Regulation of T lymphocyte function by galectin-1 and its dependence on the carbohydrate binding activity of lectin

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1. Introduction

1.1. Glycosylation and lectins

The glycosylation of proteins and lipids is a common posttranslational modification that regulates many physiological processes, like cell to extracellular matrix, cell to cell adhesion, assembly and activation of receptor complexes and protein trafficking (1). The role of glycosylation in cell physiology becomes better understandable with the characterization of the lectins, proteins that have binding sites for the specific mono- di- or oligosaccharides linked to proteins or lipids (1). There are several types of lectins:

1) The C-type lectins need calcium for recognition of their ligands and fulfill a variety of functions predominantly in the immune system. Selectins, for example, are C-type lectins known to mediate interaction between the blood and endothelial cells (1).

2) The P-type lectins are mannose-6-phosphate receptors that participate in the trafficking of enzymes to prelysosomal compartments (2, 3).

3) The I-type lectins or siglecs are carbohydrate-binding proteins belonging to the immunoglobulin superfamily (4). One of them is CD22, which is expressed on mature B-cells and mediates interactions with glycoproteins expressed on B- or T-cells.

4) The galectins are described below in details.

5) The L-type lectins are involved in protein sorting in luminal compartments of animal cells (5).

6) The M-type lectins are a recently described family of intracellular lectins involved in glycoprotein trafficking (6, 7). They resemble mannosidases found in the endoplasmic reticulum (ER), but they lack the key catalytic residues.

7) The R-type lectins contain ricin-like carbohydrate recognition domains (CRD) (8). These lectins are involved in enzyme targeting and glycoprotein hormone turnover.

8) Calnexin and calreticulin are parts of the quality control system for glycoproteins in the ER (9) They bind to the terminal glucose residues on N-linked oligosaccharides and retain misfolded glycoproteins in the ER.

Table 1.1 is a brief summary of existing lectin families, their ligands, and function.

Lectin Group	Typical Ligands	Examples of functions
C-type lectins	Various	Cell adhesion (Selectins). Glycoprotein clearance. Innate immunity (Collectins).
P-type lectins	Man 6-phosphate	Post Golgi protein sorting.
I-type lectins	Sialic acid	Cell adhesion (Siglecs).
Galectins	β-Galactosides	Various. Discussed below in details.
L-type lectins	Various	Protein sorting in the endoplasmic reticulum
M-type lectins	Man_8	ER-associated degradation of glycoproteins.
R-type lectins	Various	Enzyme targeting, glycoprotein hormone turnover.
Calnexin and Calreticulin	Glc_1Man_9	Protein sorting in the endoplasmic reticulum.

Table 1.1. Types of animal lectins, their ligands and function.

Many of the above mentioned lectins play an important role in the immune system.

1.2. Lectins in the immune system

The role of the lectins in the immune system may be subdivided into four separate categories, although they are not mutually exclusive:

-direct defense (antibody- and/or complement-like);

-recognition and trafficking within the immune system;

-immune regulation (suppression or enhancement);

-prevention of autoimmunity.

I-type lectins play an important role mostly in the immunity of invertebrates, but are not only confined to them. In higher animals, including humans, there are collectins (10, 11), ficolins (10), and the membrane-bound macrophage mannose receptor (12, 13), all of which recognize and directly promote the elimination of pathogens. A few lectins like 32-kilodalton macrophage membrane protein from Candida albicans (14) or ovgal11 from sheep (15) are considered to resemble complement components more than immunoglobulins.

The selectins function in cell trafficking, promoting extravasation via the ‘‘rolling’’ capture of leukocytes, as was shown in selectin knock out mice (16). Several other adhesion molecules with lectin activity have been implicated in cell trafficking: CD44 has a role in the lymphocyte recirculation (17), CD22 mediates the homing of murine B cells to the bone marrow (18), CD11b/CD18 mediates monocyte and neutrophil adhesion to the endothelium

and thus promotes extravasation of neutrophils to the sites of inflammation (19). CD31 also influences neutrophil migration in response to inflammatory stimuli (20).

A number of cytokines, including IL-1, IL-2, IL-6, IL-12 and TNFα have lectin activity (21-23). The immunoregulatory properties of these cytokines are well known, and are mediated by protein–protein binding to their cytokine receptors. Much less is known about the role of lectin activity, but some indications of an immunoregulatory role are emerging. For example, there is some evidence that IL-2 can bind to a high mannosyl moiety on the IL-2Rα subunit to promote the formation of a high affinity complex of IL-2/ IL-2Rα/IL-2Rβ/IL-2Rγ subunits leading to cellular signaling (24). Other lectins that may be involved in modulating the immune response include sarcolectin, which has been implicated in growth regulation (25), and the I-type lectins. There are now 10 members of the latter group of adhesion molecules (26,), and while their function(s) has not been clearly defined, several are believed to have a signal within the immune system. The plant lectins, mistletoe lectins and wheat germ agglutinin (WGA), have been implicated in cancer therapy because of the modulation of an immune response (27, 28). One possible target for WGA is the CD45 receptor tyrosine phosphatase, since binding of WGA to CD45 results in the decrease of phosphatase activity (29).

Members of the galectin family of animal lectins are important regulators of processes in immune system.

1.3. Galectin lectin family

The galectins are the beta-galactoside binding lectins with a carbohydrate recognition domain that has many conserved sequence elements. In addition to the galectins expressed in vertebrates (fish (30), birds (31), amphibians (32), and mammals (reviewed in ref. 33)), galectins have also been found in invertebrates (worms (34) and insects (35)) and even in protists (sponge (36) and fungus (37)). Utilizing the binding of the galectins to beta-galactoside, it was possible to purify them by lactose affinity chromatography. After cloning and sequencing the first galectins novel galectin family members are now being rapidly discovered by screening for related sequences in the DNA data banks (38). For instance, while fourteen human galectin proteins are already characterized, another eight related genes have been identified in the human genome by sequence similarity (39). The CRD of the galectins folds as a globule of two antiparallel β-sheets formed by five and six beta strands (Fig. 1.1.) (39).

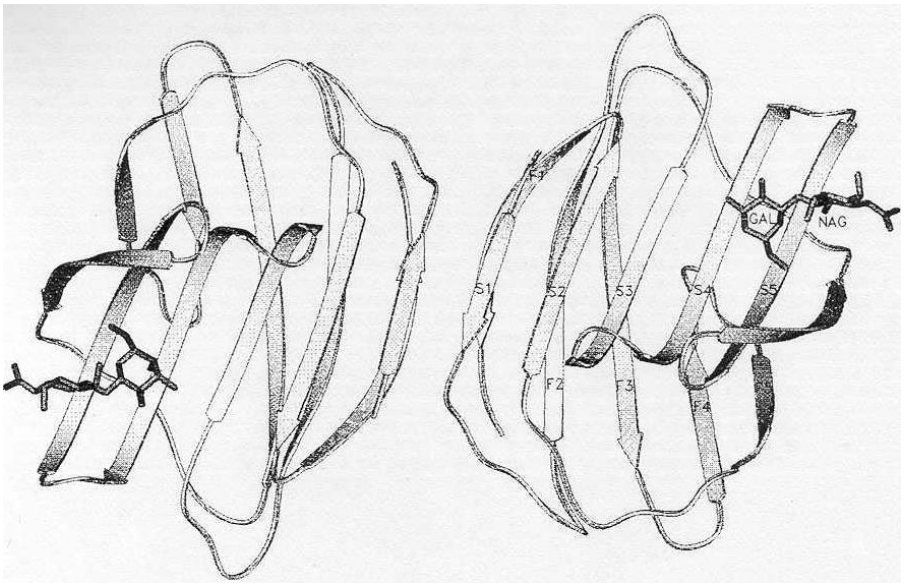

Fig.1.1. Crystal structure of galectin-1 dimer complexed with lactose (from Ref. 40).

Although galectins are often reported to be present on the cell surface (41) or in the extracellular matrix (42), they lack recognizable secretion signal sequences and do not pass through the standard ER/Golgi pathway when secreted (43, 44), with the possible exceptions of a sponge galectin (36) and several other invertebrate galectins (38). Instead, most galectins have characteristics of cytoplasmic proteins, such as an acetylated N-terminus, free sulfhydryls, and lack of glycosylation. Indeed, intracellularly they appear to be cytosolic (45), submembranous (46), or sometimes nuclear (47), but not compartmentalized inside the classical secretory compartments. Nevertheless, there is strong experimental evidence that at least some galectins are secreted, albeit by novel nonclassical mechanisms (43, 44). In this regard, galectins belong to a small category of proteins (including FGF-1 and –2 (48)) that are secreted by mechanisms thar are distinct from the classical vesicle-mediated exocytosis (Fig. 1.2).

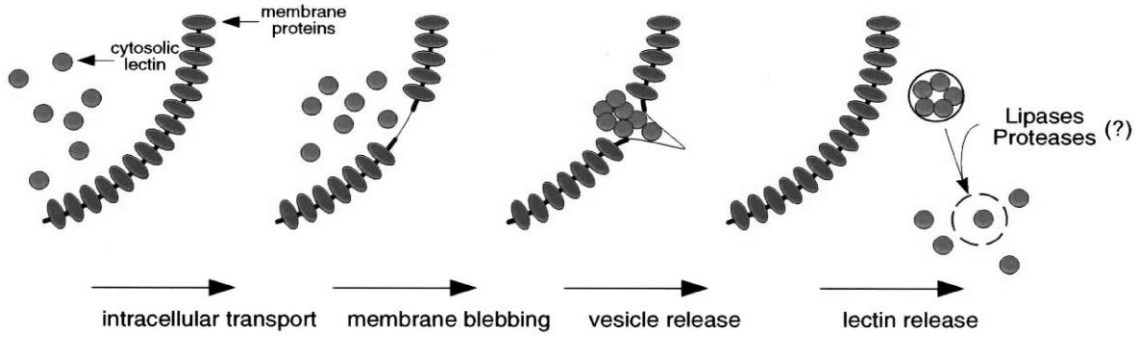

Fig.1.2. A possible mechanism of galectin secretion. Expressed protein accumulates near the cell membrane; when concentration of galectin near membrane becomes high it induces membrane blebbing and formation of a vesicle; disruption of a vesicle leads to galectin release (from Ref. 49).

All galectins identified to date can be structurally classified into three basic types (Fig. 1.3).

The ‘‘prototype’’ galectins contain a single CRD. Most of these proteins are able to self-associate to form homodimers. Chicken galectins C-14 and C-16 (50) resemble mammalian galectin-1, but the former one occurs as a monomer, while the latter one forms homodimers. The prototype galectins dimerize through their N- and C-terminal regions as was shown by mutagenesis (51) and crystallographic studies (52). Thus, the CRD pockets and any bound ligands face away from each other. The ‘‘tandem-repeat’’ galectins are composed of two nonidentical core galectin domains joined either directly or via a linker peptide of variable length (53, 54, 55). Crystal structures have not yet been obtained for any tandem-repeat class galectins. The only known member of the ‘‘chimeric’’ galectins, galectin-3, is composed of a galectin domain joined to a N-terminal non-lectin domain. The N-terminal domain of galectin-3 contains proline, glycine, and tyrosine rich repeats followed by a C-terminal CRD. The repetitive domain is similar to the ones found in certain other proteins, such as synexin and synaptophysin, where it is proposed to function in self-aggregation (56).

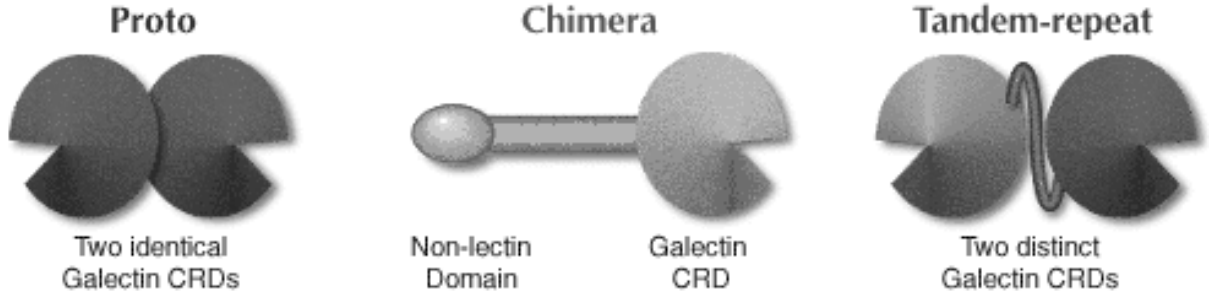

Fig.1.3. Three structural types of galectins. The “prototype” galectins are composed of a single CRD; the “chimeric” galectin-3 has nonlectin domain fused to galectin CRD; the “tandem-repeat” galectins have two distinct CRDs linked together by a linker sequence. (taken from http://www.glycoforum.gr.jp/science/word/lectin/LEA01E.html)

The presence of galectins in so many species including very ancient ones suggests that they evolved to play fundamental roles in cell biology, while the presence of multiple galectins within different species suggests that they have diverged to fulfill a variety of more specific functions. Mammalian galectins identified so far are presented in Table 1.2.

Galectin	Type	Localization	Glycoconjugate ligands
1	Prototype (monomeric/dimeric)	Most organs, lymph nodes, spleen, thymus, placenta, prostate, macrophages, B cells, T cells, tumors.	Matrix glycoproteins: laminin and fibronectin, 90K/Mac-2BP. Cell surface receptors: CD45, CD43, CD7, CD2, CD3 and GM_1
2	Prototype (monomeric/dimeric)	Gastrointestinal tract, tumors	
3	Chimera-type	Mainly in tumor cells, macrophages, epithelial cells, fibroblasts and activated T cells	Matrix glycoproteins: laminin and fibronectin, LAMPS, 90K/Mac-2BP, MP20 and CEA.
4	Tandem repeat-type	Gastrointestinal tract	
5	Prototype	Erythrocytes	
6	Tandem repeat-type	Gastrointestinal tract	90K/Mac-2 BP
7	Prototype	Skin and tumors of epidermal origin	
8	Tandem repeat-type with novel prototype isoforms	Liver, prostate, kidney, cardiac muscle, lung and brain	
9	Tandem repeat-type	Thymus, T cells, kidney and Hodgkin´s lymphoma	
10	Prototype	Eosinophils and basophils	
11	Prototype and dimeric	Lens	
12	Tandem repeat-type	Adipocytes	
13	Prototype	Placenta	
14	Prototype	Eosinophils	

Table. 1.2. Galectin family of animal lectins, localization and glycoconjugate ligands.

1.4. Properties and functions of galectin-1

Galectin-1 was the first member of the galectin family to be described (57). It specifically recognizes beta-galactosides and has conserved sequence elements characteristic of galectins. The chain of 134 amino acids folds into the typical galectin CRD globule (Fig. 1.1). Two identical galectin-1 globules can form a dimer (58). Dimerization involves self-association of monomer subunits via hydrophobic surfaces opposite to the sugar-binding pocket (59). The hydrophobic surfaces are formed by the amino acidcs 4-7 of the N terminus and the amino acids 126, 128, and 131 of the C-terminus (Fig.1.4) Mutations of hydrophobic amino acids in these regions lead to the inability of galectin-1 to form dimers.(51). The dissociation constant (Kd) of the dimer is 7μM (60). Although galectin-1 has six cysteines (Fig.1.4), disulfide bonds are not formed in the native protein and they exist as free

sulfhydryls (40). Cysteine pairs 2 and 130, 16 and 88, 42 and 60 can form disulfide bonds in an oxidizing environment (61). Mutation of Cys2 makes the protein significantly more resistant to oxidation (62). Amino acids, which interact directly with sugar, were deduced from the crystal structure of galectin-1 (Fig.1.4.). A single mutation in any of the amino acids - His44, Asn46, Arg48, Asn61, Glu71, or Arg73 - abolishes binding to asialofetuin (63, 64). The amino acids are crucial in forming the pocket which binds Galβ1-4GlcNAc via 4-OH and 6-OH of galactose and 3(4)-OH of Glc (NAc) (63). It also shows high affinity for complex N-glycans (63). Affinity increases with the number of branches up to triantennary N-glycans (63). The surface loop, comprising amino acid residues 25–30 (Fig.1.4), and joining two internal strands, forms part of the growth-inhibitory site, as was revealed by mutagenesis studies (65).

