Three early observations made on nuclear galectin-3 provided initial hints that it may play a role in RNA biogenesis: (a) in permeabilized 3T3 cells, it was released from the nucleus by ribonuclease A, but not by deoxyribonuclease I, treatment; (b) in Cs_2SO_4 gradients, galectin-3 in nuclear extracts banded at densities (1.3–1.35 g/ml) corresponding to ribonucleoprotein (RNP) complexes; and (c) affinity selection on carbohydrate columns isolated galectin-3, other polypeptides, as well as RNA species [55]. On this basis, experiments were carried out to test for a role of the protein in pre-mRNA splicing. In the course of these studies, galectin-1 was also identified to be a splicing factor. These experiments are summarized below.

3.1. Galectin-1 and -3 are redundant splicing factors

The criteria of depletion and reconstitution of splicing activity, assayed in a cell-free system, were used to show that galectin-1 and -3 are factors involved in pre-mRNA processing. This conclusion is based on the following key findings [56–58]:

- Nuclear extracts (NE) from HeLa cells used for cell-free splicing assays contain galectin-1 and -3;
- Cell-free splicing is inhibited by addition of saccharides that bind to galectins;
- Antibody depletion of either galectin-1 or galectin-3 did not result in loss of splicing activity, and the splicing activity of extracts depleted of either of the galectins retained sensitivity to saccharide inhibition;
- Depletion of both galectins from NE abolished splicing activity and arrested spliceosome formation at an early stage (H/E complex) in the splicing pathway;
- In NE depleted of galectins, reconstitution of splicing activity and transition of spliceosomes from H/E complex

to active spliceosomes was achieved by addition of either recombinant galectin-1 or galectin-3 in a dose-dependent and saccharide-inhibitable manner.

Co-localization studies provide additional evidence that galectins are splicing factors [22,57]. Immunofluorescence microscopy revealed nuclear speckled structures containing both galectin-3 and known splicing factors (i.e., the Sm proteins of the snRNPs and a non-snRNP splicing factor SC35). Galectin-1 staining was detected in similar speckled nuclear structures.

The domain structure for galectin-1 and -3 suggested that the homologous CRD was necessary and sufficient for splicing activity. Experimental evidence confirmed this prediction: galectin-1 alone or the isolated CRD of galectin-3 reconstituted splicing activity in a galectin-depleted NE, although intact galectin-3 was ~10 times more efficient in splicing reconstitution than either CRD alone [57].

Interestingly, addition of the proline-, glycine-rich N-terminal domain of galectin-3 to a complete splicing extract resulted in a dose-dependent inhibition of splicing activity and concomitant block in the formation of active spliceosomes [24]. In contrast, intact galectin-3 or its CRD (C-terminal domain) did not affect splicing activity or spliceosome formation. This dominant negative effect of the N-terminal domain on pre-mRNA splicing suggests that galectin-3 forms oligomers or interacts with another splicing component. Indeed, several studies have documented that galectin-3 can self-associate through either the N-terminal domain [59,60] or the C-terminal domain [61].

3.2. Association of galectin-1 and -3 with Gemin4 in SMN complexes

To identify proteins that interact with nuclear galectins, a yeast two-hybrid screen was performed with galectin-1 as "bait" and a HeLa cell cDNA library as "prey" [24]. One positive clone isolated by this method showed complete sequence identity with the C-terminal 50 amino acids of Gemin4 (Gemin4(C50)). Gemin4 is one component of a macromolecular complex containing SMN, Gemin2, Gemin3, some of the Sm core proteins of snRNPS and other, as yet, unidentified polypeptides. These complexes are implicated in processes directly or indirectly related to pre-mRNA splicing [62,63]. A direct interaction between galectin-1 and Gemin4(C50) was demonstrated in pull down assays using the fusion protein containing glutathione *S*-transferase (GST) and Gemin4(C50). Using this assay, galectin-3 also was shown to interact with Gemin4(C50) [24].

