Cell Cycle Regulation by Galectin-12, a New Member of the Galectin Superfamily

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Galectins are a family of β-galactoside-binding animal lectins with conserved carbohydrate recognition domains (CRDs). Here we report the identification and characterization of a new galectin, galectin-12, which contains two domains that are homologous to the galectin CRD. The N-terminal domain contains all of the sequence elements predicted to form the two β-sheets found in other galectins, as well as conserved carbohydrate-interacting residues. The C-terminal domain shows considerable divergence from the consensus sequence, and many of these conserved residues are not present. Nevertheless, the protein has lactose binding activity, most likely due to the contribution of the N-terminal domain. The mRNA for galectin-12 contains features coding for proteins with growth-regulatory functions. These include start codons in a context that are suboptimal for translation initiation and AU-rich motifs in the 3′-untranslated region, which are known to confer instability to mRNA. Galectin-12 mRNA is sparingly expressed or undetectable in many tissues and cell lines tested, but it is up-regulated in cells synchronized at the G1 phase or the G1/S boundary of the cell cycle. Ectopic expression of galectin-12 in cancer cells causes cell cycle arrest at the G1 phase and cell growth suppression. We conclude that galectin-12 is a novel regulator of cellular homeostasis.

In mammals, 11 members have been designated as galectins (galectin-1 through -11); a large number of additional members are likely to be discovered, and many identifiable homologues are present in the published data base (6). Proteins containing sequence similarities with galectins but without demonstrable lectin activity also exist. For example, a novel protein structurally related to galectins, but lacking β-galactoside binding activity, has been identified in the lens and designated as GRIFIN (galectin-related interfiber protein) (7).

Diverse biological functions have been demonstrated for various galectins in vitro, including extracellular functions that are consistent with the lectin properties. For example, galectin-1 and -3 have been shown to activate various cell types, through cross-linkage of appropriate cell surface glycoproteins, and to modulate cell adhesion, probably through interactions with cell surface glycoproteins, including cell adhesion molecules (reviewed in Refs. 3 and 5). Galectin-3 has been shown to promote neurite growth (8) and induce differentiation and angiogenesis of endothelial cells (9). Galectin-3 is also a chemottractant for monocytes (10) and endothelial cells (9), while galectin-9 is a chemottractant for eosinophils (11). Galectin-1 and -9 have been demonstrated to induce apoptosis of T-cells and thymocytes (12–14), via recognition of cell surface glycoproteins. Intracellular functions of galectins are also noteworthy. Consistent with the absence of a classical signal sequence, many galectins are found to primarily reside intracellularly. Moreover, the presence of some, such as galectin-1 and -3, in the nuclei of proliferating cells has been reported (15, 16). Both galectin-1 and -3 have been shown to be active in vitro in inducing pre-mRNA splicing (17, 18). Galectin-3 has been found to suppress apoptosis, possibly through interaction with Bel-2, a well characterized antiapoptotic protein with which it shares sequence similarity (19–21). Galectin-7, on the other hand, has been demonstrated to promote apoptosis in transfected cells (22) and may be responsible for the proapoptotic function of p53 (23). Relevant to the intracellular functions is the fact that galectins have been shown to recognize intracellular proteins, including Bel-2 (19), nuclear lectins (24), and cytokeratins (25).

Another notable feature of galectins is their association with neoplastic transformation. Galectin-3 is overexpressed in some types of cancers, in which the normal parental cells do not express the protein, including specific types of lymphomas (26, 27), thyroid carcinoma (28, 29), and hepatocellular carcinoma (30). However, it is down-regulated in other kinds of neoplasms, including colon carcinoma (31, 32), breast carcinoma (33), ovarian carcinoma (34), and uterine carcinoma (35). Galectin-7, which is highly inducible by p53 (23) and normally expressed in stratified epithelia, is down-regulated in squamous cell carcinomas (36). Galectin-8 is overexpressed in prostate cancer (37), and galectin-9 is overexpressed in Hodgkin’s lymphomas (38, 39). Whether the altered expression of galec-
tins contributes to the neoplastic transformation or is a consequence of the transformation awaits elucidation. Studies of cells transfected with galectin-3 cDNA or treated with specific antisense oligonucleotides, however, have provided evidence for the involvement of galectin-3 in tumor development and metastasis (40, 41). Galectins may play a major role in the biological behaviors of cancer cells in which the lectins are either up-regulated or down-regulated because of the various functions of galectins mentioned above.

Cell cycle control is a major theme of normal development and differentiation. Most cancers arise due to faulty cell cycle regulation (42). Under genotoxic assaults, cell cycle arrest prevents transmission of damaged genomic DNA that could otherwise cause widespread mutations of vital genes controlling cell growth in daughter cells, a major cause of cancer (43). In conditions not permissive for productive proliferation, e.g., growth factor deficiency, cells invoke G₁ arrest to stay alive until conditions improve. Premature entry into the cell cycle leads to apoptosis (44, 45). Cell differentiation is also usually associated with irreversible cell cycle exit (46).