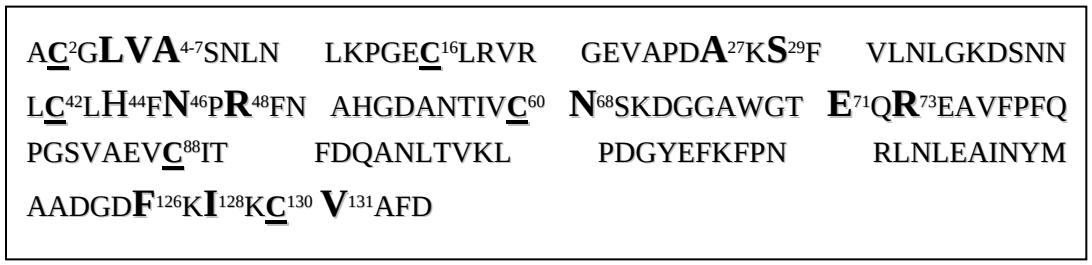

Fig.1.4. Protein sequence of galectin-1. The cysteines are shown in bold and underlined. Amino acids crucial for sertain functions are shown by large letters and in bold. 4-7, 126, 128, 131 are important for dimer formation; 27,29 are parts of the growth inhibitory site; 44, 46 ,48, 68, 71, 73 are in direct contact with the sugar ligand.

The activity of the galectin-1 gene is strongly modulated under a variety of physiological or pathological conditions. A small genomic region of no more than 100 base pairs surrounding the transcription start site (-50/+50), accounts for most of the transcriptional activity of the galectin-1 gene (66). Both upstream and downstream position-dependent cis-elements are necessary for efficient transcriptional activity. Using the bisulfite genomic sequencing technique, it was found that the primary mechanism controlling the cell-specific expression and reactivation of the galectin-1 gene is the transition from a fully methylated to a fully unmethylated state of the 11 CpG sites lying around the transcription start site (67) Moreover, the *in vitro* methylated galectin-1 promoter is inactive when transfected into mouse fibroblasts. Also in normal mouse and rat tissues the rate of DNA methylation of the small CpG island surrounding the start site correlates with transcription activity (68). While in cell lines differences in DNA methylation are spread over a large area, in tissues differences in CpG modification are confined to the cluster of CpG surrounding the transcription start site.

In this case, the methylation profiles in non-expressing tissues are highly heterogeneous and the density of methyl-CpGs rather than site-specific methylation, distinguishes the non-expressing from the expressing alleles (68).

The expression pattern of galectin-1 varies among different adult tissues, changes during development and is altered in many neoplastic transformations and other pathological conditions. The spatio-temporal distribution of the lectin in human and mouse embryogenesis has been thoroughly investigated (69). It is first expressed in the trophoectoderm cells of the implanting embryo and has been implicated in the process of implantation. In mouse embryos prominent expression of galectin-1 in the myotomes of the somites has been reported (69). In human embryogenesis, it is found prevalently in connective tissues and some epithelia, such as in the basal layers of the skin and in the epithelial cells of the gonads (70). In adult tissues, galectin–1 is abundantly expressed in many cell types, such as skeletal, smooth and cardiac muscle and other cells of mesenchymal origin. Galectin-1 might play an important role in skeletal muscle differentiation, since it was shown to be implicated in the conversion of dermal fibroblasts to muscle (71). High galectin-1 expression has been detected in sensory, motor and olfactory neurons and it has been demonstrated to participate in the regulation of olfactory axon fasciculation and targeting (72). In hematopoietic and lymphoid organs galectin-1 is expressed by stromal cells. In murine thymus, the lectin is expressed by thymic epithelial cells (73) and is detected throughout the stromal framework of the thymus.

The expression patern of the galectin-1 gene in cell lines reproduces the one found in the tissues of origin. Galectin-1 is highly expressed in cells of mesenchymal origin and weakly or not expressed in epithelial cells. However, some conditions can dramatically modulate the expression of the lectin gene in cultured cells. The most important is the cellular transformation obtained by transfection of different oncogenes. Galectin-1 expression correlates with the degree of malignancy in rat thyroid cell lines transformed with several cellular or viral oncogenes (74). Its mRNA levels increase 20-fold in low tumorigenic and up to 100-fold in high tumorigenic cells. A transient increase in galectin-1 expression is also achieved upon treatment of thyroid cells with thyroid stimulating hormone (75). In human head and neck carcinoma cells lectin expression is stimulated by butyrate treatment (76). In this case, the expression correlates with growth inhibition and soft agar colony formation inhibition. Because galectin-1 expression is also increased upon treatment with other inhibitors of histone deacetylase, it has been proposed that expression is linked to the status of histone acetylation (77). In human endothelial cells, lectin synthesis is up-regulated by endothelial cell activation (77). Activation of the galectin-1 gene can also be achieved by

fusing non-expressing cells with tumor cells or by the treatment of non-expressing cells with a DNA demethylating agent (78).

Galectin-1 expression is strongly affected in human tumors. Lectin mRNA levels are very low in normal thyroid non-neoplastic goiters, adenomas and follicular carcinomas, while they are increased (5-20-fold) in a large proportion of papillary carcinomas and in almost all the aggressive anaplastic carcinomas (74, 79). In immunohistochemical studies, however, a higher galectin-1 content relative to normal thyroid (20-30-fold) has been reported in all analyzed follicular and also in some medullary carcinomas (80). The data on galectin-1 expression in tumors and its role in cancer cell adhesion has been the subject of a detailed analysis (81).

Targeted disruption of the galectin-1 gene alone, or galectin-1 and galectin-3 genes together, was performed in mice. Galectin-1-/-, or galectin-1-/- together with galectin-3-/-, homozygous null mutants appear normal and fertile (82, 83). The potential role of galectin-1 in the developing olfactory system was investigated in newborn mice. This analysis revealed that the absence of lectin results in major defects in primary olfactory neuron outgrowth (72). Because galectin-1 has been postulated to participate in regulating immune function (84), including T-lymphocyte selection in the thymus, complete blood counts were compared for control and galectin-1 “null” mutant mice. No significant differences were found in white cell numbers or differential distribution (85). However, potential differences in the prevalence of lymphocyte subtypes have not been investigated.

Galectin-1 exhibits dual localization, both in the intracellular and extracellular compartments. Immunohistochemical studies demonstrated the presence of lectin in cytoplasm of smooth muscle (86) and thymic epithelial (73) cells, as well as in the nucleus of the same smooth muscle cells(86) and osteoblasts (87). Indirect immunofluorescence studies showed the presence of galectin-1 on the surface of K562 cells (41). The protein is also present in the extracellular environment in thymus(71) and bone marrow (88). In accordance with the dual localization, galectin-1 functions can be divided intracellularly and extracellularly.

1.4.1. Extracellular function of galectin-1

Extracellularly, galectin-1 participates in the regulation of cell activation, proliferation, adhesion and apoptosis. A topic of great interest is the regulation of these processes by galectin-1 in the immune system.

Modulation of adhesion by galectin-1

Galectin-1 has been implicated in cell to cell and cell to extracellular matrix interactions. It may have anti- or pro-adhesive effect on the interaction. For large cell lymphoma the positive regulation of cell adhesion has been shown; the lectin was present on the surface of the endothelial cells and partially mediated the adhesion of RAW117-H10 large cell lymphoma cells to liver microvessel endothelial cells (89). The same positive effect was observed in human prostate carcinoma cell lines transfected with galectin-1 (90). The interaction of galectin-1 with the tumor secreted antigen, 90K has been shown to contribute to the formation of multicellular aggregates of human melanoma cells (91). It has also shown proadhesive effects toward other cell types such as olfactory neurons (92) For other cells, the negative regulation of cell adhesion was demonstrated. The lectin has been shown to inhibit myoblast interactions with laminin by blocking $\alpha_7\beta_1$ integrin (93). It also inhibited A121 ovarian carcinoma cell line adhesion to extracellular matrix (94). Presence of the lectin was shown to inhibit IL-2 activated T-cell adhesion to intact ECM, laminin and fibronectin, and to a lesser extent to collagen type IV, in a dose-dependent manner (95). This effect was specifically blocked by anti-galectin-1 antibody and was dependent on the lectin's carbohydrate-binding properties. Galectin-1 could promote cell attachment or detachment according to cell type, cell activation status, or cell developmental stage (reviewed in ref. (96)). Moreover, the binary actions of galectin-1 could also be associated to the high or low concentrations of galectin-1 in the extracellular milieu or the glycosylation state of the counter-receptors.

Galectin-1 regulates cell growth

Galectin-1 inhibits growth of different cell types: phytohemagglutinin (PHA)-activated human T cells (95, 98), concanavalin A (Con A)-stimulated rat T cells (99), human neuroblastoma cells (100), human leukemia T cells (101), and murine fibroblasts (102). The growth inhibitory effect may depend on the carbohydrate-binding activity or be independent of the lectin-sugar interactions. A pioneer study conducted by Wells and Mallucci in 1991 (102) showed that galectin-1 acts as an autocrine-negative growth factor and inhibits the growth of mouse embryonic fibroblasts in a carbohydrate-independent manner. Inhibition of proliferation of Con A-stimulated rat T cells was dose- and carbohydrate-dependent (99). Further investigation revealed that galectin-1 secreted by CD8+ T cells reduces clonal expansion of stimulated CD8+ T cells and interleukin (IL)-2 induced proliferation of PHA-activated T lymphocytes in an autocrine manner by arresting the cells in the S and G2/M

phases of the cell cycle (97, 103). The negative regulation of cell growth by galectin-1 has been extended to some tumor cells. It was shown that cell density-dependent growth inhibition of human neuroblastoma cells is initiated by increased ganglioside sialidase activity, leading to increased cell-surface expression of the ganglioside G_{M1}, which acts as a ligand for galectin-1 (100).

It has been speculated that the growth inhibitory properties of this lectin are highly dependent on cell type, cell-activation status, and concomitant environmental signals.

Galectin-1 induces apoptosis

Galectin-1 induces apoptosis of T cells during their development in the thymus and after immune stimulation of the peripheral activated (104, 105, 84), but not resting (84, 106), T cells. The triggering of cell death does not occur via the T cell antigen receptor (TCR), Fas or tumor necrosis factor (TNF) receptor (84). As an early cell response, the lectin initiates tyrosine phosphorylation in T and B cell lines. Tyrosine kinase and phosphatase inhibitors, genistein and phenylarsine oxide or sodium vanadate, respectively, diminish the phosphorylation and the apoptotic effect, pointing out the role of the tyrosine phosphorylation events in the induction of apoptosis (R.Fajka-Boja, unpublished observations). Screening of a large panel of mAbs that would block galectin-1 binding to MOLT-4 human T cells revealed thatt Abs recognizing CD45, CD43, CD8, CD7, CD4, and CD3 inhibited galectin-1 binding to the cells (107). Separation of the galectin-1 binding glycoproteins directly from membrane proteins of MOLT-4 cells was performed by Triton X-100 extraction and subsequent galectin-1 affinity chromatography (107). The isolated proteins: CD45RA, CD45R0, CD43, CD4, CD7, actin, and the proteins of CD3 complex. Only three galectin-1 binding proteins were isolated from ARR cells, CD45R0, CD43, and CD7 (107). Because the ARR cell line is susceptible to galectin-1 (107), these data suggested that CD4, CD3, and CD8 are not involved in the death signal and, at most, CD45, CD43, and CD7 are directly involved in delivering the death signal following galectin-1 binding. The coimmunoprecipitation experiment indicated that CD45, CD43, and CD7 are either the primary or the most accessible glycoprotein receptors for galectin-1 on the cell surface, implying that galectin-1 death involves one or more of these three glycoproteins (107). The relevance of CD45 in mediating apoptosis was supported by the fact that CD45-positive Jurkat T cells were more susceptible to the apoptotic effect than were CD45-negative cells (84, 108). It was later shown that CD45 does not mediate the apoptotic signal of galectin-1 in T cells (109). Binding of the lectin leads to a decrease in phosphatase activity of CD45 and downregulation of Src kinase Lyn (Fig.1.5)

(110) that could lead to various downstream effects. Further investigation revealed that CD7 delivered the apoptotic signal of galectin-1. HUT78 cells, not sensitive to galectin-1 and not expressing CD7, were transfected with CD7 cDNA (110). CD7 expression rendered the cells sensitive to galectin-1 induced apoptosis (111).

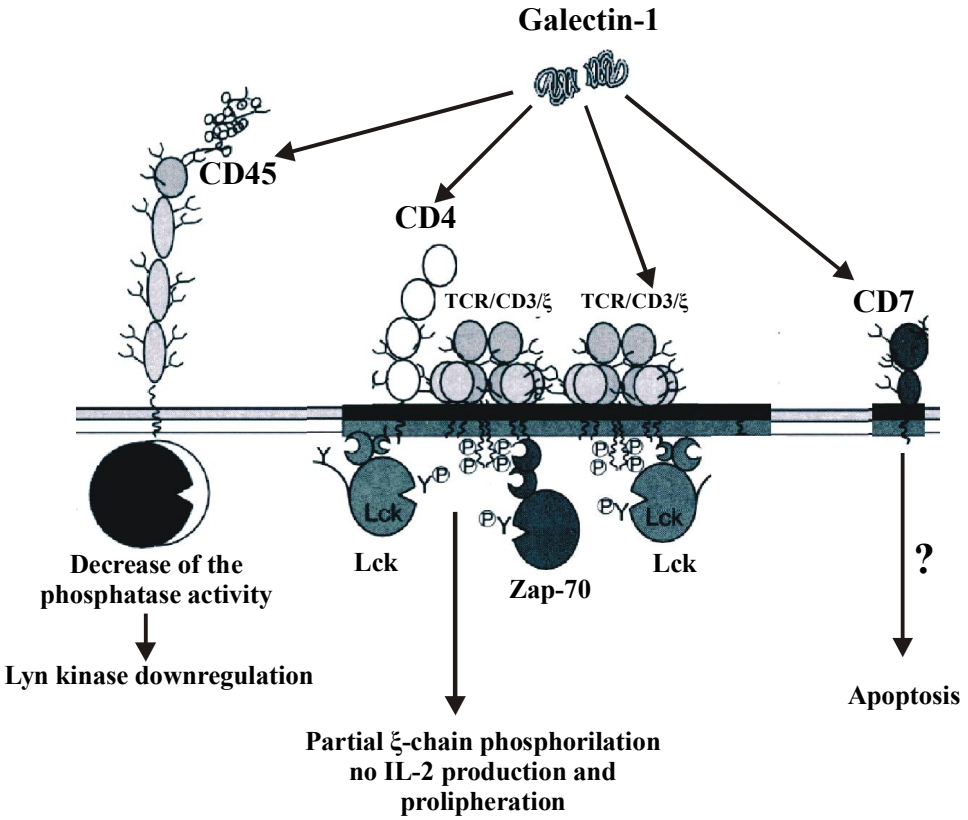

Fig.1.5. Galectin-1 binding molecules on T cell surface and mechanisms of modulation of lymphocyte signalling and apoptosis induction (from Ref. 112 with modifications).

It was demonstrated that binding of galectin-1 to T cells results in a dramatic redistribution of CD3, CD7, CD43, and CD45 into segregated membrane microdomains on the cell surface (111). Whereas CD45 and CD3 colocalize on apoptotic blebs, CD7 and CD43 are distributed in small patches away from the membrane blebs (111). The galectin-1-induced CD45 sequestration could functionally remove and/or inhibit the protein tyrosine phosphatase domain of CD45 from a pro-apoptotic signaling complex. Because susceptibility to galectin-1 induced apoptosis may be regulated by the presentation of lactosamine on the specific oligosaccharide structures created by the glycosyltransferase enzymes, the requirement of these enzymes for the apoptotic process was investigated. Galvan and coworkers demonstrated that expression of the core 2-beta-1,6-*N*-acetylglucosaminyltransferase (C2GnT) is necessary to create a branched structure on the *O*-glycans of T-cell surface

receptors and is critical for galectin-1 to kill activated T cells (113). Overexpression of C2GnT increased the susceptibility of double-positive thymocytes to galectin-1 death signals (113).

Investigations were performed regarding the downstream mechanisms of signal transduction induced by galectin-1. A marked increase in the binding of nuclear extracts to synthetic oligonucleotides containing the activator protein 1 (AP-1) consensus sequence, was detected by an electrophoretic mobility shift assay, when T cells were cultured for 30 min in the presence of the galectin-1 (114). This DNA-binding activity was preceded by a rapid increase in the levels of *c-Jun* mRNA, as determined by Northern blot analysis. Evidence was also provided by Western blot analysis, showing that galectin-1 inhibits Concanavalin A (Con A) induction of Bcl-2 expression (114). These facts suggest that apoptosis induced by galectin-1 requires the activation of the AP-1 transcription factor and modulation of Bcl-2 protein expression (114). Two mutant Jurkat cell lines, lacking the $p56^{lck}$ (JCaM) or the ZAP70 (P116) kinases, did not respond with tyrosine phosphorylation upon galectin-1 treatment and they were much less sensitive to galectin-1 induced apoptosis (R.Fajka-Boja, unpublished observation). Recent studies have provided evidence that galectin-1 cooperates with T cell antigen receptor (TCR) engagement to enhance extracellular signal-regulated kinase-2 activation in a T-cell hybridoma and freshly isolated mouse thymocytes (106). It was proposed that galectin-1 acts as a partial ligand that antagonizes TCR responses known to require costimulation and complete tyrosine phosphorylation, such as IL-2 production, but permits those TCR responses that require only partial signals, such as CD69 up-regulation and apoptosis (Fig.1.5) (115).