Significantly, antibodies to galectin-1 immunoprecipitated from NE, along with galectin-1, a complex containing, SMN, galectin-3, Gemin2, and the B/B′ and D polypeptides of the Sm core proteins of snRNPs [24]. SMN is encoded by the gene identified for the disease spinal muscular atrophy [64]. SMN was the first identified component of a set of macromolecular complexes containing ~12 proteins found in both nuclear and cytoplasmic compartments. Several members of the SMN complex bind core proteins of snRNPs and a cytoplasmic SMN complex plays a role in biogenesis of snRNPs before their re-entry into the nucleus [65]. In the nucleus, SMN and associated proteins are found in gems (gemini of coiled bodies) [66] and coiled bodies, while Gemin4 is also found in nucleoli [63]. Nuclear SMN-containing complexes are thought to recycle/supply snRNPs to the H/E complex initially formed in the splicing pathway or to rejuvenate snRNPs after their dissociation from active spliceosomes [62]. The findings that galectin-1 and -3 interact with Gemin4 and are co-immunoprecipitated with components of the nuclear SMN complex confirm their classification as splicing factors.

More importantly, the identification of galectin-1 and -3 in the SMN complex also offers mechanistic insights regarding their role in the splicing pathway. Three lines of experiments converge at the H/E complex to implicate it as the locus of action of the galectins in spliceosome assembly. First, nuclear extracts depleted of galectins assemble pre-mRNAs into H/E complexes, but show no conversion of these complexes into higher order structures [56]. Second, the addition of the N-terminal domain of galectin-3 to a competent splicing extract acts as a dominant negative mutant to arrest spliceosome formation at the H/E complex and inhibits splicing activity [24]. Finally, galectin-1 and -3 are found in SMN-containing complexes, which are required for pre-mRNA splicing by supplying functional snRNPs to the H/E complex in the pathway of spliceosome assembly [62].

4. Regulation of cell growth and apoptosis

Several early studies provided correlative evidence for an association between galectin expression and cell proliferation. Cultures of mouse 3T3 fibroblasts, made quiescent either by density inhibition or by serum deprivation, expressed low levels of galectin-3. Stimulation of the same cells into a proliferative state was accompanied by increased: (a) transcription of the gene; (b) accumulation of mRNA; and (c) expression of the protein [31,67]. In serum-stimulated 3T3 cells, galectin-3 is an immediate-early gene, whose transcription was not dependent on de novo protein synthesis. In addition, galectin-3 was expressed at high levels in a wide range of neoplasms, including spontaneous, viral, ultraviolet and chemically induced tumors [68]. Among related tumor cell variants of the K-1735 melanoma, the UV-2237 fibrosarcoma, and the A31 angiosarcoma, the expression of galectin-3 was correlated with metastatic potential [69]. [See related article in this issue on Galectins and Cancer by Danguy et al. [70]]. These observations suggested the possibility that the galectins may have a role in regulation of cell growth.

Studies from a number of research groups have indeed provided evidence for some galectins to have such a role. An important development closely related to this is that a number of galectins have been found to regulate apoptosis. This section reviews the literature on the function of galectins in regulation of cell growth and apoptosis. We will primarily focus on those studies dealing with galectins functioning intracellularly.

4.1. Galectin-3

The role of galectin-3 in regulation of cell growth has been demonstrated with various cell types by different approaches. Transfectants of the human T lymphoma Jurkat cells ectopically expressing galectin-3 were found to grow much better than control transfectants not expressing this lectin, especially under suboptimal growth conditions (e.g., in culture medium containing only 1% fetal bovine serum) [38]. Evidence for a role of endogenous galectin-3 in regulation of cell growth has been provided by a study in which galectin-3 expression is suppressed by an antisense oligonucleotide [71]. Thus, the human breast cancer MDA-MB435 cells transfected with antisense galectin-3 cDNA exhibited significantly decreased cell proliferation, as measured by thymidine incorporation, compared to control transfectants.