Identification of new cell cycle regulation proteins is therefore important for the understanding of normal cellular homeostasis and pathogenesis of neoplasms as well as the development of new methods for diagnosis and treatment of cancers. Here we report the isolation of a cDNA for such a protein from human T-cells in G₁ phase, identify that this protein is a member of the galectin family, and demonstrate that its ectopic expression arrests the cell cycle at the G₁ phase.

**Experimental Procedures**

Cells and Cell Lines—All cell lines used were from ATCC (Manassas, VA). The breast cancer cell line MCF-7 was grown in Dulbecco’s modified Eagle’s medium-F-12 supplemented with 10% fetal bovine serum (FBS). Human T-leukemic lines and U937 were cultured in RPMI 1640 medium containing 10% FBS and 2 mM glutamine. HeLa cells were grown in McCoy’s 5A medium with 10% FBS and 2 mM glutamine. The human tumor cell lines HBL-100, and MCF-7. Human blood was obtained from three unrelated healthy volunteers following informed consent and pooled. Mononuclear cells were prepared by dextran sedimentation and isolated from the high density cell pellet that also contained erythrocytes, followed by removal of erythrocytes by osmotic lysis. Polymorphonuclear cells were prepared by isopycnic centrifugation on Histopaque 1077 (Sigma). Premature entry into the cell cycle at the G₁ phase.

**Cloning of cDNAs for Full-length Galectin-12—Randomly selected EST clones from a G₁ phase Jurkat T-cells cDNA library (47) were sequenced with the ABI PRISM Rhodamine Terminator Cycle Sequencing Ready kit (PE- Applied Biosystems, Foster City, CA) on the ABI PRISM 310 Genetic Analyzer per the manufacturer’s protocols. Three positive clones were found to contain the same insert, whose’ regions were identical to the HJACE54 cDNA.

In the EST data base, we searched for a mouse homolog of the above described cDNA was found. Screening the entire EST clone, however, revealed that it contained additional 5′ sequence not present in the human clones. In GenBank™, a cosm id clone (CSR187d6; GenBank™ Accession Number U73641) from human chromosome 11 was found to contain the genomic DNA for the cloned cDNA. In this cosm id DNA, a segment homologous to the leading sequence found in the mouse homolog was identified. The full-length cDNA was then obtained by PCR of a human retina Marathon cDNA with an Advantage 2 PCR kit (CLONTECH), using the upstream primer (AGTGGAGAGTGCCCCCACC), specific from the upstream cosmid sequence, and the downstream primer GSP. The PCR product was cloned into pcDNA3 with the pcDNA3-amp Cloning kit (Stratagene) to generate the pcDNA3-Gal12 and sequenced as described above.

**Analysis of Galectin-12 mRNA Expression—**PCR on Multiple Tissue cDNA panels (CLONTECH) was carried out per the manufacturer’s protocol using the following primers: for galectin-12, CACGCTCCTAGGTGTTGTAGGATTGCACCAG (nucleotides 942–979 in Fig. 1) and TAGTCTCTAAGCTCTGCTTGTAGCTAGCCAGC (antisense to nucleotides 1211–1248 in Fig. 1); for galectin-3, CTTAATAACCATGTGCATCGCATGCAAGTTG (sense to nucleotides 41–59 in the HJACE54 cDNA) and related to nucleotides 41–59 in the HJACE54 cDNA) and AGCCGCCTATGTCTGCACATGGG (antisense to nucleotides 951–972 in Fig. 1) primers for galectin-12 and upstream (AGTGGAGAGTGCCCCCACCAGC) and downstream (CACTTCTCAGAGTGGACACAGC) primers for β-actin. Products were visualized by ethidium bromide staining.

**Cell Cycle Synchronization—**Synchronization of Jurkat cells was performed by treatment of the cells (in logarithmic growth) overnight with 0.1 mM thymidine or 1 mM hydroxyurea to obtain cells at the G₁/S boundary (48), with 0.1 μM nocoazole to block cells at mitosis, or with 0.2 mM theophylline plus 0.5 mM dibutyryl cAMP to obtain cells at G₁ (49). RNA was extracted, and reverse transcription was done as described above. PCR was performed by using Taq polymerase (Promega, Madison, WI) together with upstream (AGTTGCCTCGCTCAATGCTG; nucleotides 581–601 in Fig. 1) and downstream (CACTTCTCAGAGTGGACACAGC) primers for galectin-12 and upstream (CTCTTGGCTACAGGAGAAC) and downstream (GAAGCATTGCGCTGGTACAG) primers for β-actin. Products were visualized by ethidium bromide staining.