1.4.1.1. Extracellular function of galectin-1 in the immune system

Role of galectin-1 in the generation of immunological tolerance

An important role of galectin-1 is that it participatesc in the generation of central and peripheral tolerance utilizing its growth inhibitory and apoptotic properties (Fig.1.6). Human thymic epithelial cells express galectin-1, which binds to core 2-O-glycans on immature cortical thymocytes and modulates their survival (105). Galectin-1 binding correlates with the activation state of the cells, as immature cortical thymocytes bind more galectin-1 than mature medullar thymocytes (105) due to different glycosylation patterns in the cell populations. Galectin-1 killed non-selected and negatively selected immature thymocytes (105). Induced apoptosis was independent of that induced by steroids, but was synergistic with TCR

engagement (105). In this sense the epithelial lectin participates in positive and negative selection growth and cytotoxic signals in the thymus.

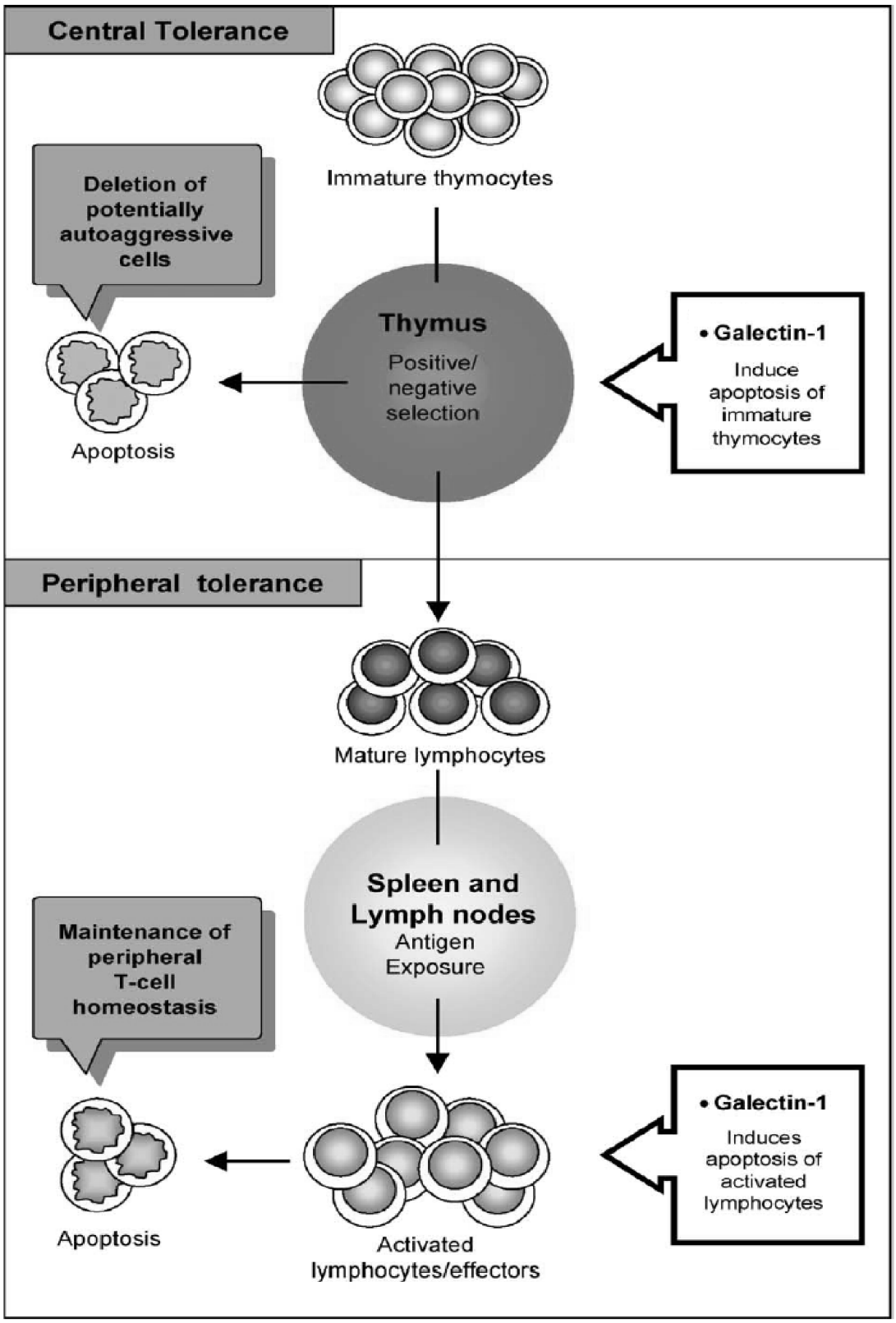

Fig.1.6. Role of apoptotic function of galectin-1 at the central and peripheral levels of the adaptive immune response (from Ref. 116 with modifications).

The peripherial T cells are also exposed to the cytotoxic effect of galectin-1 as has been shown in *in vitro* experiments. It shows specific growth inhibitory properties towards different cell types, such as phytohemagglutinin (PHA)-activated human T cells (117,118), concanavalin (ConA)-stimulated rat T cells (119), activated chicken lymphocytes (120), human leukemia T cells (121). Following an antigenic challenge, galectin-1 is secreted from activated T cells and reduces clonal expansion of antigen-stimulated CD8+ T cells and IL-2 production in an autocrine manner (106, 122, 117, 118). It has been demonstrated that under certain inflammatory conditions, activated macrophages (104, 123), antigen-stimulated T cells (118), activated B cells (98) and alloreactive T cells (124) secrete high level of galectin-1, and downregulate the immune response.

The immunosuppressive and anti-inflammatory effects of galectin-1 have been demonstrated in several experimental models of autoimmunity and chronic inflammation. Galectin-1 ameliorates phospholipase A2-induced edema in a selective and dose-dependent manner, when pre-injected or co-injected together with the enzyme (125). The lectin inhibits the release of arachidonic acid and prostaglandin production from lipopolysaccharide-stimulated macrophages and blocks neutrophil extravasation, mast cell degranulation and nitric oxide synthesis (125). It prevents clinical and histopathological manifestations of autoimmune encephalomyelitis (EAE) (126), ameliorates the inflammatory and autoimmune response in collagen-induced arthritis by increasing T cell susceptibility to activation induced cell death (AICD) (122). Galectin-1 pre-treatment prevented both liver injury and T cell liver infiltration induced by ConA (127). This immunosuppressive effect was associated with the inhibition of TNF-α and TNF-γ production. Galectin-1 purified from electric eel prevented the development of experimental autoimmune myasthenia gravis in rabbits (128).

Galectin-1 is expressed in immune privileged tissues, such as retina (129), placenta (130-133), testis (134, 135) and ovary (60, 136). Immune privileged tissues do not encounter the differentiating immune cells, therefore they are potential targets for the immune response. They have to protect their integrity, prohibiting the survival and proliferation of specific lymphocytes and thereby the inflammation reaction in these tissues. Galectin-1 might contribute to immune privileged mechanisms, ensuring the rapid elimination of the autoreactive T-cells by an apoptotic pathway.

Questions remain open about the mechanisms of the galectin-1 function in the immune system. One of the most important is the involvement of its sugar binding activity in extracellular function, particularly in the induction of apoptosis. As has been mentioned above, T cells lacking C2GnT are resistant to galectin-1 induced cell death (113). This

enzyme is responsible for creating the branched structure on O-glycans of T cell surface glycoproteins (137). Blocking of the CRD by lactose gives controversial results, inhibiting (84) or not inhibiting (R.Fajka-Boja, unpublished observation) the lectin induced apoptosis. Experiments where the lectin CRD specificity is abolished would give additional valuable data on importance of sugar binding in the induction of apoptosis.

1.4.2. Intracellular function of galectin-1

In spite of the more or less well characterized functions of secreted galectin-1, its intracellular function remains poorly defined. The lectin has been implicated in the regulation of the membrane localization of oncogenic H-Ras(12V) (138). Overexpression of galectin-1 results in cell transformation, while antisense RNA inhibits such transformation (138). Farnesylthiosalicylic acid, which disrupts Ras membrane anchorage, disrupts H-Ras(12V)-galectin-1 interactions as well (138). Further studies demonstrated that the lectin diverts the Ras signals to Raf-1 at the expense of PI3 kinase (139).

Galectin-1 is also involved in pre-mRNA processing. Nuclear extracts (NE) from HeLa cells used for cell free splicing assays contain galectin-1 (140). Depletion of galectin-1 and galectin-3 from NE inhibits splicing, and reconstitution of the proteins reconstitutes splicing activity (140). A component of the splisosome complex, Gemin4 has been shown to interact with galectin-1 in this process (139). This function is inhibited by lactose (139). Intracellular galectin-1 has also been shown to promote proliferation of human neuroblastoma cells by an unknown mechanism (141).

2. Aims of the study

Our aim was to study two principal aspects of galectin-1 function:

1. It has not been clearly defined whether the carbohydrate binding of galectin-1 is essential for the apoptotic function of the protein. The main reason for this is that the purification of the non-binding mutants with classical sugar-matrix affinity chromatography encountered difficulties. Therefore our purpose was:

a) To establish a system that would provide a way to isolate this kind of mutant proteins.

b) To analyse whether or not mutants characterized previously as sugar "non-binding" are able to trigger apoptosis of T lymphocytes.

c) To investigate how particular mutations of amino acids, that directly bind carbohydrate, affect galectin-1 induced apoptosis.

2. To obtain a deeper insight into the intracellular function of galectin-1 we have also asked the questions:

a) How *de novo* expressed galectin-1 modulates T cell function?

b) What is the role of the lectin property of galectin-1 in intracellular function?

3. Materials and Methods

3.1. Recombinant protein production

Human wild type and mutant galectin-1 cDNAs (GAL1) were a generous gift from J. Hirabayashi and K. ichi Kasai Teikyo University, Japan (61). Figure 3.1. shows the sequence of the protein and the positions of the mutations. Wild type and R48H (Arg48 changed to His), E71Q (Glu71 changed to Gln), R73H (Arg48 changed to His) mutant GAL1s were excised from pUC540 vector with NcoI, and inserted into a bacterial expression vector pETHis (142) at the NcoI site, in the sense direction. The resulting protein has a His_6 tag at the N-terminus and three linker amino acids. The His tag allows for affinity purification of the proteins on a Ni-resin. Wild type GAL1 cDNA, after excision from pUC540, was also inserted into bacterial expression vector pQE60 (QUIAGEN, Hilden, Germany) at the NcoI site. The proper orientation of the cDNA inserts was confirmed by restriction enzyme analysis. Quality of the cloning and "in frame" position of the His_6 tag in pETHis vector was confirmed by sequencing.

ACGLVASNLN LKPGECLRVR GEVAPDAKSF VLNLGKDSNN LCLHFNP(R48H)FN AHGDANTIVC NSKDGGAWGT (E71Q)Q(R73H)EAVFPFQ PGSVAEVCIT FDQANLTVKL PDGYEFKFPN RLNLEAINYM AADGDFKIKC VAFD

Fig.3.1. Protein sequence of galectin-1 with indicated mutations. Each mutated version of galectin-1 carries one of the substitutions.

GAL1 subcloned to pQE60 or to pETHis was transformed into BL-21 (BF^-*dcm ompT hsdS*($r_B^-m_B$) gal λ(DE3)) (Stratagene), inoculated from a single colony into 2ml of LB medium containing 200 μg/ml ampicillin, and grown overnight. The obtained culture in 1:1000 dilution was inoculated in LB medium containing 200 μg/ml ampicillin and grown for 8 hours at 37^0C.

3.2. Purification of recombinant galectin-1 (rGal1)

200ml of bacterial culture producing rGal1 was centrifuged at 2500g for 30 min at 4^0C in a Sorvall RC3B centrifuge, washed twice with 80ml of lysis buffer (2500g 15 min, 4^0C, Hettich Universal 30RF, rotor 1424A) containing 4 mM beta-mercaptoethanol, then disrupted at 4^0C by "French" press (AMINCO, Maryland, USA) in 50ml of lysis buffer supplemented

with 1 mM PMSF. All following steps were done at 4^0C. The lysate was spun at 10 000g for 30 min, the supernatant was filtered through GF/C membrane, and loaded on an alpha-lactose gel (EY Laboratories) column pre equilibrated with 20ml of lysis buffer supplemented with 1mM PMSF and 4mM beta-mercaptoethanol. The lysate was recirculated three times and the bound protein was washed with 20 ml of washing buffer supplemented with 0,25 mM PMSF and 4mM beta-mercaptoethanol. Protein was eluted with 20ml of elution buffer supplemented with 0,1M iodoacetamide, and 1ml fractions were collected. Eluted fractions were analyzed on sodium dodecyl sulfate 12% polyacrylamide gel electrophoresis (SDS/12%PAGE) and dialyzed against phosphate buffered saline (PBS) containing 100 μM beta-mercaptoethanol. Protein concentration was estimated with Bradford assay reagent (Biorad) and by the absorbance at 280nm.

3.3. Purification of recombinant His-tagged galectin-1 (rHisGal1)

200ml of bacterial culture producing rHisGal1 or its mutants was processed as described in section 3.2. All following steps were done at 4^0C. Lysate was spun at 10 000g for 30 min, supernatant was filtered through GF/C membrane (Whatman), and supplemented with iodoacetamide to final concentration of 0,1M. Then the lysate was loaded onto a Ni-NTA-agarose (Invitrogen) column pre equilibrated with 20ml of lysis buffer supplemented with 1mM PMSF. It was recirculated three times and washed with 20ml of washing buffer containing 0,1 M iodoacetamide. Bound protein was eluted with 20ml of elution buffer . Collected 1ml fractions were tested on SDS/12%PAGE and dialyzed against PBS containing 100μM beta-mercaptoethanol. Protein concentration was estimated with Bradford assay reagent (Biorad) and by the absorbance at 280nm.

3.4. Alpha-lactose binding assay

Purified recombinant wild type rHisGal1 and its mutants in PBS containing 100μM beta-mercaptoethanol were incubated with alpha-lactose gel (EY Laboratories) at 4^0C for 1 hour on a rotating platform, washed with washing buffer three times. Resin, together with bound protein, was subjected to SDS/12%PAGE and Western blotting (see below) with rabbit anti-galectin-1 antibody produced in our laboratory.

3.5. Cell Culture and transfection

The human Jurkat T cell line was cultured in RPMI 1640 (GIBCO) tissue culture medium supplemented with 5% heat inactivated FCS and glutamine . Human embryonic

kidney epithelium based cell lines ProPak-A (PPA) (143) and Phoenix gp (generous gift of G. Nolan) were cultured in DMEM (Life Technologies, Gaithersburg, MD) supplemented with 10% heat inactivated FCS and glutamine . All cell lines were maintained at 37°C in a humidified atmosphere of 95% air and 5% CO_2. GAL1 and mutant (R48H) GAL1 cDNA were excised from pETHis with BamHI/NcoI, and ligated into EcoRI/BamHI treated mammalian expression vector pLXSN together with preannealed oligonucleotides (G-1.Kozak"+": 5’ - AAT TCT CTT CGC TTC AGC TTC AAT - 3'; G-1.Kozak"-"5' - CAT GAT TGA AGC TGA AGC GAA GAG - 3'). The proper orientation and position of the cDNA inserts was confirmed by restriction enzyme analysis and sequencing. Phoenix gp cell line was transfected by conventional Ca/phosphate method (144) with obtained constructs together with pCiGLwt vector containing viral envelope cDNA (kindly provided by G. Veres). Virus containing supernatant was collected 48 hours after transfection, filtered through 0.45 μm filter (Corning, SFCA membrane) and used for infection of PPA cells by centrifugation (145) in the presence of 4μg/ml polybrene. Infected PPA cells were selected on 400μg/ml of G418 (Invitrogen) for two weeks. Selected cells were grown to confluence, and supplied with a fresh medium. Cells were fed with fresh medium and supernatant was collected after 16 hours culture. The Jurkat cells were transduced by centrifugation at 1000g for 90 minutes with virus containing supernatant (146) in presence of 4μg/ml polybrene and selected for two weeks on 1mg/ml of G418. Protein expression was analyzed by Western blotting (section 3.6).

3.6. Western blotting

Triton X-100 soluble extracts prepared from 2×10^5 Jurkat cells per sample or proteins after binding to alpha-lactose gel (EY Laboratories) were separated on a SDS/12%PAGE and transferred to nitrocellulose membrane (Schleicher & Schuell) in transfer buffer . The membranes were blocked using Tris buffered saline (TBS) containing Tween20 (TBST) , 3% Teleostean gelatin and subsequently probed with 20μg/ml of rabbit anti-galectin-1 antibody in TBST for 1 hour at 4^0C and with 275μg/ml biotinylated goat anti-rabbit IgG (DAKO) in TBST for 1 hour at 4^0C, and then with 18,75 μg/ml streptavidin-HRPO conjugate (DAKO) in TBST for 1 hour at 4^0C. Immunoreactive proteins were visualized by an enhanced chemiluminescence (ECL plus) detection system (Amersham Pharmacia Biotech). Prestained molecular weight marker was purchased from GIBCO-BRL.