Galectin-3 was found to be induced when T lymphocytes were activated by anti-CD3 antibody or IL-2, IL-4, and IL-7, all of which cause cell proliferation. Inhibition of the expression of galectin-3 in these cells by specific antisense oligonucleotides rendered the cells less responsive to these mitogenic stimuli [72], suggesting that galectin-3 plays a role in proliferation of activated T lymphocytes.

The above results could be due to galectin-3's function in either promoting cell growth or inhibiting cell death. Indeed, the function of galectin-3 in inhibiting apoptosis has been demonstrated by a number of research groups. Galectin-3 transfectants of Jurkat cells mentioned above were found to survive significantly longer than control transfectants when they were treated with anti-Fas receptor antibody, which cross-links Fas receptor and causes apoptosis, or staurosporine, a protein kinase inhibitor [38]. The anti-apoptotic activity of galectin-3 has been confirmed in other cell types. For example, expression of galectin-3 in the human breast carcinoma BT549 cells inhibited *cis*-diamminedichloroplatinum (cisplatin)-induced apoptosis [39]. Stable transfectants of a human breast carcinoma cell line, Evsa-T, overexpressing galectin-3 were found to survive longer than control transfectants, when exposed to apoptotic stimuli, including cycloheximide/TNF-α and UVB irradiation [73].

The effect of overexpression of galectin-3 on the survival of human breast carcinoma cells has also been studied using a mouse liver ischemia/reoxygenation model. In this system, ischemia was induced in the liver by clamping the portal vein and hepatic artery. The liver was then excised, dissected, and added to the culture containing galectin-3 or control transfectants. Nearly all galectin-3 transfectants survived under these conditions, while the majority of control transfectants died during the same period. Furthermore, galectin-3 transfectants were more resistant than control transfectants to apoptosis induced by SNAP (*S*-nitroso-*N*-acetylpenicillamine), which is an NO donor known to induce apoptosis in many cell types, and mimics the situation during hepatic ischemia/reperfusion. The decreased apoptosis due to galectin-3 overexpression in this case is accompanied by increased mitochondrial integrity, decreased cytochrome *c* release, and caspase activation [74].

Galectin-3 has also been shown to protect cells against apoptosis induced by the loss of cell anchorage (anoikis). Thus, in contrast to wild-type BT549 cells, which readily underwent anoikis, transfectants overexpressing galectin-3 responded to the loss of cell adhesion by cell cycle arrest without detectable cell death [75]. Similar results were noted in terms of response of these transfectants to genistein, which is a natural isoflavonoid compound found to be a competitive inhibitor of protein tyrosine kinase and other critical enzymes involved in signal transduction, and induces apoptosis in some cells. Galectin-3-transfected cells underwent cell cycle arrest without appreciable apoptosis when treated by genistein, whereas the parental cells underwent apoptosis without detectable cell cycle arrest under the same conditions [76]. Recently it was reported that galectin-3 phosphorylation is required for galectin-3's anti-apoptotic function [77]. Thus, it appears that galectin-3 may be involved in an intracellular apoptosis-regulating pathway; it is phosphorylated by certain kinases and then exerts its effect by regulating other downstream molecules.

Studying cells from galectin-3-deficient mice has provided additional convincing evidence for the anti-apoptotic function of galectin-3. When peritoneal macrophages from galectin-3-deficient and wild-type mice were treated with IFN-γ and LPS, a procedure known to induce apoptosis in these cells, galectin-3-deficient cells died more rapidly [78]. Bone marrow-derived mast cells from galectin-3-deficient mice were also found to be more prone to apoptosis than those from wild-type mice, when cultured in the absence of growth factors (e.g., IL-3) (Sharma, B. and Liu, F.T., unpublished observations).