**Preparation of Anti-galectin-12 Antibody and Immunoblot Analysis—**A peptide corresponding to residues 162–175 in the linker region of the leukemic line MOLM-14 (50) was purified with an adsorbent made by conjugating the peptide through the sulfhydryl group to sulfolink gel using the kit provided by the manufacturer (Pierce). The specific antibody was affinity-purified with an adsorbent made by conjugating the peptide through the sulfhydryl group to sulfolink gel using the kit provided by the manufacturer (Pierce). The immunoblot analysis of galectin-12 was performed by using this antibody at 0.2 μg/ml. The immunoblot analysis for detecting HA-tagged proteins was performed by using the mouse monoclonal antibody C12A5 (Roche Molecular Biochemicals) at 0.2 μg/ml. All blots were developed with the Supersignal West Femto Maximum Sensitivity Substrate Kit from Pierce.

**Lactose-binding Assay—**This was performed as described (51).

**Constructs for Mammalian Expression—**The insert from pcDNA3-Gal12 (see “Cloning of cDNAs for Full-length Galectin-12”) was directly cloned into pEF (51) to generate pEFGal12. pCMV was generated by deleting the internal ribosome entry site and neo gene from pEFIRE (CLONTECH), cDNA for HA-tagged galactin-9 was generated by PCR using ACCATGATACCATACACACGCTCCAGACTAGCTTACTGCAGCTTCCAGGCCAG and ACCGGCCTATGTCTGCACATGGG as primers and the plasmid DNA from clone HTTPBR22 as template. cDNA for HA-tagged galectin-12 was generated by PCR using ACATGATACCATACACACGCTCCAGACTAGCTTACCATGCAGCTTCCAGGCCAG and GSP as primers and pcDNA3-Gal12 as template. PCR were used.
cloned into pCR-Script and sequenced to generate pCR-HA9 and pCR-HA12, inserts from which were finally cloned into pCMV to generate pCMV-HA9 and pCMV-HA12, respectively. pCMV-puro was made by replacing the BamHI–XhoI fragment in pIRES1neo with the BamHI–SalI fragment from pWE3 (ATCC).

Transfection and Cell Cycle Analysis—Cells from subconfluent cultures in the log growth phase were transfected by electroporation following published protocols with some modifications (52, 53). HeLa cells at 70–80% confluency were split 1:3 the day before transfection. On the day of transfection, cells were trypsinized and resuspended in culture medium (McCoy's 5A medium with 10% FBS and glutamine). Aliquots of 0.4 ml of cell suspension containing 0.75–1.3.10^6 cells were transferred to 0.4-cm electroporation cuvettes. Plasmid DNA (a total of 30 m g) was added and electroporation was carried out on a Gene Pulser-II electroporator (Bio-Rad) at 250 V, 1070 microfarads. Cells were then resuspended in 5 ml of complete tissue culture media and cultured for 24 h. Cells were subsequently cultured in the presence or absence of 0.1 mg/ml nocodazole for an additional 24 h before harvest. For cell cycle analysis, trypsinized cells were fixed in 70% ethanol at 4 °C overnight. Nuclear DNA was then stained for 20 min at 37 °C with 50 mg/ml propidium iodide (Calbiochem) in phosphate-buffered saline containing 50 mg/ml RNase A. Samples were analyzed by flow cytometry for propidium iodide width versus area fluorescence, which allowed the exclusion of doublets and weakly fluorescent cell debris. The above gated cells were acquired into a red fluorescence area histogram on a FACS Calibur (Becton-Dickinson, Franklin Lakes, NJ) and analyzed with the CellQuest software (Becton-Dickinson).

RESULTS

Cloning of Galectin-12 cDNA—One of the EST clones from a human T-cell line, Jurkat, at the G1 phase was found to have sequence homology with galectins. This clone, HJACE54, has an insert of 865 bp containing an open reading frame of 402 bp (134 amino acids). A search of an EST database with this sequence revealed that the gene is also expressed in human retina. We then attempted to clone the full-length cDNA from a human retina cDNA library by 5′-RACE, and a clone with an open reading frame coding for 275 amino acids was obtained. A BLAST search showed that the deduced protein is homologous to the galectin family members with two CRDs separated by a linker sequence. The N-terminal domain of this protein, however, appeared to be incomplete, because it lacks the sequence to form the S1b-strand that is present in all other known galectins.

Sequence comparisons between our cDNA and homologous genomic DNA in a cosmid clone and with a mouse cDNA homolog, both identified in the nonredundant GenBank™ data base, revealed the complete 5′ sequence of the human cDNA. This complete sequence, however, did not contain the 5′ sequence of the RACE product obtained from the retina cDNA library. Our complete sequence, designated as galectin-12, is presented in Fig. 1. Like all other known galectins, the sequence of galectin-12 does not contain a transmembrane domain or a classical signal sequence.