3.7. Indirect immunofluorescence

Jurkat cells were washed once in PBS, then incubated for 1 hour at 4^0C in PBS containing 20μg/ml of the indicated monoclonal antibody, washed three times with cold PBS containing 0,1% NaN_3, and then incubated for 30 min at 4^0C with goat anti-mouse FITC conjugate. Finally, the cells were washed three times with cold PBS containing 0,1% NaN_3 and subjected to cytofluorimetric analysis with FACScalibur (Becton Dickinson). The dead cells were discriminated by propidium iodide (PI) uptake (1μg/ml) prior measurement.

3.8. Assays for cell growth

To assess the growth and proliferation of Jurkat transfectants, cells were plated at a density of 10^5 cells/ml in RPMI 1640 medium containing 5% FCS in triplicates. Viable cell numbers were determined on day 4 after plating by the 1-(4,5-Dimethylthiazol-2-yl)-3,5-diphenylformazan (MTT) assay (145) and by trypan blue exclusion counting. MTT assay is based on a reduction of tetrazolium salt to formazan by mitochondrial dehydrogenase. It was performed as follows. MTT solution was added to each sample to a final concentration of 1mg/ml. Samples were incubated for 4 hours at 37^0C then the medium was removed and a solution of 0,05M HCl in isopropanol was added for 10 min to solubilize formazan crystals. Absorbance of the samples was measured at 570nm. Trypan blue exclusion counting was performed as follows. Cell suspension was diluted 1:10 in trypan blue solution for cell counting and the living cells were discriminated by trypan blue exclusion. Average, standard deviation, and Student test parameters were calculated.

3.9. Cell cloning

The Limiting dilution method was used for cloning. Transfected cells were diluted to 10, 100, 1000 cells/ml in RPMI 1640 tissue culture medium supplemented with 5% FCS and antioxidants (147) , then plated in 96-well flat bottom plates in 100 μl/well. After three weeks growing colonies were expanded and analyzed.

3.10. Measurement of intracellular Ca^{2+} concentration

Jurkat cells were suspended in 10^7 cells/ml concentration in RPMI-1640 supplemented with 5% FCS and incubated with 7.5 μM of Fluo-3AM and 7.5 μM of FuraRed-AM (Molecular Probes, Eugene, OR, USA) for 30 min at 37°C. Cells were then adjusted to a final concentration of $5x10^5$ cells/ml with the addition of cell culture medium and incubated for a further 30 min at 37°C. The cells were washed twice with RPMI and resuspended in fresh cell

culture medium at a concentration of 10^6 cell/ml. Intracellular Ca^{2+} was measured using FACScalibur (Becton Dickinson) and data were presented as the ratio of Fluo-3 and FuraRed fluorescence intensity at 530 and 650 nm, respectively, versus time.

3.11. Apoptosis assay

For the apoptosis assay we used cytofluorimetric analysis of the DNA content and sub-G1 cell population was considered as apoptotic cells. The cells (1-3 10^6 cells/ml) were treated with various apoptosis inducing agents for 16 hours in RPMI 1640 medium containing 2% FCS in a CO_2 thermostat. After washing with PBS, the cells were resuspended at a concentration of 1×10^6 cells/ml with staining solution . The samples were then incubated at room temperature for 20 min in the dark and analyzed by flow cytometry (FACScalibur; Becton Dickinson). CellQuest (Becton Dickinson) and ModFit LT (Verity Software House, Topsham, ME) were used for the analysis.

3.12. Binding assay to cell surface glycoconjugates

Jurkat cells were incubated for 1 hour at 4^0C in PBS containing 25μg/ml of rHisGal1 or its mutants, then washed with cold PBS or PBS containing 100mM lactose and incubated further at 4^0C in PBS containing 20μg/ml of rabbit anti-galectin-1 antibody for 30 minutes. After washing with cold PBS containing 0,01% of NaN_3, the cells were stained with 20μg/ml of goat anti-rabbit biotin conjugate (DAKO) in PBS at 4^0C for 30 min, then it was washed out and they were stained with the 2μg/ml of streptavidin-FITC in PBS for 30 min. Finally, the cells were washed three times with cold PBS containing 0,01% NaN_3 and subjected to cytofluorimetric analysis with FACScalibur (Becton Dickinson). Propidium iodide was added to cells to the final concentration of 1μg/ml to discriminate the dead cells.

3.13. Mitochondrial dehydrogenase activity assay

The cells were plated in triplicates at a concentration 10^6 cells/well in flat bottom 96-well plates in RPMI supplemented with 5% FCS and glutamine. MTT assay was performed as described in the section 3.8.

4. Results

4.1 Role of the carbohydrate-binding activity in the extracellular function of galectin-1

4.1.1. rGal1 induced apoptosis is not diminished by lactose

To test if, in our system, apoptosis induced by galectin-1 could be inhibited by lactose, we performed the following experiment. Jurkat cells were treated with rGal1 for 24 hours in the presence or absence of 100mM lactose and apoptosis was estimated by measuring the DNA content (Fig. 4.1). Apoptosis induced by rGal1 was not significantly inhibited by lactose.

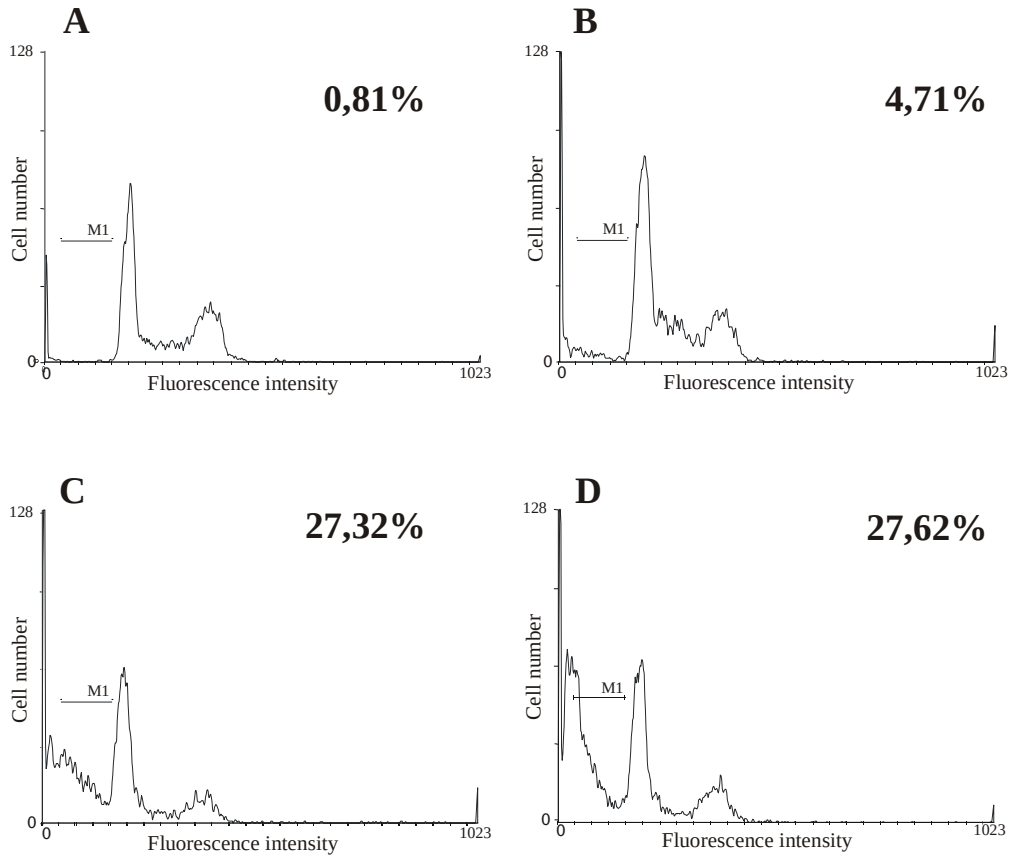

Fig.4.1. FACS analysis of the DNA content of PI stained Jurkat cells (see section 3.11 for details) after incubation for 24 hours with a dilution of the PBS containing 100μM beta-mercaptoethanol for control (**A**), or with 100mM lactose (**B**), or with 25μg/ml of the rGal1 in abscence(**C**) or presence of lactose (**D**) is shown.

4.1.2. rHisGal1 is purified on Ni-NTA agarose resin

Galectin-1, mutated at the sites responsible for direct binding to its ligands, were obtained from J. Hirabayashi and K. ichi Kasai Teikyo University, Japan (61, 63). We subcloned wild type cDNA and cDNA of the R48H, E71Q, R73H mutants into pETHis vectors in order to be able to purify the recombinant proteins via His_6-tag independently of

their carbohydrate-binding activity. Wild type and mutant proteins were named rHis-Gal1 for wild type, rHisGal1.71 for E71Q, rHisGal1.73 for R73H, rHisGal1.48 for R48H. SDS 12%PAGE analysis of the purification fractions of recombinant proteins is shown on Fig. 4.2.

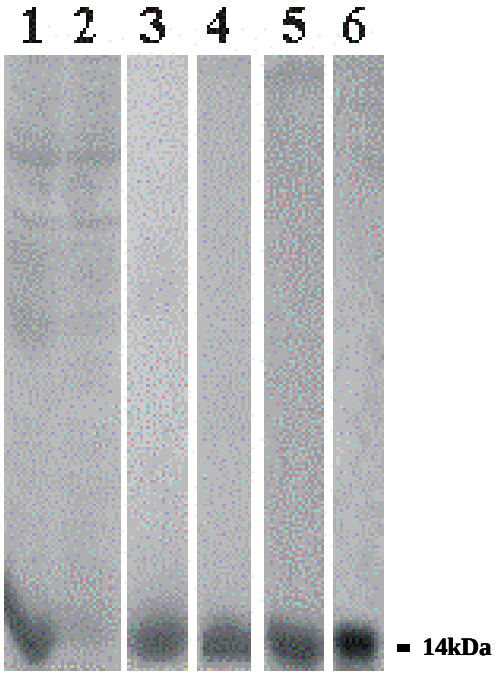

Fig.4.2. 1 - bacterial lysate, 2 – flow through after binding to the resin, 3 – 6 elution fractions: 3 - rHisGal1, 4 - rHisGal1.48, 5 - rHisGal1.71, 6 - rHisGal1.73

4.1.3. Rabbit anti-galectin-1 antibody recognizes both His-tagged and non-His-tagged galectin-1 and its mutants

The wild type rHisGal1 and the mutants contain an extra nine amino acids, six histidines and three linker amino acids, in the protein sequence compared to the non-His-tagged rGal1. To test whether the extra sequence affected the antigenic properties of rHisGal1, we performed immunoblotting analysis using rabbit anti-galectin-1 antibody (Fig.4.3). The antibody equally recognized both proteins. This suggested that the antigenic properties of rHisGal1 were not changed compared to rGal1 and the antibody can be used to detect rHisGal1.

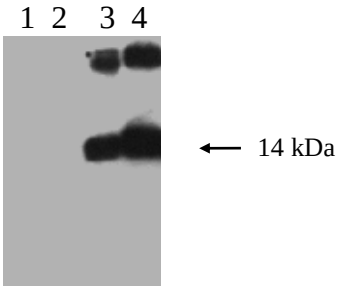

Fig.4.3. Rabbit anti-galectin-1 antibody recognizes rHisGal1. rGal1 (1,3) and rHisGal1(2,4) were run on a SDS/12%PAGE, transferred onto a nitrocellulose membrane and stained with control rabbit IgG (1,2) or rabbit anti-Gal1 (3,4) antibody (see section 3.6. for details).

Antigenic properties of the recombinant His-tagged mutants were tested in comparison to wild type rHisGal1 with Western blotting (Fig. 4.4.). The antibody to rGal-1 recognized the mutant rHis-Gal1 proteins similarly to the wild type protein.

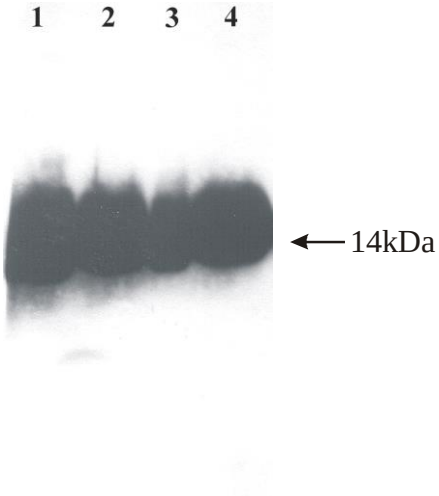

Fig.4.4. The proteins were separated on a SDS12%PAGE, transferred onto a nitrocellulose filter and hybridized with rabbit anti-galectin-1 antibody: **1** – rHisGal1.71, **2** – rHisGal1.73, **3** – rHisGal1.48, **4** – rHisGal1.

4.1.4. rHisGal1 binds to lactose and cell surface glycoproteins, and induces apoptosis of Jurkat cells

To test whether addition of the His-tag affected the biological activity of rHisGal1, we compared the lactose binding ability of rGal1 and rHisGal1. As it is shown on Fig.4.4. rHisGal1 bound to lactose.

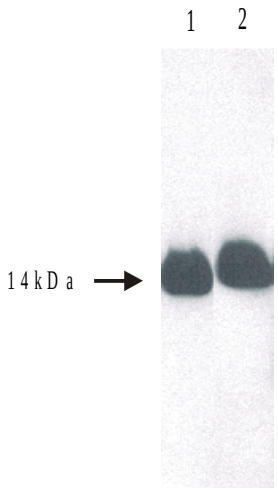

Fig 4.5. Recombinant proteins were subjected to alpha-lactose binding assay, then alpha-lactose-bound proteins were eluted in SDS sample buffer, run on a SDS/12%PAGE, transferred to a nitrocellulose membrane, and blotted with anti-galectin-1 rabbit antibody. **1** - rGal1, **2 -** rHisGal1.

Both proteins, rGal1 and rHisGal1, bound to the surface glycoconjugates of Jurkat cells as was shown by indirect immunofluorescence staining (Fig. 4.6).

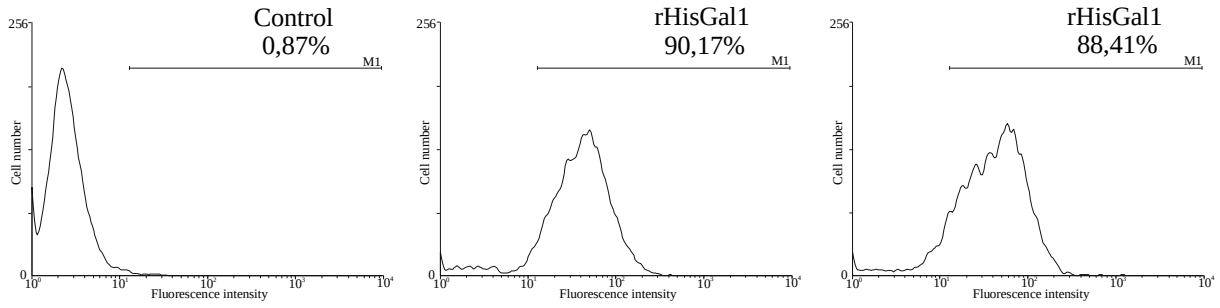

Fig.4.6. Jurkat cells were incubated with a protein to be bound, stained with anti-galectin-1 rabbit antibody, then with goat anti-rabbit biotin conjugate and streptavidin FITC. The percentage of cells that bound rGal1 or rHisGal1 is shown.