The mechanism underlying galectin-3's anti-apoptotic activity remains to be elucidated. There is evidence that the anti-apoptotic protein Bcl-2 may be involved. First, galectin-3 shares significant sequence similarity with Bcl-2. Both proteins are rich in proline, glycine, and alanine in the N-terminal region and contain an NWGR quartet in the C-terminal part. This quartet is found in the BH-1 domain of Bcl-2 and shown to be critical for the anti-apoptotic function of this protein. This sequence is highly conserved among galectin-3 from different animal species and is essential for the carbohydrate-binding activity of this lectin. Substitution of glycine to alanine in this motif abrogates its anti-apoptotic activity [39]. Furthermore, like other Bcl-2 family members, which form heterodimers with Bcl-2, galectin-3

binds Bcl-2 in vitro [38]. However, whether these two proteins interact with each other inside the cell is not known. Also, the sequence similarity is limited to Bcl-2 and is not found between galectin-3 and other Bcl-2 family members. No similarity exists between other galectins and Bcl-2 either. It is noted also that galectin-3 expression does not alter the expression levels of Bcl-2 family members, including Bcl-2, Bcl-xL, and Bax [39].

Another possible mechanism for galectin-3's anti-apoptotic activity has been provided by studying mast cells from galectin-3-deficient mice. These cells are more prone to apoptosis than wild-type cells, as mentioned earlier. These cells were also found to be defective in the expression of c-jun N-terminal kinase (JNK), which is one of the mitogen-activated kinases (MAPK), and to have much reduced c-jun kinase activity [B. Sharma, et al., The FASEB Journal, 15 (5) Abstract-778.1]. Since JNK is known to be a major regulator of apoptosis, it is possible that galectin-3 exerts its anti-apoptotic activity by regulating the JNK level. However, other cell types in galectin-3-deficient mice are not defective in JNK expression and, therefore, this mechanism, even if operative in mast cells, cannot be universally applicable to all cell types.

The identification of human galectin-3-binding proteins from a Jurkat cell cDNA library by the yeast two-hybrid method has also provided significant insights into galectin-3's anti-apoptotic activity (Chang, E.-Y. and Liu, F.T., unpublished results). One of these proteins is a human homolog of ALG-2 linked protein x (Alix) or ALG-2-interacting protein-1 (AIP-1), which has been shown to interact with ALG-2 and presumably contribute to the latter's pro-apoptotic function [79,80]. Alix/AIP-1 contains a proline-, glycine-, alanine-, and tyrosine-rich sequence in the C-terminal region, which is highly homologous to the tandem repeat sequence in the N-terminal part of galectin-3.

Based on the various studies mentioned above, it appears that galectin-3 regulates cell survival by functioning inside the cell and interacting with some components of the apoptosis-signaling pathways. However, the possibility of some extracellular mechanisms needs to be addressed. First, it was found that galectin-3 expression resulted in enhanced adhesion to laminin, fibronectin, and vitronectin in one study [73]. Since increased cell adhesion is known to protect cells from apoptosis, the resistance to apoptosis resulting from galectin-3 overexpression could be due to increased cell adhesion. This mechanism, however, cannot be a universal explanation of the decreased apoptosis rates of galectin-3 transfectants, since in some of the studies, apoptosis was induced in cells in suspension, where this type of adhesive interactions is not relevant.

Second, it is possible that galectin-3 protects cells from apoptosis through an extracellular mechanism by binding to cell surface glycoconjugates. In fact, galectin-3 has been shown to stimulate growth of fibroblasts [81], as well as mesangial cells [82], and promote outgrowth of neurites from dorsal root ganglia explants [83]. These effects are dependent on its lectin properties and were demonstrated by exogenously added galectin-3. However, the apoptosis rate of galectin-3 transfectants of Jurkat cells is not affected by inclusion of lactose in the culture medium, which is known to be effective in inhibiting various biological activities of galectin-3 manifested extracellularly. Moreover, recombinant galectin-3 does not affect apoptosis of Jurkat cells, when added to the cells exogenously (Yang, R.-Y. and Liu, F.T., unpublished observations). Therefore, at least the effect of galectin-3 expression on apoptosis in Jurkat cell transfectants cannot be explained by this type of extracellular mechanism.