Apoptosis inducing ability of rHisGal1 was not affected by the his-tag and was similar to that of rGal1 (Fig. 4.7)

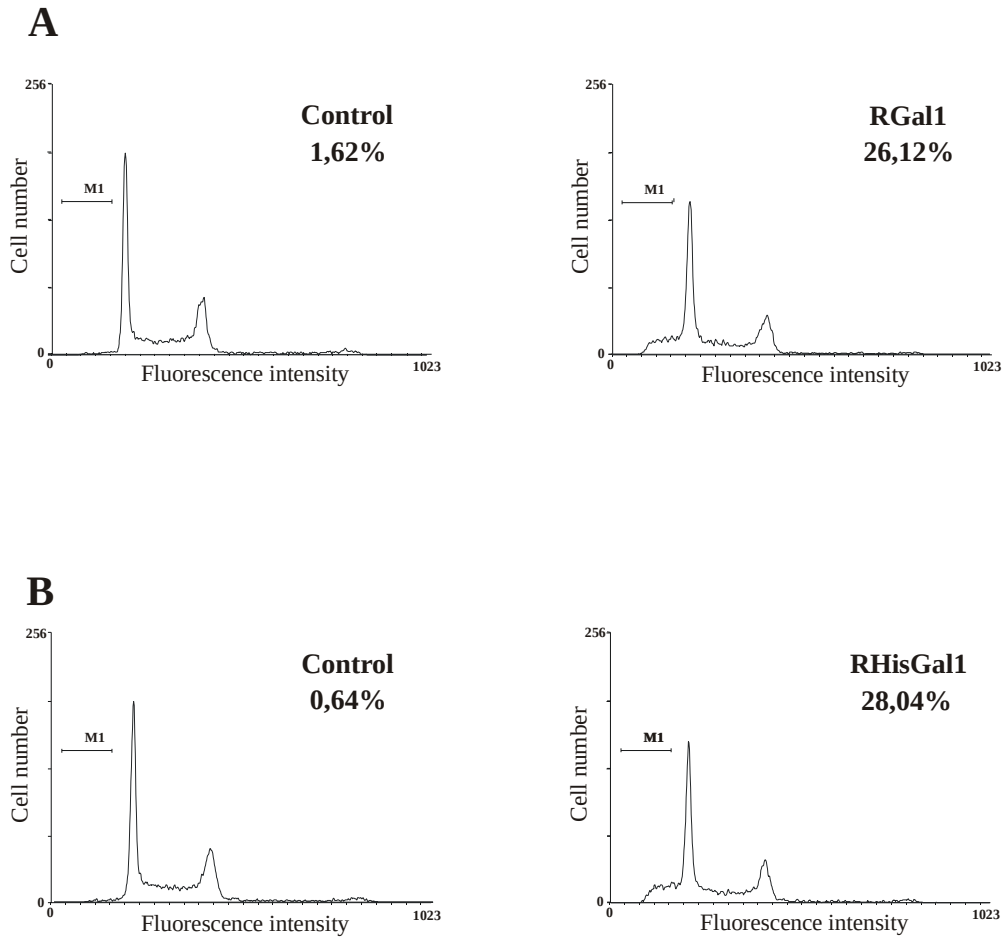

Fig.4.7. FACS analysis of the DNA content of PI stained Jurkat cells (see section 3.11 for details) after incubation for 16 hours with a dilution of the PBS containing 100μM beta-mercaptoethanol for control , or with 25μg/ml of the recomninant proteins is shown. **A –** apoptosis induced by rGal1, **B** - apoptosis induced by rHisGal1.

4.1.5. rHisGal1 mutants, R48H, E71Q, R73H, have reduced or abrogated ability to bind alpha-lactose, no binding activity towards cell surface glycoconjugates, and have reduced or abrogated ability to induce apoptosis

To test the ability of rHisGal1 and the mutants to bind lactose, we performed an alpha-lactose binding assay (see section 3.4 for details). As Fig.4.8 shows, rHisGal1.71 and rHisGal1.73 had a slightly reduced and rHisGal1.48 an abrogated lactose-binding activity.

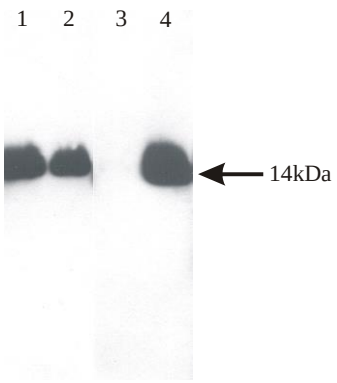

Fig. 4.8. Recombinant proteins were subjected to alpha-lactose binding assay and then alpha-lactose-bound proteins were eluted in SDS sample buffer, run on a SDS/12%PAGE, transferred to a nitrocellulose membrane, and blotted with anti-galectin-1 rabbit antibody. **1** - rGal1.71, **2** - rHisGal1.73, **3** - rHisGal1.48, **4** - rHisGal1.

Recombinant wild type and mutant proteins were tested for their ability to bind to cell surface glycoconjugates of Jurkat cells. The "non-binding" mutants, rHisGal1.73, rHisGal1.71, and rHisGal1.48 did not bind to the cells (Fig.4.9). Cell bound galectin could be washed off by PBS containing 100mM lactose (data not shown).

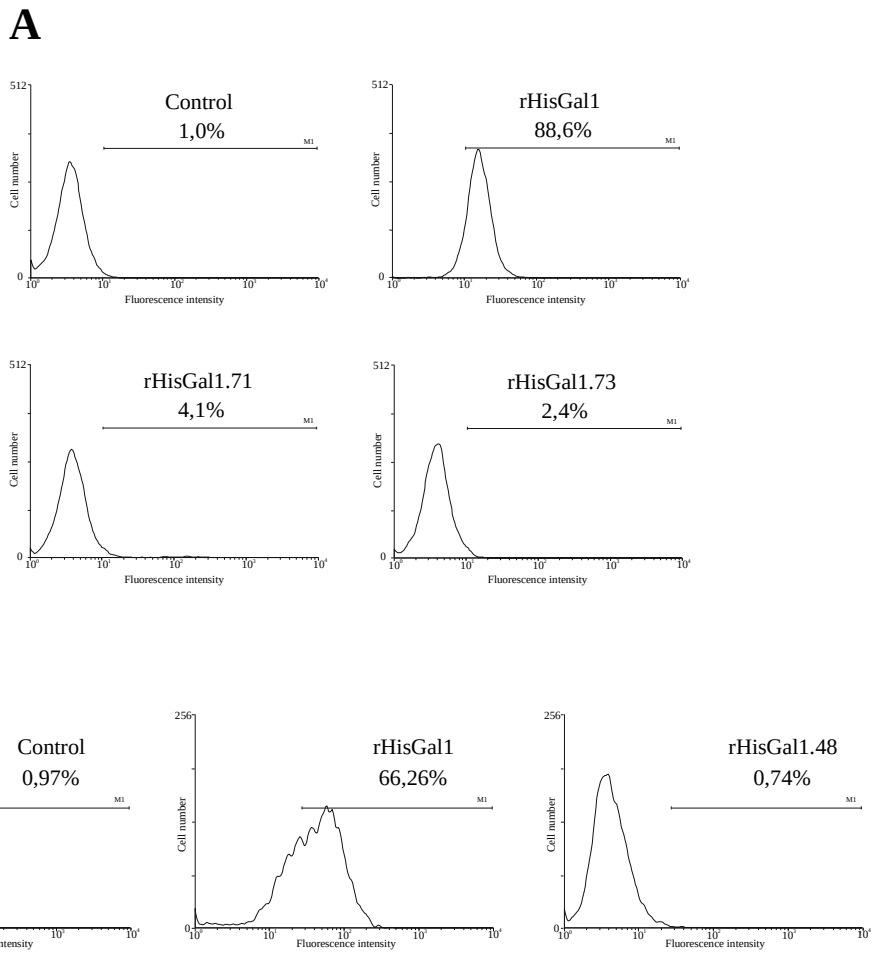

Fig.4.9. Jurkat cells were incubated with a protein to be bound, stained with anti-galectin-1 rabbit antibody, then with goat anti-rabbit biotin conjugate and streptavidin FITC. The percentage of the cells that bound a protein is shown. **A** – binding of rHisGal1.71 and rHisGal1.73; **B** – binding of rHisGal1.48.

Apoptosis inducing ability of the recombinant wild type and mutant proteins was tested on Jurkat cells. Recombinant HisGal1 and rHisGal1.73 induced apoptosis, although rHisGal1.73 triggered a lower level of apoptosis. Recombinant HisGal1.48 did not induce a significant degree of apoptosis (Fig. 4.10).

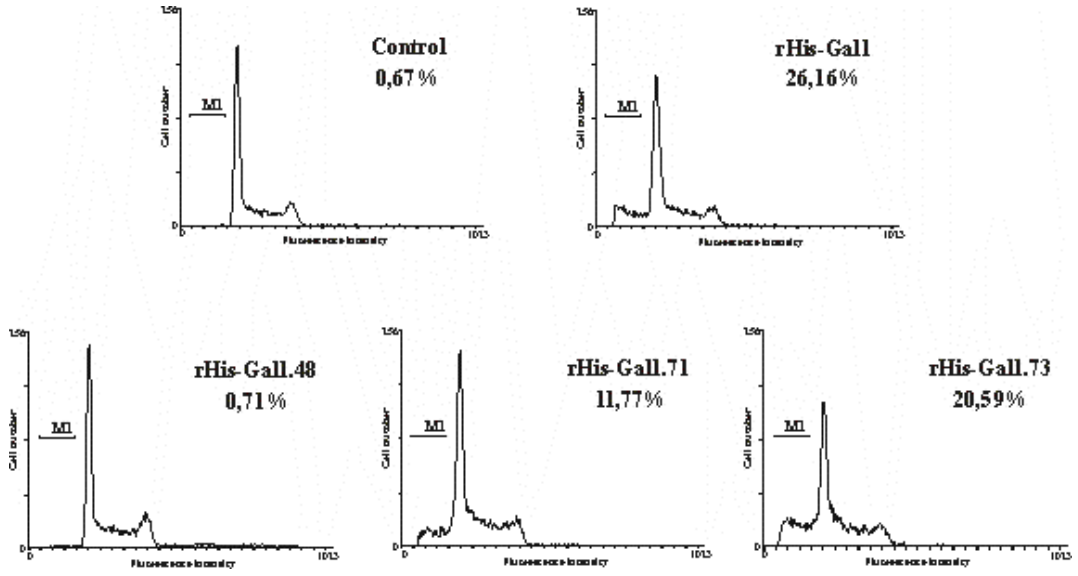

Fig. 4.10. FACS analysis of the DNA content of PI stained Jurkat cells (see section 3.11 for details) after incubation for 16 hours with a dilution of the PBS containing 100μM beta-mercaptoethanol as the control , or with 25μg/ml of the recombinant proteins, is shown. The percentage of apoptotic cells is indicated.

4.2. An intracellular function of galectin-1

Intracellular function and possible connection of this function and the sugar-binding ability of galectin-1 is poorly or not studied. We subcloned wild type or mutant (R48H) GAL1 into the mammalian retroviral expression vector, pLXSN, and transfected the constructs into Jurkat cells, that do not express endogenous galectin-1.

4.2.1. Galectin-1 expression in stably transfected Jurkat cells

For the investigation of intracellular function of galectin-1 and its dependence on carbohydrate binding activity, we transfected Jurkat cells with wild type and R48H mutant galectin-1 using retroviral transfection (see section 3.5. for details). The mutant R48H has no ability to bind its carbohydrate ligands (Fig. 4.8 and 4.9). In the control, Jurkat cells were transfected with an empty vector. The transfectants were named: Jmock – for Jurkat transfected with pLXSN vector, Jgal – for Jurkat transfected with galectin-1 cDNA cloned into pLXSN vector,

and Jgal48 – for Jurkat transfected with R48H mutant galectin-1 cDNA cloned into pLXSN vector. After selection of the transfected cells expression of galectin-1 was detected by Western blotting (Fig. 4.11, A) using rabbit anti-galectin-1 antibody. Later on, the selected cells were cloned. 60 clones of the Jmock cells, 59 clones of the Jgal cells, and 56 clones of the Jgal48 cells were obtained. The clones were analysed by Western blotting for galectin-1 expression (data not shown). Western blotting analysis of the clones selected for further experiments is shown in Fig. 4.11, B.

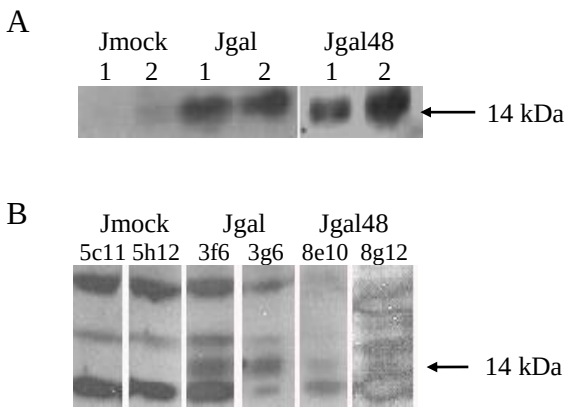

Fig.4.11. Wild type and mutant galectin-1 are expressed in transfected Jurkat cells. Western blotting analysis with rabbit anti-galectin antibody is shown. **A** – uncloned transfectants; 1 and 2 - two independent transfections; **B -** analysis of the selected cloned transfectants: 5c11 and 5h12 – Jmock clones; 3f6 and 3g6 - Jgal clones, 8e10 and 8g12 - Jgal48 clones.

4.2.2. Galectin-1 is not present on the surface of transfected cells

In order to determine whether galectin-1 is present on the surface of expressing cells, we performed indirect immunofluorescence staining of the surface proteins with the rabbit anti-galectin-1 antibody (Fig. 4.12). The exogenously added rGal1 was bound to the surface of Jurkat cells (Fig. 4.12, A). However galectin-1 produced by the transfectants did not appear on the surface of Jgal cells (Fig. 4.12, C). Jmock cells were used as negative control (Fig. 4.12, B).

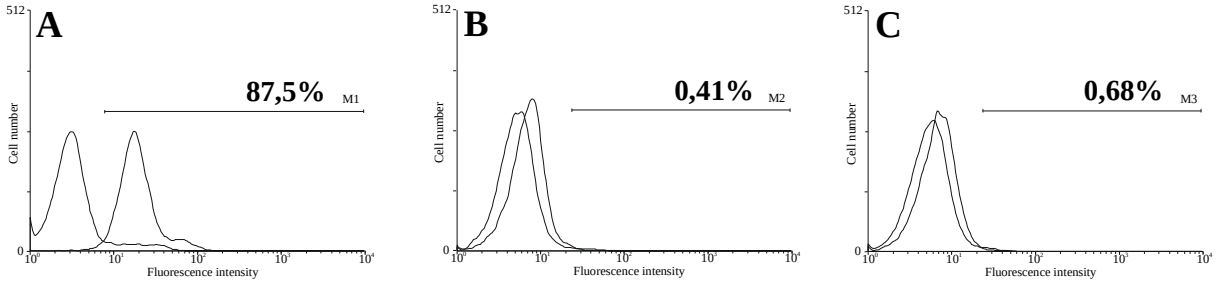

Fig.4.12. The cells were treated (**A**) or were not treated (**B, C**) with 25µg/ml of rGal1 then stained with anti-galectin-1 rabbit antibody, then with goat anti-rabbit biotin conjugate and streptavidin FITC (see sections 3.7 and 3.12 for details) and analysed by a cytofluorimeter. The thin line is a control treatment with rabbit IgG, the thick line is a sample treated with rabbit anti-galectin-1 antibody. **A** - rGal1 bound to the surface of Jurkat cells; **B** – Jmock; **C** - Jgal. Increase in fluorescence intensity is indicated in percent.

4.2.3. Presence of intracellular galectin-1 influences the proliferation of Jurkat cells

Both Jgal and Jgal48 showed significantly lower proliferation compared to the Jmock cells (Fig. 4.13) in a four day proliferation assay. The result was obtained with two different methods. The MTT assay (Fig. 4.13, A) represents the metabolic activity of the living cells, and the trypan blue exclusion assay (Fig. 4.13, B) shows the number of the viable cells. Expression of the wild type galectin-1 in the Jurkat cells resulted in a more dramatic effect on proliferation then did the mutant protein, since galectin-1 caused a 38% decrease while the mutant only a 20% decrease in the cell division compared to the proliferation of mock transfected cells measured with MTT.

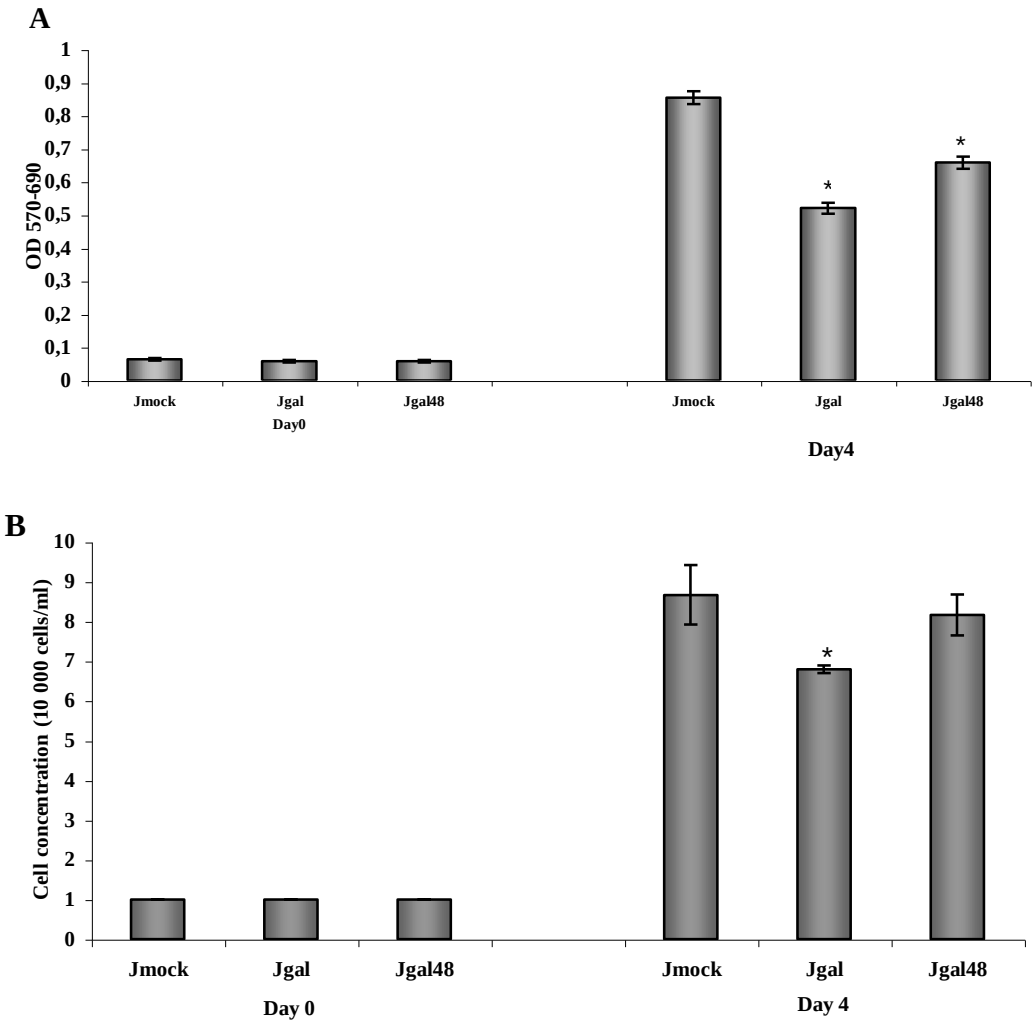

Fig.4.13. The cells expressing wild type galectin-1 have changed proliferation activity. ***A*** - MTT assay, optical density at 570 nm wave length was measured on day 0 and day 4; ***B*** - the cell counting by trypan blue exclusion method, cells were counted at day 0 and day 4. Significant difference (n=3, P<0,01) between Jmock and other transfectants is marked with a star.

4.2.4. Galectin-1 expression upregulates cell surface level of TCR

Expression of the surface molecules determines T cell function. We examined the expression of the most important surface markers. The expression levels of CD45, CD95, CD2, LFA-1, and MHC1 were not changed on Jgal compared to Jmock, as was shown by the indirect immunofluorescence labeling with respective antibodies (Fig. 4.14). In contrast, TCR expression level was upregulated by galectin-1 expression as Fig. 4.15 shows. Expression of the mutant galectin-1 in the Jurkat cells resulted in an intermediate elevation of TCR expression between the TCR level of mock and wild type galectin-1 transfected cells (Fig. 4.15).

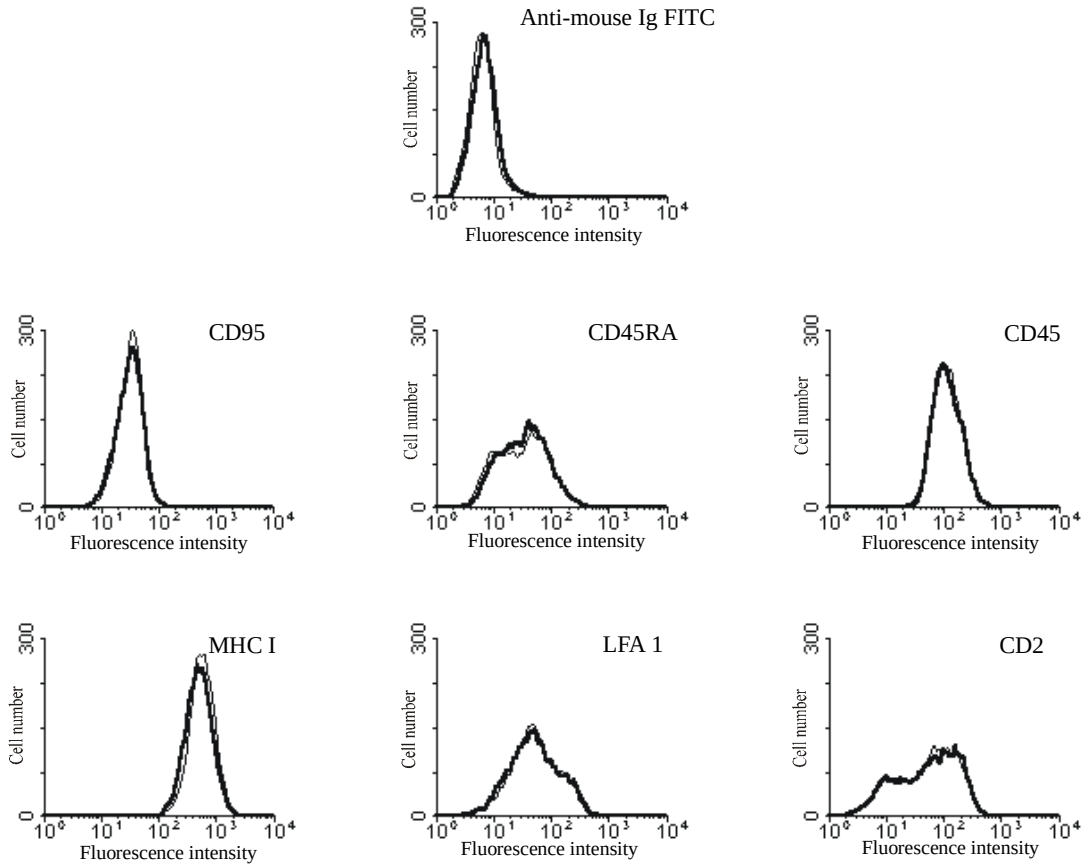

Fig.4.14. Expression of the examined T cell surface markers on Jgal cells is not changed in comparison to Jmock. Thin line – Jmock, thick line –Jgal. FACS analysis of the indirect immuno fluorescence labeling with respective antibodies is shown.

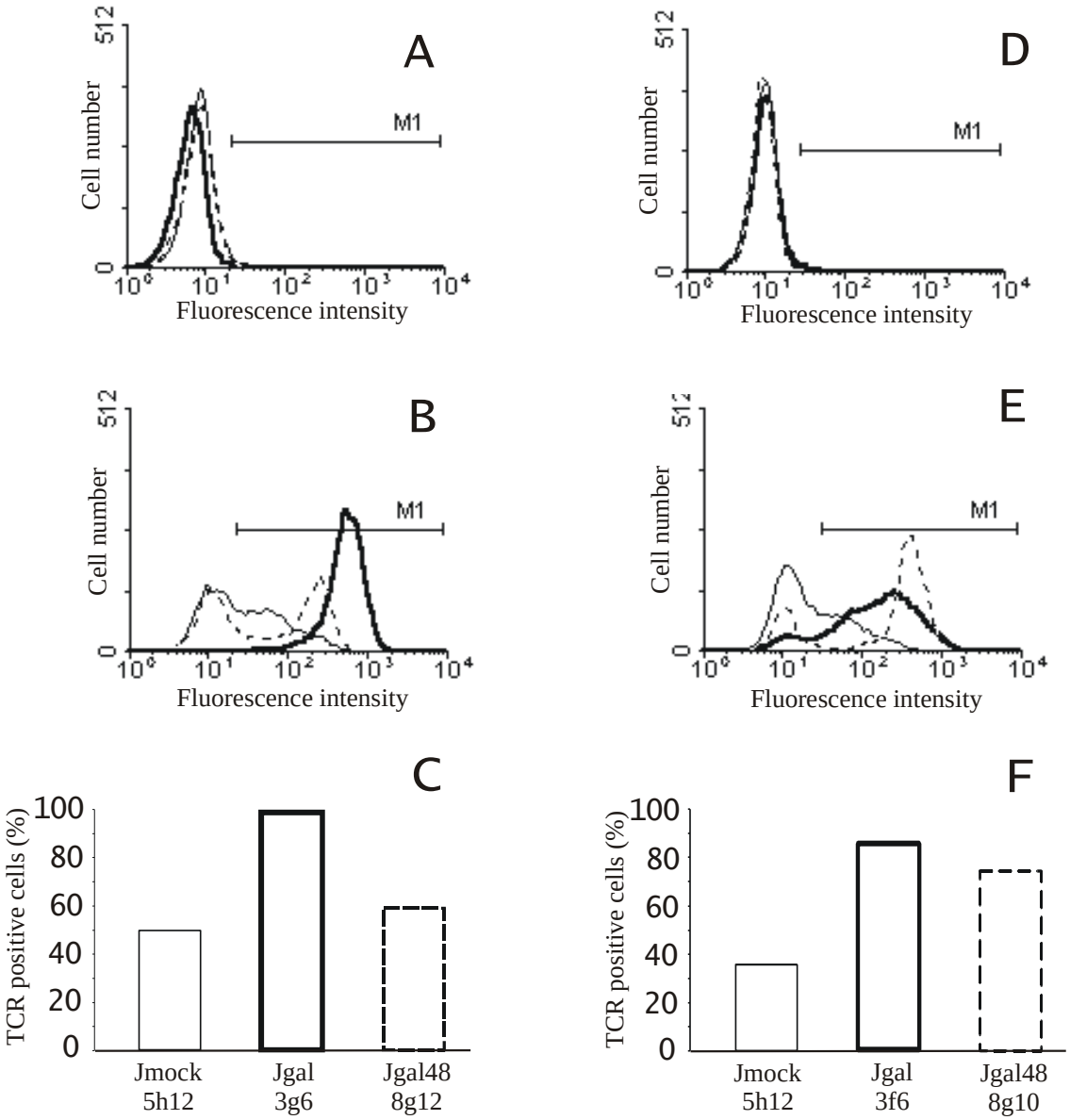

Fig.4.15. T cell antigen receptor expression level is higher on Jgal in comparison to Jmock and Jgal48. ***A-C*** – clones 5h12 for Jmock (thin line); 3g6 – for Jgal (thick line); 8g12 - for Jgal48 (dashed line); ***D-F*** – clones 5h12 for Jmock (thin line); 3f6 for Jgal (thick line); 8g10 for Jgal48 (dashed line); ***A, D*** – histograms of staining only with anti-mouse-FITC conjugate; ***B, E*** – histograms of staining with monoclonal OKT3 antibody and with anti-mouse-FITC conjugate as a second antibody; ***C*** and ***F*** –percentage of TCR positive cells under the markers set on histograms ***B*** and ***E*** respectively.

4.2.5.Ca^{2+} influx is affected by galectin-1 expression

To analyse whether galectin-1 expression affected the TCR function, we performed the measurement of the intracellular Ca^{2+} concentration in response to TCR stimulation. Elevation of the intracellular Ca^{2+} concentration is one of the responses to stimulation of T cells through the TCR. The calcium acts as a second messenger molecule directly and

indirectly regulating the function of various proteins, such as calmodulin, calcineurin, transcription factors, and others. The Jmock, Jgal, and Jgal48 cells were activated via the TCR with anti-TCR antibody, OKT3 (Fig. 4.16, A, B). Clone 3g6 (Jgal) cells had a higher elevation of intracellular Ca^{2+} concentration in response to TCR stimulation then clones 5c11 (Jmock) and 8g12 (Jgal48) (Fig. 4.16. A). The Jgal and Jgal48 transfected cells expressed higher level of TCR that the Jmock transfected cells (Fig. 4.15). To show whether the elevation of Ca^{2+} level is a result of this, we used a calcium ionophore, which acts independently of TCR in triggering Ca^{2+} influx. The calcium ionophore treatment resulted in Ca^{2+} elevation with a similar pattern to TCR stimulation (Fig. 4.16. C), indicating that Jgal cells give a higher response to the stimulations triggering Ca^{2+} influx. The increase of intracellular calcium concentration may come from two sources: the release of Ca^{2+} from intracellular stores or from the extracellular space. We added EGTA to the tissue culture medium to deplete the extracellular Ca^{2+} and to inhibit the latter source. Release from intracellular stores observed in absence of extracellular Ca^{2+} depleted by 5mM EGTA was also higher in 3g6 Jgal cells in either induced via the TCR or with calcium ionophore suggesting that Ca^{2+} comes from both sources (Fig. 4.13, B and D). Analysis of the second set of clones 5h12, 3f6, and 8e10 gave a similar result (data not shown).

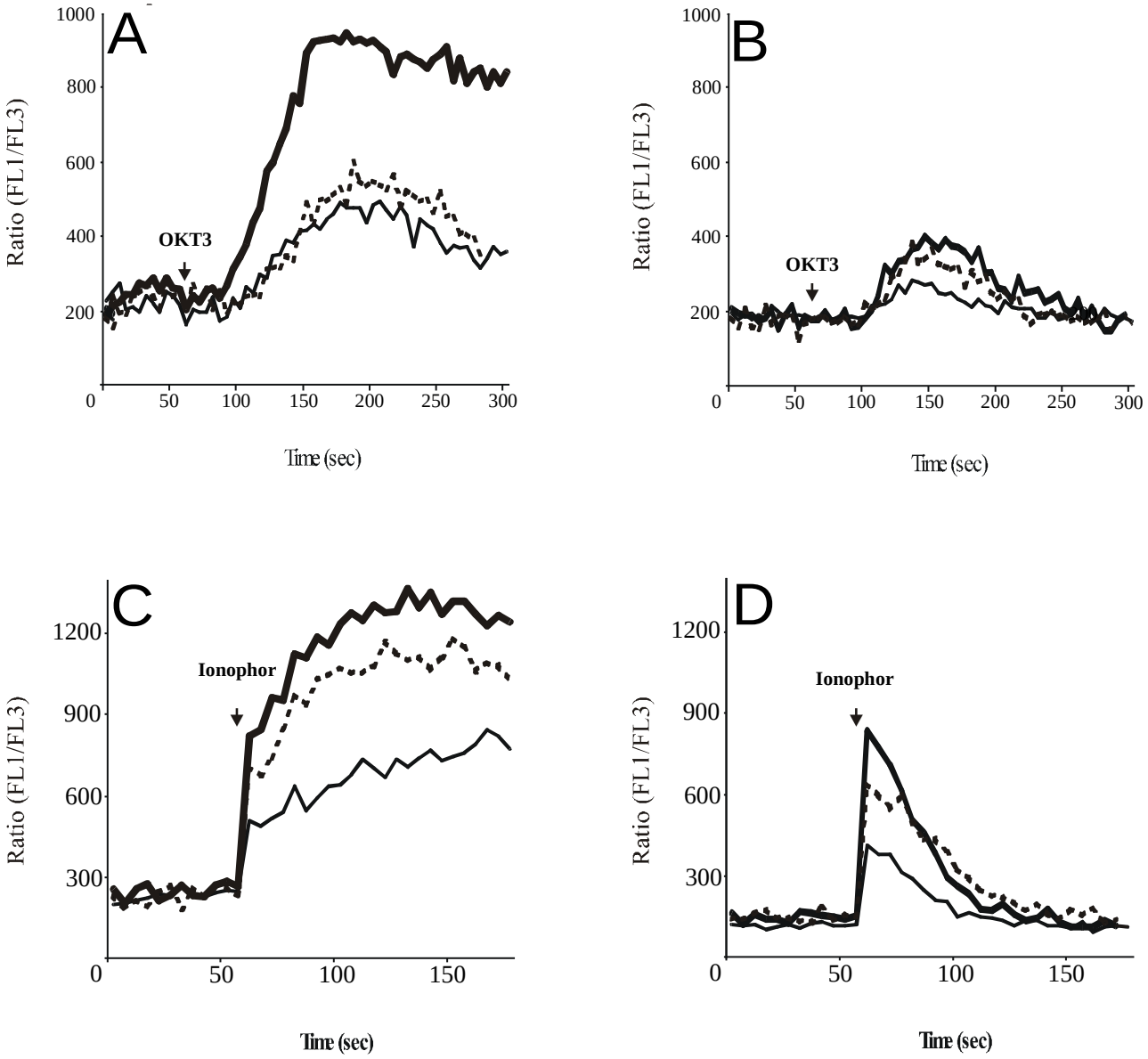

Fig.4.16. The cells were loaded with Fluo3 and FuraRed as described in section 3.10, and anti-TCR antibody (OKT3, 3μg/ml) (**A** and **B**) or calcium ionophore (**C** and **D**) were added in the abscence (**A** and **C**) or presence (**B** and **D**) of the 5mM EGTA. Elevation of Ca^{2+} concentration is plotted as ratio of FL1/FL3 against time. Thin line – 5c11 (Jmock), thick line – 3g6 (Jgal), dashed line – 8g12 (Jgal48).