4.2. Galectin-7

Galectin-7 contains a single CRD and is specifically expressed in stratified epithelia [84], but its expression is dramatically reduced in carcinoma cell lines derived from these epithelial cells. In the effort to identify gene products that are induced when the colon carcinoma DLD cells are transfected with the tumor suppressor p53, it was found that galectin-7 mRNA is one of the most highly induced mRNA [85]. It was thus speculated that galectin-7 might play a role in the pro-apoptotic function of p53 [85]. Subsequently, it was found that the amounts of galectin-7 mRNA and protein were increased in epidermal keratinocytes after UVB irradiation, paralleling p53 stabilization [86]. Furthermore, UV-induced apoptotic cells expressed a higher level of galectin-7 compared to non-apoptotic cells and keratinocytes overexpressing galectin-7 had a higher tendency to undergo apoptosis. The results suggest that galectin-7 is a pro-apoptotic protein.

The mechanism of galectin-7's proapoptotic function has been studied in detail [43]. HeLa cell transfectants overexpressing galectin-7 were found to exhibit a higher rate of apoptosis compared to control transfectants, when exposed to various apoptotic stimuli, including UV-irradiation, actinomycin D, etoposide, camptothecin, and a combination of TNF-α and cycloheximide. The results suggest that galectin-7 acts at a common point of apoptosis-signaling pathways. The following results were obtained with regard to the mechanism. First, galectin-7 transfectants undergoing apoptosis showed increased cleavage of procaspase-3, a precursor of caspase-3, which is a cell death executioner activated in apoptosis. Second, apoptosis in galectin-7 transfectants, like control transfectants, was inhibited by the pan-caspase inhibitor zVAD-fmk, suggesting that the enhanced apoptosis in galectin-7 transfectants is largely caspase dependent. Third, significantly more cytochrome *c* was released in galectin-7 transfectants compared to control cells undergoing apoptosis, suggesting that galectin-7 functions upstream of cytochrome *c* release. Fourth, zVAD-fmk had no inhibitory effect on the enhanced cytochrome *c* release found in galectin-7 transfectants, suggesting that caspases are not involved in this lectin's apoptosis-signaling pathway

upstream of cytochrome *c* release. Finally, following exposure to the apoptotic stimuli, galectin-7 transfectants exhibited a significant rise in the activity of JNK, an important regulator of apoptosis.

DNA microarray comparisons of the gene expression pattern between galectin-7 and control transfectants have revealed a number of genes whose expression is affected by galectin-7 [43]. Among them, monoamine oxidase B and ryanodine receptor 2, which are up-regulated by galectin-7, and glutathione *S*-transferase Mu 3, which is down-regulated, are most relevant to the apoptosis susceptibility of galectin-7 transfectants. These gene products have been linked to regulation of apoptosis, and significantly, are redox-related. Since redox is intimately related to apoptosis, the finding suggests a possibility that galectin-7 might regulate the expression of gene products that modulate the redox status of the cell, resulting in promotion of apoptosis.

While all the mechanisms discussed above implicate intracellular pathways, it is possible that galectin-7 secreted from cells or released by damaged cells can enhance cell death by interacting with cell surface apoptosis receptors. However, several observations (cited in Ref. [43]) speak against this possibility. First, there was no detectable galectin-7 in the culture supernatant of UV-treated galectin-7-transfectants. Second, this supernatant did not cause appreciable apoptosis when added to HeLa cells. Third, recombinant galectin-7 up to 1 μM did not cause HeLa cell apoptosis, although higher doses (e.g., 5 μM) caused appreciable apoptosis. The total amount of galectin-7 in galectin-7 transfectants is 2 ng/10^5 cells; thus even if all the galectin-7 protein is released by these cells, the concentration of this lectin in the supernatant would be 0.13 nM, which is well below the amount that can cause apoptosis. Finally, immunofluorescence analysis of HaCaT cells (transformed keratinocytes) and galectin-7-transfected HeLa cells showed that galectin-7 is localized in the nucleus and cytoplasm. This subcellular distribution is consistent with an intracellular function of the protein.