4.2.6. Susceptibility to apoptosis is increased in Jurkat cells expressing galectin-1

Galectin-1 induces apoptosis of activated T cells when applied extracellularly. It is not known, however, how endogenous expression of galectin-1 influences the susceptibility of T cells to apoptosis. We examined the transfected cells for their ability to respond to extracellular rGal1 stimulation. Treatment with recombinant galectin-1 induced a higher degree of apoptosis in cells expressing galectin-1 (Fig. 4.17).

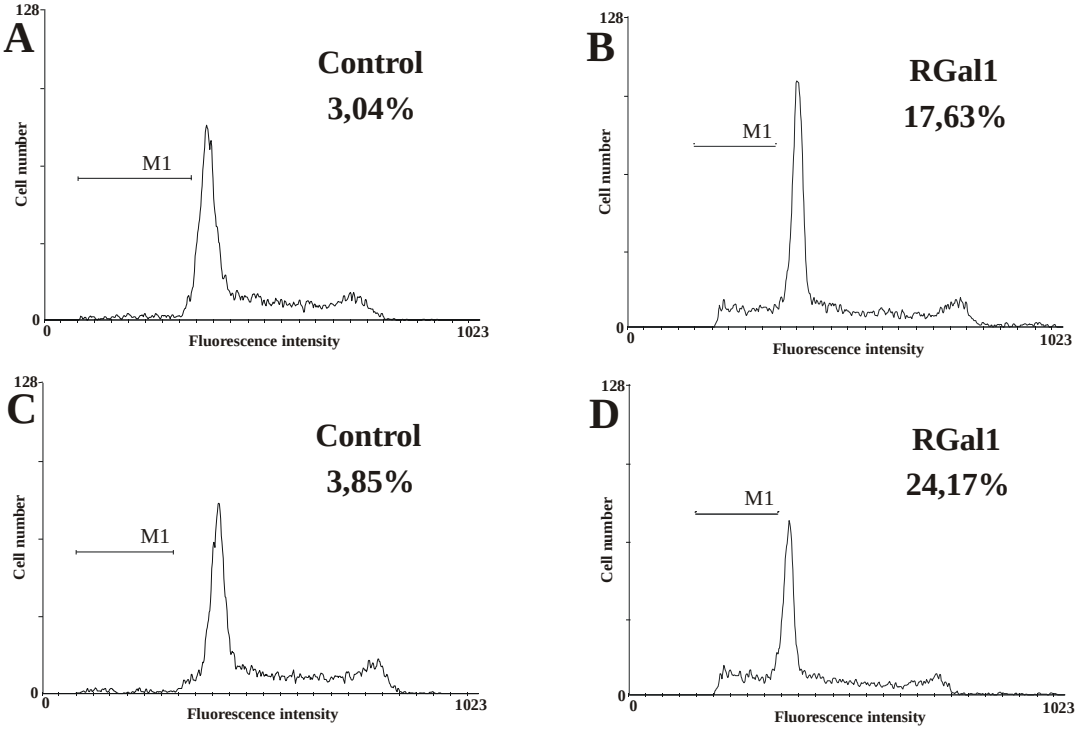

Fig.4.14. Galectin-1-expressing cells are more susceptible to apoptosis induced by galectin-1. Jmock (**A** and **B**) and Jgal (**C** and **D**) cells were treated with PBS containing 100μM beta-mercaptoethanol for the controls (**A** and **C**), or 25μg/ml of rGal1 (**B** and **D**), and DNA content was estimated as described in section 3.11.

4.2.7. Mitochondrial activity is higher in the cells expressing galectin-1

Mitochondria are a target for galectin-1 induced apoptosis, as was shown in our laboratory (G. Ion et al. unpublished observation). To examine whether endogenously expressed galectin-1 affected mitichondrial activity, a possible reason for the increased sensitivity to galectin-1 induced apoptosis of the Jgal cells, we analysed mitochondrial activity by labeling the cells with MTT. Expression of galectin-1 resulted in an increase in the activity of the mitochondrial dehydrogenase.

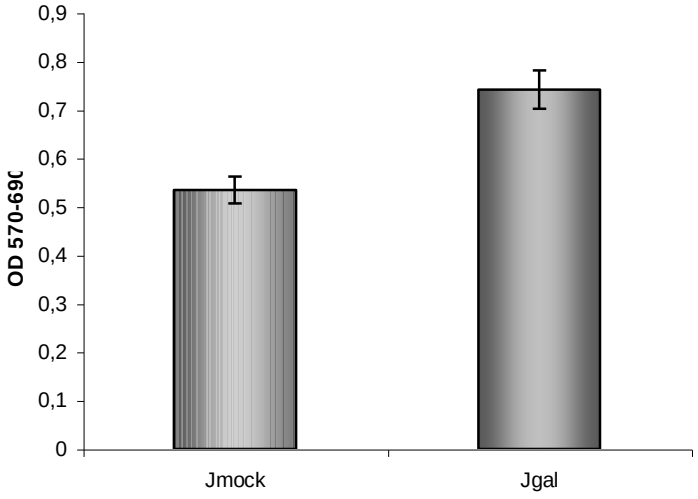

Fig.4.15. Jurkat cells expressing galectin-1 have an increased activity of mitochondrial dehydrogenase. The cells were plated at the same concentration of 10^6/ml, incubated in presence of 1μg/ml MTT, lysed and colour was measured on 570nm wavelength, indicating the activity of the mitochondrial dehydrogenase (see section 3.13).

5. Discussion

5.1. Role of the carbohydrate binding activity in the apoptotic function of galectin-1

The previous data have suggested that galectin-1 induces apoptosis in a lactose inhibitable fashion (83). This was supported with the following observation: presence of lactose in concentration of 10mM inhibited calciunm signalling induced by galectin-1 in Jurkat cells (148). In contrast, we showed that the presence of the disaccharide competitor, lactose during the treatment of the cells with galectin-1 did not inhibit the cell death. To resolve this controversy between our and others' results, we studied the role of sugar binding in the apoptosis using sugar "non-binding" mutants of galectin-1. The three point mutants R48H, E71Q, R73H, generated by Hirabayashi et. al. (62, 64), have been described as unable to bind asialofetuin. Because these proteins could not be purified by the classical sugar affinity chromatography, they were not further analyzed for biological activity. In our experiments, the wild type, and mutant galectin-1 proteins were expressed in fusion with His-tag and purified with Ni^{2+} affinity chromatography. In agreement with the results of Hirabayashi et al (62, 64), none of the mutants bound to cell surface complex glycoconjugates. However rHisGal71 and rHisGal73, but not rHisGal48 coupled to solid phase lactose, indicating that the former mutants retained the capacity to bind to a simple but not complex carbohydrates. This was a remarkable change in the specificity, since the wild type galectin-1 showed higher affinity toward complex sugars with two orders of magnitude, as to lactose (63). Analyzing the apoptotic effect of the mutants compared to the wild type protein, we found that the mutants retaining the affinity to lactose (e.g. rHisGal73) induced apoptosis, in contrast to the "null binding" form, rHisGal48. These findings can be explained in several ways: 1. The lectin property of galectin-1 contributes to the induced cell death, but the high affinity binding to complex sugars is not indispensable. 2. The receptor(s) transmitting the apoptotic signal by coupling to galectin-1 has not been characterized yet. The glycosphingolipid, lactosyl ceramide (CDw17) that contains disaccharide facing to the extracellular environment can be such a potential receptor. If this is the case we can understand why those mutants that bound to lactose but not to complex glycogonjugates, are able to stimulate apoptosis. On the other hand, the result that the presence of high concentration of lactose during induction of apoptosis does not support this theory.

Although many galectin-1-binding cell surface glycoproteins were identified on T cells (107), olny CD7 was proposed as the one mediating the apoptotic signal. It is intriguing

whether lactosyl-ceramide can be the another “apoptotic receptor”. It is noteworthy that our latest results indicate that galectin-1 regulates cell viability via intracellular ceramide (an apoptotic sphingolipid second messenger) release. In the future we intend to investigate the binding the galectin-1 to lactosyl ceramide.

5.2. Function and the role of the carbohydrate binding activity of intracellular galectin-1

The intracellular function of galectin-1 have been much less studied than the extracellular one. Previous reports showed that the galectin-1 modulated the cell cycle, proliferation and viability of certain cell types. These activities were demonstrated by adding recombinant protein to the cell cultures and thus were likely to be exerted by an extracellular mechanism (102, 105, 84, 106). However the intracellular galectin-1 may also operate as an regulator of the above cell functions. It is not without examples in galectin family members: intracellular pro-apoptotic function was demonstrated for galectin-7, that is a prototype galectin similarly to gallectin-1. Galectin-1 and galectin-7 are not co-expressed in the same cells since the later is present mainly in keratinocytes. The overexpression of galectin-7 in keratinocytes and tumor cell lines increased their susceptibility to ultravioletB (UVB) or actinomycin-induced apoptosis (149). Upon UVB irradiation the amount of galectin-7 mRNA and protein dramatically increased and these cells were more sensitive to undergo apoptosis. In contrast to galectin-7, galectin-3 exerted an intracellular anti-apoptotic function, therefore cells overexpressing this protein were more resistent to apoptotic stimuli, such as UVB irradiation or cycloheximide/TNFα treatment (150).

To analyze the consequence of the expression of intracellular galectin-1 and to understand its possible function in T cells, we chose the approach transfecting Jurkat cells which did not express galectin-1 with either wild type or with “non-binding” mutant cDNA. The transfected cells produced but did not secrete the lectin. Expression of galectin-1 affected the proliferation rate in the following order: Jgal (expressing wild type galectin-) < Jgal48 (transfected with carbohydrate non-binding mutant) < Jmock (transfected with empty vector). We characterized the tansfectants from the point of view of the modulation of different cell surface receptors. The pesence of the intracellular galectin-1 did not affect the expression of the investigated membrane proteins (CD45, CD95, CD2, LFA-1, MHC1), however the T cell receptor complex was upregulated highly in Jgal and to the lesser extent in Jgal48, compared to the mock transfected Jurkat cells. The increase of the membrane TCR level may result in the T cell responsiveness to TCR stimulation, as it has been shown in several studies (151-

153). Indeed, when Jgal cells were activated with TCR antibody (that mimics the antigen), a higher elevation of the intracellular Ca^{2+} concentration occurred as compared to Jgal48 or Jmock. However the increase of the TCR level did not clearly explain the higher calcium response, because the Ca-ionophor that acts independently of the TCR also generated a similar pattern of Ca^{2+} signal as TCR stimulation did: the highest in Jgal, intermediate in Jgal48 and lowest in Jmock cells.

The mechanisms that regulate Ca^{2+} signaling in T lymphocytes are diverse and complex. The events initiated by TCR ligation lead to inositol-1,4,5-trisphospate production and result in regulated release and influx of Ca^{2+} ions from the intracellular stores and from the extracellular space, respectively to the cytoplasm. The high intracellular Ca^{2+} concentration culminates in the activation of calcineurin and protein kinase C by Ca^{2+}/calmodulin and Ca^{2+}/diacylglycerol, respectively (154). Finally, calcineurin and the serine/threonine kinases activate a set of transcription factors that promote gene expression (154). It has been shown for B cells that different amplitude and duration of Ca^{2+} influx results in activation of distinct transcription factors (155). Similarly, the increased Ca^{2+} response in galectin-1 transfected Jurkat cells may direct the cells to different responses. Alternatively, the expression of galectin-1 in Jurkat T cells may decrease the treshhold for the same stimulus thereby increasing the sensitivity of the cells.

Regarding the necessity of the carbohydrate binding activity of the intracellular galectin-1 we think that it is early to draw final conclusion from these experiments. The Jurkat cells transfected with the carbohydrate non-binding mutant galectin-1 gave a response between the wild type and mock transfected cell lines for the stimuli, including proliferation rate, Ca^{2+} signal to TCR or ionophore stimulation. The experiments carried out using uncloned cells, where the expression of the wild type and mutant proteins was on the same level, suggested that these effects of intracellular gal1 were at least partially dependent on the sugar binding activity. In the case of cloned transfectants, used for the TCR level determination and Ca^{2+} influx measurement, it is difficult to decide whether the effect is caused by absence of carbohydrate binding activity or lower expression of the mutant galectin-1 in Jurkat cells.

Increased amplitude of the Ca^{2+} influx could also influence apoptotic signaling rendering the cells, expressing galectin-1, more sensitive to apoptosis (156). The addition of extracellular galectin-1 triggered a more remarkable cell death in Jgal cells compared with Jmock cells. The elevation of the sensitivity to galectin-1 induced apoptosis could be explained with the following findings: 1. We showed here, that the activity of the

mitochondrial dehydrogenase was higher in the Jgal than in the Jmock cells suggesting that there are more mitochondria in Jgal cells or the mitochondria are more active in this cells. 2. The galectin-1 induces apoptosis through the mitochondrial pathway as it was shown by G. Ion et. al (unpublished observation).

Summarizing our results we following model. Intracellular expression of galectin-1 has been shown to increase following antigenic stimulation of effector T cells (157). The galectin-1 is then secreted and induces apoptosis in an autocrine manner. In accordance with this, we found that activation of protein kinase C (PKC), that is an early consequence of TCR stimulation (158), results in augmenting the amount of intracellular and secreted galectin-1 level (G. Ion et. al., unpublished observation) (see Fig. 5.1. A, B, and C as a model). If the stimulation of TCR and the activation of PKC are sustained, the apoptosis induced with the secreted or exogenously added recombinant galectin-1 will be inhibited (Fig. 5.1. D and C) as it has been shown by G. Ion et. al. (unpublished observation). If the other enviromental signals are appropriate (e. g. the TCR is downregulated after antigen stimulation and PKC is not activated), the galectin-1 induces apoptosis of activated and inflammatory T lymphocytes and therefore it can contribute to the peripheral T cell homeostasis and tolerance by eliminating these cells (see the model on Fig. 5.1. E).

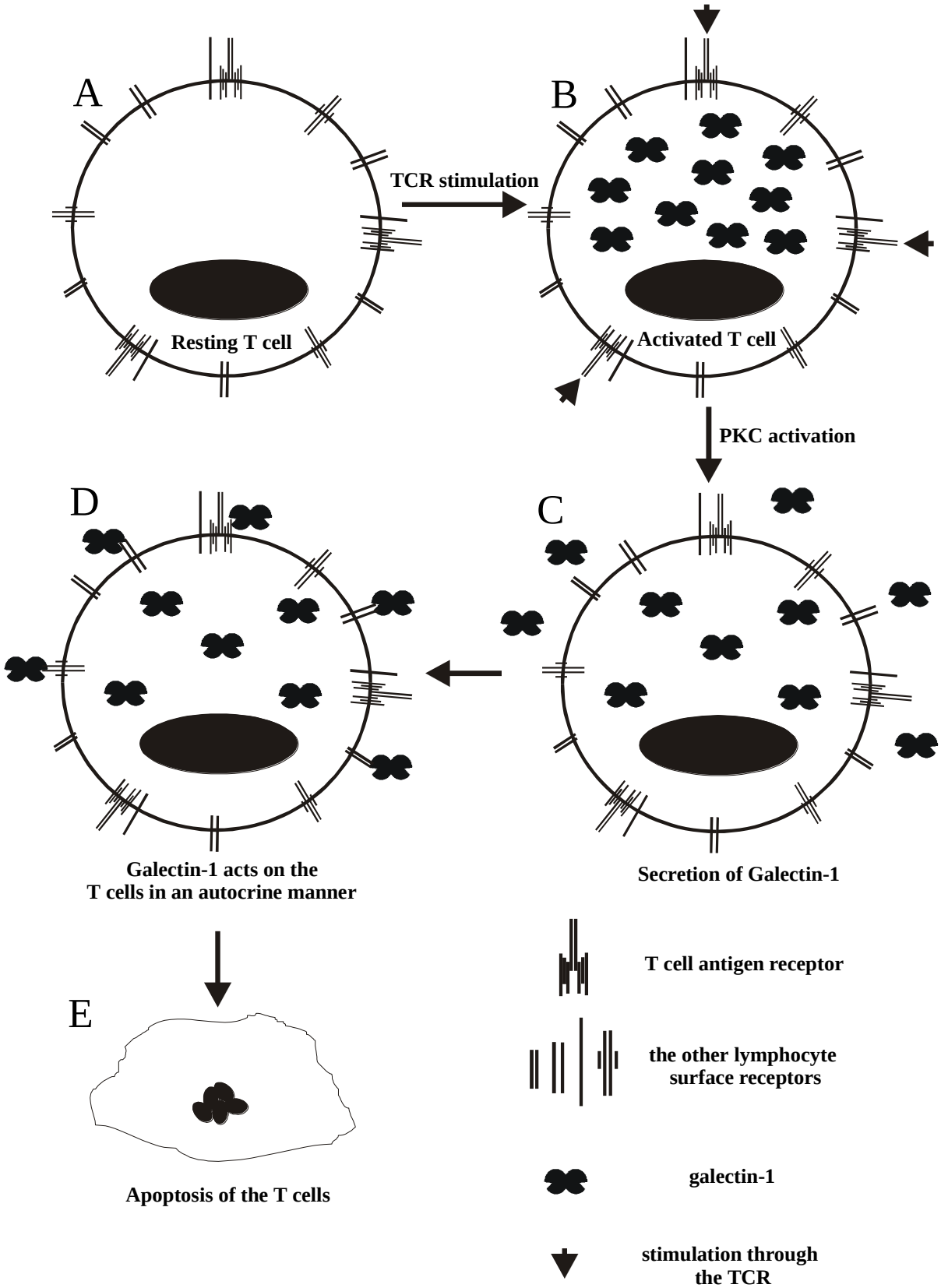

Fig.5.1. Proposed model of the regulation of T cell function by galectin-1 (see text for details).

6. Concluding remarks

1. To study the structure-function relationship of the extracellular galectin-1, we fused galectin-1 to His_6-tag, cloned and expressed in E. Coli and purified with Ni^{2+} affinity chromatography. The His-tagged wild type recombinant protein retained the affinity to the disaccharide, lactose and complex glycoconjugate ligands and induced apoptosis on Jurkat cells. The wild type and the mutant his-galectin-1 forms, rHisGal1.48, rHisGal1.71, rHisGal1.73 were recognized by the rabbit anti-galectin-1 antibody similarly to the recombinant galectin-1 without his-tag.

2. Two mutants, rHisGal1.71 and rHisGal1.73 but not rHisGal1.48 coupled to solid phase lactose but not to complex glycoconjugates on the surface of the Jurkat cells. The mutants maintaining the lactose binding capacity, induced apoptosis of the Jurkat cells, in contrast to the "null-binding" mutant, rHisGal1.48.

3. For analysis of the function of the intracellular galectin-1, the cDNA was stably transfected and overexpressed in Jurkat cells. The *de novo* expressed galectin-1 caused the upregulation of TCR expression but did not effect the expression of CD45, CD2, LFA1, MHCI, and CD95.

4. The galectin-1 expressing Jurkat cells had lower proliferation rate, higher amplitude of the Ca^{2+} response upon TCR stimulation or using Ca ionophore, and higher susceptibility to galectin-1 induced apoptosis in comparison to galectin-1 non-expressing cells.

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8.Appendix

Reagents and antibodies

All restriction and modification enzymes were purchased from MBI Fermentas (Vilnius,Lithuania).

All other reagents if not otherwise indicated were purchased from Sigma-Aldrich Kft. (Budapest, Hungary).

Antibodies used:

1. OKT3 (Ortho, Raritan, USA) recognizes CD3ε chain;
2. GB3 (produced in our lab(159))-recognizes all isoforms of CD45;
3. KD3 (produced in our lab(159))-recognizes CD45RA;
4. TMB6-5 (produced in our laboratory) – recognizes MHC class I;
5. Anti-CD95 (BectonDickinson) – recognizes CD95;
6. BT11 (generous gift of Michael Crumpton, ICRF) – recognizes CD2
7. ITM3 (produced in our laboratory) – recognizes LFA1;
8. Rabbit anti-galectin-1 antibody (produced in our laboratory) – recognizes human galectin-1;

Buffers and Solutions

RPMI cell culture medium:

1,04% (w/v) RPMI 1640 powder (Gibco BRL)

0,2% (w/v) $NaHCO_3$

10 NE/ml penicillin (Biogal)

0,1 mg/ml streptomycin (EGIS)

after sterilization 0,63 mg/ml L-glutamine, 5% (v/v) or 10% (v/v) heat inactivated foetal calf serum (FCS, Protein GMK, Gödöllő) were added.

DMEM cell culture medium:

1,34% (w/v) DMEM powder (Gibco BRL)

0,37% (w/v) $NaHCO_3$

10 NE/ml penicillin (Biogal)

0,1 mg/ml streptomycin (EGIS)

after sterilization 0,63 mg/ml L-glutamine, 5% (v/v) or 10% (v/v) heat inactivated foetal calf serum (FCS, Protein GMK, Gödöllő) were added.

Trypan Blue solution for cell counting:

0,16% (w/v) trypanblue

0,9% (w/v) NaCl

Antioxidants:

1 mM sodium pyruvate;

50 mM a-thioglycerol (a-TG);

20 nM bathocuproindisulfonic acid disodium salt (BCS).

LB:

10 g/l tripton

5 g/l yeast extract (Difco)

5 g/l NaCl (Merck)

pH 7,0

Buffers for alpha-lactose gel (EY Laboratories) column purification:

Lysis buffer:

50 mM Tris/HCl pH 7.5

10 mM EDTA

Washing buffer:

50 mM Tris/HCl pH 7.5

Elution buffer

50 mM Tris/HCl pH 7.5;

100 mM lactose.

Buffers for Ni-NTA-agarose column purification:

Lysis buffer

50mM NaH_2PO_4, pH8.0;

300mM NaCl;

20mM Imidasole, pH8.0.

Washing buffer:

50mM NaH_2PO_4, pH6.0;

300mM,NaCl;

200mM Imidasole, pH6.0;

10% Glycerol.

Elution buffer:

50mM NaH_2PO_4, pH6.0;

300mM NaCl;

400mM Imidasole, pH6.0;

10% Glycerol.

PBS, pH 7,4:

1,8 mM NaH_2PO_4

10 mM Na_2HPO_4

135 mM NaCl

3 mM KCl

Propidium Iodide Stock Solution:

50 μg/ml propidium iodide,

0,1% (w/v) sodium citrate.

Staining buffer for apoptosis assay:

PBS supplemented with:

0,1% (v/v) Triton X-100

0,1% (w/v) Na-citrate

10 μg/ml RNase

10 μg/ml propidium-iodide (Sigma, Fluka)

Solutions for Ca/phosphate transfection:

2.5M calcium chloride ($CaCl_2$)

2X HBS:

280mM NaCl

50mM HEPES acid

1,5mM Na_2HPO_4

Titrate to pH 7.05 - 7.12

Filter sterilize with 0.2 μm membrane

Reducing SDS sample buffer stock:

125 mM Tris/HCl pH 6,8;

20% (v/v) glycerine;

4% (w/v) SDS;

2% (v/v) β-mercaptoethanol;

bromfenolblue (Bio-Rad).

Stacking gel:

5% (w/v) acrylamide / 0,13% (w/v) bis-acrylamide (Bio-Rad);

125 mM Tris/HCl pH 6,8;

0,1% (w/v) SDS;

0,033% (w/v) ammonium-peroxydisulfate (APS);

0,07% (v/v) N, N, N, N'- tetrametil-ethylene-diamine (TEMED) (Bio-Rad);

Running gel:

7-15% (w/v) acrylamide 0,182-0,39% (w/v) bis-acrylamide (Bio-Rad)

125 mM Tris/HCl pH 8.8;

0,1% (w/v) SDS;

0,033% (w/v) APS;

0,07% (v/v) TEMED (Bio-Rad).

Running buffer:

25 mM Tris/HCl pH 8,3

194 mM glicin

0,1% (w/v) SDS

Coomassie Brilliant Blue G-250 for staining of protein gels:

0,1% (w/v) Coomassie Brillant Blue G-250;

50% (v/v) methanol;

10% (v/v) acetic acid.

Protein gel destainer:

10% (v/v) acetic acid

Transfer buffer:

25 mM Tris-HCl pH 8,3;

194 mM glicin;

20% (v/v) methanol.

TBST:

10 mM Tris-HCl pH 7,5;

150 mM NaCl;

0,05% (v/v) Tween 20.

Abbreviations

AICD – activation-induced cell death

AP-1 - activator protein 1

C2GnT - core 2-beta-1,6-*N*-acetylglucosaminyltransferase

GAL1 – galectin-1 gene

CD -cluster of differentiation

CIA - collagen-induced arthritis

GF/C - glass microfibre/C series(1.2μm)

G_{M1}(Galβ1–3GalNAcβ1–4[Siaα2–3]Galβ1–4Glcβ1-Cer) - receptor for cholera toxin

Con A – concavalin A

CRD – carbohydrate recognition domain

ECL - enhanced chemiluminescence

EDTA - ethylenediaminetetraacetic acid

EGTA - ethylnglycol-bis-N, N, N' ,N'-tetraacetic acid

ER – endoplasmatic reticulum

FACS - fluorescent activated cell sorter

FCS – foetal calf serum

HRPO - horse-radish peroxidase

His(H) - histidine

IFN - interferon

Ig - immunoglobulin

IL – interleukin

Kd – dissociation constant

LFA – lymphocyte function antigen

M-phase – phase of mitosis during cell cycle

MTT - methylthiazoletetrazolium

NF-AT - nuclear factor of activated T cells

NFκB - nuclear factor κB

NTA - nitrilo-triacetic acid

PAGE - polyacrylamide gel electrophoresis

PBS - phosphate buffered saline

PHA - phytohemagglutinin

PI – propidium iodide

PI3K - phosphatidylinositol 3-kinase

PKC – protein kinase C

PLCγ – phospholipase Cγ

PMSF - phenylmethhylsulfonyl fluoride

Ras – rat sarcoma

rGal1-1 – recombinant human galectin-1

rHisGal1 – recombinant human His_6 tagged galectin-1

rHisGal1.48 - recombinant human His_6 tagged galectin-1 with amino acid substitution at position 48

rHisGal1.71 - recombinant human His_6 tagged galectin-1 with amino acid substitution at position 71

rHisGal1.73 - recombinant human His_6 tagged galectin-1 with amino acid substitution at position 73

SDS - sodium dodecyl sulfate

TBS - tris-buffered saline

TBST - tris-buffered saline containing Tween20

TCR – T cell antigen receptor

WGA – wheat germ agglutinin.

9. List of publications

1. **Demydenko D.** Galectin-1 expression in tumors and its role in cancer cell adhesion. Review. Exp Oncol 2002 Sept; 24: 163-166.

2. Fajka-Boja R, Hidvegi M, Shoenfeld Y, Ion G, **Demydenko D**, Tomoskozi-Farkas R, Vizler C, Telekes A, Resetar A, Monostori E. Fermented wheat germ extract induces apoptosis and downregulation of major histocompatibility complex class I proteins in tumor T and B cell lines. Int J Oncol 2002 Mar; 20(3):563-70.

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11. Summary

The human galectin-1 belongs to the family of N-acetyllactosamine binding lectins. It specifically binds to glycoconjugates via its carbohydrate recognition domain (CRD) that is highly homologous within the members of the galectin family. The galectin-1 is expressed in a wide range of different tissues. Most importantly, immune cells, such as activated T cells and macrophages, express the lectin at the site of the immune reactions where galectin-1 acts as a strong immunomodulator. The immunomodulatory function of the galectin-1 is attributed to its apoptosis inducing capacity on activated T lymphocytes. The role of the carbohydrate recognition of galectin-1 in the induction of apoptosis has been disputed for a long time. It is also poorly understood whether the *de novo* expressed intracellular galectin-1 affects the cellular functions, including the sensitivity to different apoptotic stimuli of the activated T lymphocytes.

Our **aim** was to study the following main questions:

1. It has not been clearly defined whether the carbohydrate binding of the galectin-1 is essential for the apoptotic function of the protein. The main reason for this is that the purification of the non-binding mutants with the classical sugar-matrix affinity chromatography encountered difficulties. Therefore our purpose was:

a) To establish a system that would provide a way to isolate this kind of mutant proteins.

b) To analyze whether or not these mutants, characterized previously as “sugar non-binding” are able to trigger apoptosis of T lymphocytes.

c) To investigate how are particular amino acid mutation, that is directly couple to carbohydrate, affect the galectin-1 induced apoptosis.

2. To obtain a deeper inspite into the intracellular function of the galectin-1 we studied:

a) How the de novo expressed galectin-1 modulates the T cell functions.

b) The role of the lectin property of the galectin-1 in its intracellular function.

The **results** obtained in this work are as follows:

1. A single mutation in the gal1 gene at the positions E71Q, R73H, resulted in a reduced and R48H an abrogated lactose-binding capacity. None of these mutants bound to cell surface glycoconjugates.

2. The two mutant with retained lactose-binding (rHisGal1.71 and rHisGal1.73) but not the "null-mutant", rHisGal1.48 induced apoptosis in the Jurkat cells.

3. Presence of intracellular galectin-1 caused

a) a slower proliferation; b) an elevated TCR expression; c) a higher Ca^{2+} response induced via the TCR or with Ca^{2+} ionophore; d) an increase in the mitochondrial activity and e) a higher sensitivity of the Jurkat cells toward extracellular galectin-1 triggered apoptosis.

The **conclusions** drawn from this study are:

1. The results obtained in regard to the extracellular function of galectin-1 with carbohydrate non-binding mutants indicate that this quality is only partially required for the induction of apoptosis in T cells.

2. The expression of intracellular galectin-1 results in the upregulation of the cell surface TCR, the inducibility of a Ca^{2+} response with TCR or ionophore stimulation, an increased mitochondrial activity and likely as a result an elevated sensitivity of the extracellular galectin-1 induced apoptosis.

12. Summary in Hungarian

A humán galektin-1 az N-acetillaktózamin kötő lektinek csoportjába tartozik. Szénhidrátkötő doménjén (carbohydrate recognition domain, CRD) keresztül specifikusan kapcsolódik a különböző glikokonjugátumokhoz. A CRD szakasz nagyfokú homológiát mutat a galektin családba tartozó lektinek között. A galektin-1 expresszióját sokféle szövetben kimutatták. Az immun rendszer sejtjei, mint például az aktivált T limfociták és a makrofágok, az immunreakciók helyén nagy mennyiségben expresszálják, így a galektin-1 erős immunmoduláló hatása érvényesülhet. Gyulladáscsökkentő és az autoimmun folyamatokat gátló hatása nagy mértékben köszönhető annak, hogy az aktivált T limfociták apoptózisát okozza. A szénhidrát felismerő domén apoptózis indukcióban betöltött szerepe régóta a vizsgálatok tárgya.
A de novo expresszált intracelluláris galektin-1 szerepe különböző sejt funkciókra, beleértve az aktivált T-limfociták által a különböző apoptotikus szignálokra adott válasz érzékenységét azonban kvéssé vizsgálalt kérdés.

Célul tűztük ki a következő problémák vizsgálatát:
1. Még nem eldöntött, hogy szükséges-e a szénhidrátkötő domén az apoptózis indukcióhoz. Ennek legfőbb oka, hogy a szénhidrátkötésre képtelen mutáns formák laktozil-agaróz oszlopon történő tisztítása nem lehetséges. Ezért:
A, Olyan rendszer beállítását tűztük ki célul, amely lehetővé teszi a szénhidrát nem-kötő mutáns formák tisztítását.
B, Vizsgálni kívántuk, hogy a “cukor-nemkötő”- mutánsként karakterizált formák indukálnak-e apoptózist T-limfocitákon.
C, Meg akartuk határozni, hogy azok az aminosavak, amelyek közvetlenül kapcsolódnak a szénhidráthoz, befolyásolják-e a galektin-1 által indukált apoptózist.

2. A sejtek által expresszált intracelluláris galektin-1 funkciójának megértéséhez a következő kérdésekre kerestük a választ:
A, Hogyan szabályozza a de novo expresszált galektin-1 a T sejtek funkcióit.
B, Mi a galektin-1 lektin (szénhidrát kötő) funkciójának intracelluláris szerepe.

A munka során elért eredmények a következők:

1. A galektin-1 gén E71Q és az R73H mutációi csökkent laktózkötő képességet eredményeztek, míg az R48H mutáció a laktóz kötés teljes hiányát okozta. Egyik mutáns sem kötődött a sejt felszíni cukorkonjugátumokhoz.
2. A két csökkent laktóz kötést mutató mutáns (71 és a 73) apoptózist indukált Jurkat sejteken, míg a "null-mutáns" 48-as forma nem.
3. A galektin-1 intracelluláris jelenléte csökkent sejtproliferációt, megnövekedett TCR expressziót, TCR vagy Ca^{2+} ionofór által kiváltott, a kontrollhoz képest megnövekedett mértékű Ca^{2+} választ, mitokondrium aktivitás növekedést és az extracelluláris galektin-1 kezelésre adott megnövekedett apoptotikus választ okozott.

A vizsgálatokból levonható következtetések:

1. A szénhidrát nem kötő mutánsok vizsgálatából kiderült, hogy a szénhidrátkötőképesség csak részben szükséges a galektin-1 általi apoptózis indukcióhoz T sejteken.
2. A galektin-1 intracelluláris expressziója megnövekedett sejtfelszíni TCR expressziót, megnövekedett TCR ill. ionofor által stimulált Ca^{2+} választ, megnövekedett mitokondriális aktivitást, valamint az extracelluláris galektin-1 által indukált apoptózissal szembeni megnövekedett érzékenységet eredményez.