In conclusion, galectin-7 is a pro-apoptotic protein that functions intracellularly upstream of JNK activation and cytochrome *c* release. It may operate by affecting the expression of some gene products that are related to the redox status of the cell.

4.3. Galectin-12

The N-terminal domain of galectin-12 contains all the sequence elements predicted to form the two β-sheets as well as the conserved carbohydrate-contact residues present in all known galectins. The sequence of the C-terminal domain, however, strays significantly from the consensus sequence and many of these conserved residues are not present. Consistent with these features, full-length galectin-12 exhibits lactose-binding activity, whereas the C-terminal domain fragment lacks such activity [87]. Thus, the carbohydrate-binding activity appears to be contributed mainly by the N-terminal domain.

An important feature in galectin-12 mRNA is that the 3'-untranslated region contains five AU-rich motifs (AUUUA), which were initially identified in inflammatory cytokine mRNAs and are also detectable in many other mRNAs coding for proteins of growth regulatory functions, such as oncoproteins and growth factors. These motifs are known to confer instability to mRNA [88,89]. Consistent with this feature, Northern blot analysis showed that galectin-12 mRNA was nearly undetectable in many tissues tested, in contrast to galectin-3 mRNA, which was detected in almost all tissues [87]. Subsequent studies noted that the protein is predominantly expressed by adipose tissue and is found in the adipocyte cell line 3T3-L1 [47].

The effect of galectin-12 expression on cell growth was observed in gene transfection experiments. When HeLa cells were transfected with galectin-12 or galectin-9 constructs containing a puromycin selection marker and cultured in the presence of puromycin, it was noted that while galectin-9 transfectants continued to grow, galectin-12 transfectants failed to proliferate. At the end of the 5-day culture period, the number of galectin-12 transfectants was approximately 1/6 that of galectin-9 transfectants [87].

One possible explanation for these findings is that galectin-12 promotes cell death. Indeed, it was noted that treatments that resulted in an increase in the number of apoptotic cells in adipose tissue also caused an increase in the level of galectin-12 mRNA [47]. Moreover, when COS-1 cells were transfected with galectin-12 cDNA or the vector alone, there were more apoptotic cells in galectin-12 transfectants than control transfectants [47]. However, galectin-12 can also regulate cell growth by inducing cell cycle arrest (see Section 6).

4.4. Other galectins

Both galectin-1 and -9 have been shown to induce apoptosis (reviewed in Ref. [90])[see related article in this issue on Role of Galectins in Inflammatory and Immunomodulatory Processes by Rabinovich et al. [91]]. These activities were demonstrated by adding recombinant proteins to the cell cultures and thus are likely to be exerted through an extracellular mechanism. However, on the basis of the demonstrated functions of a number of galectins described in this section, further studies may reveal that these two galectins, as well as other galectins, play a role in regulation of cell growth and apoptosis through intracellular mechanisms.

Therefore, a picture that has emerged is that galectins are a family of regulators of apoptosis. Some members are proapoptotic, while others are anti-apoptotic, much like the Bcl-2 family. The fact that galectin-3 has sequence similarity with Bcl-2 suggests the possibility that these two families might have evolved from the same ancestral gene. Thus, the survival of many cell types may be

controlled by the expression level, subcellular localization, and activation status of different galectin family members.

5. Regulation of the cell cycle

Many regulators of cell growth and apoptosis also function in controlling the cell cycle. Thus, it is not surprising that some galectins have been found to be cell cycle regulators.

5.1. Galectin-3

Earlier studies have demonstrated the dependence of galectin-3 expression on the cell cycle. Thus, the effect of galectin-3 expression on the cell growth rate could also be due to this lectin's regulation of the cell cycle. Evidence for this has been provided through the studies of galectin-3's protection of cells from apoptosis induced by the loss of cell anchorage, described in the previous section. In this case, transfectants overexpressing galectin-3 responded to the loss of cell adhesion by undergoing G1 arrest without detectable cell death [75]. This effect is associated with the galectin-3-mediated down-regulation of cyclin E and cyclin A levels (kinases associated with these cyclins are known to be activated in late G1 and S phase of the cell cycle). Galectin-3 is also found to up-regulate the levels of the inhibitory proteins (p21 and p27) for these cyclins. Moreover, retinoblastoma (Rb) protein becomes hypophosphorylated when galectin-3-overexpressing cells lose their cell anchorage. This is consistent with these cells failing to enter the S phase, since it is known that Rb is maintained at its hyperphosphorylated state through S, G2, and most of M phases. Finally, the NWGR motif is essential for galectin-3's cell cycle regulation activity, as the mutant with glycine in this motif substituted by alanine does not have this activity.

Similarly, galectin-3 expression in BT549 cells results in a different response to a cell cycle regulator, genistein: genistein induces p21 expression in galectin-3-expressing BT549 cells, but not in control BT549 cells [76]. This is consistent with the finding that genistein effectively induces apoptosis without detectable cell cycle arrest in BT549, while it induces cell cycle arrest at the G2/M phase without apoptosis induction in galectin-3-transfected cells [76].

5.2. Galectin-12

Galectin-12 was initially cloned from a cDNA library made from Jurkat cells synchronized in the G1 phase [87], suggesting the dependence of the expression of this galectin on the cell cycle. The finding that expression of galectin-12 mRNA is up-regulated in cells under conditions that induce cell stasis supports this. Thus, while Jurkat cells do not contain a detectable amount of galectin-12 mRNA, this mRNA is readily detectable when the cells are treated with hydroxyurea or thymidine that synchronizes cells at the G1/S boundary, or with theophylline plus dibutyryl-cAMP that synchronizes cells at G1 [87].

The results suggest that galectin-12 might have a role in regulation of the cell cycle. Indeed, when HeLa cells were transfected with galectin-12 cDNA, there was a considerably higher percentage of cells in G1, and a compensatory lower percentage of cells in S and G2/M, compared to cells transfected with galectin-9 cDNA [87]. The results were due to G1 arrest rather than accelerated M to G1 transition, because the increase in the percentage of cells in the G1 phase was more pronounced when nocodazole was added to the cells after transfection. This drug disrupts the formation of spindle fibers and results in a block at mitosis, thus preventing cells from recycling back to G1.

The exact mechanism of galectin-12's cell cycle regulation is largely unknown. Preliminary studies showed that galectin-12 expression resulted in a down-regulation of both Rb protein phosphorylation and cyclin A expression (Yang, R.-Y. and Liu, F.T., unpublished observations). Therefore, this lectin may control the cell cycle through these key cell-cycle regulators. Existing data do support an intracellular mechanism of galectin-12's function. First, the cell cycle distribution of galectin-12 transfectants is not affected by the presence of lactose in the culture medium (Yang, R.-Y. and Liu, F.T., unpublished observations). Since lactose is known to effectively block galectin functions that are dependent on lectin–carbohydrate interactions, the results speak against the possibility that galectin-12 released from the cell exerts its function through binding to cell surface glycoconjugates.

It appears that the lactose-binding activity is not required for the cell cycle-regulation activity of galectin-12. Cells transfected with cDNA coding for galectin-12 lacking the N-terminal amino acids that form the S1 β-strand still undergo cell cycle arrest, even though the mutant galectin-12 no longer has lactose-binding activity (note that the S1-β strand is critical for the carbohydrate-binding activity of galectins) (Yang, R.-Y. and Liu, F.T., unpublished observations).

5.3. Other galectins

Galectin-1 has been shown to inhibit growth of mouse embryonic fibroblasts at relatively low concentrations (~ 10 nM) in a carbohydrate-independent fashion [92], and induce cell cycle arrest during the S-to-G2 transition of mammary cell lines [93,94]. Galectin-1 has also been shown to inhibit the IL-2-induced proliferation of phytohemagglutinin-activated T lymphocytes as well as the IL-2-independent proliferation of T lymphoma cells, in a carbohydrate-independent manner [95]. In the latter case, galectin-1 appears to arrest the cells in the S and G2/M phases of the cell cycle. These effects are related to the extracellular functions of this galectin. However, it is possible that future studies will show that many galectins control the cell cycle through intracellular mechanisms.

6. Concluding remarks

This review has attempted to summarize the intracellular activities displayed by several members of the galectin family (Table 1). From the historical perspective, galectins were isolated as carbohydrate-binding proteins. Based on their lectin properties, these intracellular activities would surely seem unexpected. Nevertheless, these activities are consistent with the predominantly intracellular localization of these proteins, which have clearly been documented. Fascinated as we are by these revelations regarding the intracellular activities of galectins, it seems important to address three major sets of questions. First, are the apparently disparate intracellular activities of any one galectin (e.g., cell cycle control, anti-apoptosis, and RNA processing for galectin-3 (see Table 1)) a manifestation of the multifunctional nature of the protein? Or, does the galectin participate in one fundamental process (RNA processing for galectin-3), to which the other activities (cell cycle control and anti-apoptosis) can be attributed? A collaborative effort needs to be mounted to address this issue. A single cell type should be transfected to overexpress the native galectin, variants devoid of carbohydrate-binding activity, and fragments of the polypeptide that might behave as dominant negative mutants. These transfectants would then be studied for cell growth, cell death, expression of cell cycle regulatory genes, RNA metabolism in general, and expression of specific RNA transcripts.

Second, which of the intracellular activities (see Table 1), if any, involve protein–carbohydrate recognition corresponding to that expected of a lectin? Or, are the various activities derived from galectin binding to other intracellular proteins solely based on protein–protein interactions? With respect to the former, GalNAc residues on cytokeratins represent the only example of intracellular carbohydrates that have been documented to interact with galectins [42]. It remains to be shown that such carbohydrate-bearing ligands play a role in the intracellular activities of galectins. On the other hand, there is experimental evidence to implicate the carbohydrate-binding site on the galectin polypeptide for some of the intracellular activities. For example, the binding of galectin-3 to Bcl-2 is inhibitable by lactose [38] and the cell-free splicing reaction is inhibitable by high affinity saccharide ligands of galectin-3 [56]. While these observations indicate the importance of the carbohydrate-binding site of galectin-3, it is possible that saccharide binding to the carbohydrate-binding site induces a conformational change at a distal site that disrupts the interaction of the galectin with other proteins.

Finally, did the CRD of the galectins, as a family of phylogenetically conserved proteins, initially serve a carbohydrate recognition function outside of cells and then become adapted for protein–protein interactions for functioning inside cells? Or, were galectins intracellular molecules with both nuclear and cytoplasmic functions (those that we are unmasking currently), which then evolved into carbohydrate recognition molecules outside of cells? It seems possible that initially, the interactions between galectins with intracellular partners were exclusively protein–protein interactions. As abundant proteins, some were "misrouted" and interacted with cell surface carbohydrates whose structure mimicked intracellular peptide ligands. Historically, extracellular lectin–carbohydrate mediated functions predominated the literature leading to the classic definition of lectins as non-immunoglobulin, nonenzymatic carbohydrate-binding proteins. Perhaps it is appropriate to shed this paradigm and search for new ones that focus on the dominant intracellular form of this family of molecules.

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