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RT-PCR with mRNA from cell lines, using a primer corresponding to a sequence at the 5′-end of the cDNA generated by 5′-RACE, failed to yield any detectable product. In the same experiments, RT-PCR using primers specific for full-length ga-
lectin-12 resulted in predicted products (data not shown), and this is the method used for full-length cloning. The cDNA from 5'-RACE thus probably represents an alternatively spliced gene product with a retained intron, and the expression of the corresponding mRNA in cell lines may be insignificant compared with the authentic full-length mRNA.

The first ATG in the open reading frame of galectin-12 cDNA is not in a favorable context for translation initiation, according to the Kozak rule (54). Significantly, the 3'-untranslated region contains five AT-rich motifs (ATTTA) (Fig. 1), which are initially identified in inflammatory cytokine cDNAs (55) and are also detectable in many other cDNAs coding for proteins of growth regulatory functions, such as oncoproteins and growth factors (56). These motifs are known to confer instability to mRNA (56, 57).

Organization of Galectin-12 Genomic DNA—Alignment of the full-length galectin-12 cDNA sequence with the genomic DNA sequence revealed that the galectin-12 gene consists of nine exons (Fig. 2A). Comparison of the coding exon sequences of the galectin-12 gene with those of other known galectin genes revealed conservation in exon organization (Fig. 2B). This comparison allowed us to classify exons for the galectin CRD into two groups. The lengths of the three exons coding for the N-terminal CRD of galectin-12 correspond well to those coding for the N-terminal CRD of mouse galectin-6 and the single CRD of galectin-10. The lengths of the three exons coding for the C-terminal CRD of galectin-12 correspond well to those coding for the C-terminal CRD of galectin-6 and the CRD of galectin-1, -2, and -3. Therefore, galectin-12 and other known galectins appear to be derived from an ancestral gene.

Exon by Exon Protein Sequence Comparison between Galectin-12 and Other Galectins—Crystal structures of galectin-1 (58), galectin-2 (59), galectin-3 (60), galectin-7 (61), and galectin-10 (62) determined by x-ray diffraction showed that each carbohydrate recognition domain is composed of a five-stranded (F1–F5) and a six-stranded (S1–S6) β-sheet. These studies also identified residues in the proteins that are in contact with the carbohydrate ligands. The N-terminal domain of galectin-12 contains all of the sequence elements predicted to form the two β-sheets as well as the conserved carbohydrate-contact residues (Fig. 3A). The sequence of the C-terminal domain, however, strays significantly from the consensus sequence, and many of these conserved residues are not present (Fig. 3B).

Comparison of each CRD in galectin-12 with CRDs of other members of the galectin family demonstrates that the N-terminal domain has significant homologies with other galectins (Fig. 3C). The C-terminal domain (galectin-12C) displays greater divergence from other members, as evident from lower percentage identities (in comparison, among all other galectin domains listed in Fig. 3C, the median sequence identity is 38%, with the upper decile of 45% and the lower decile of 32%). These homologies are reflected in the evolutionary analysis of the galectin family as shown in the phylogenetic tree in Fig. 3D.

Galectin-12 Exhibits Lactose Binding Activity—The sequence analysis described above suggests that galectin-12 is likely to have lactose binding activity because of the contribution of the N-terminal domain. To clarify this point, lysate from Jurkat cells transfected with the galectin-12 cDNA was mixed with lactosyl-Sepharose 4B, and the bound protein was eluted with the SDS sample buffer. The eluted material was analyzed by immunoblotting using antibodies specific for an internal peptide of galectin-12. We found that galectin-12 bound to lactosyl-Sepharose 4B (Fig. 4). The binding is specific, because galectin-12 did not bind to sacrosyl-Sepharose 4B, and its binding to lactosl-Sepharose 4B was inhibited by lactose but not by sacrose (Fig. 4).

Tissue Distribution of Galectin-12 mRNA—Northern blot analysis showed that galectin-12 mRNA was nearly undetectable in many tissues tested, in contrast to galectin-3 mRNA, which was detected in almost all tissues, when the same membranes were reprobed (data not shown). However, using a more

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**Fig. 2.** Exon organization of galectin-12 gene in comparison with other cloned galectin genes. **A,** nine exons (bars) separated by introns. **B,** exon comparisons. The exon structures of galectin-12 are compared with other known galectin structures (66–70). The numbers below each closed bar indicate the lengths of exons, portions of which shown in shaded regions constitute the galectin domains.
sensitive procedure, RT-PCR, galectin-12 mRNA was detectable in the heart, pancreas, spleen, thymus, and peripheral blood leukocytes. It was also present at lower levels in the lung, skeletal muscle, kidney, prostate, testis, ovary, and colon but virtually undetectable in the brain, placenta, and liver (Fig. 5A). Galectin-12 mRNA was not detected in many cell lines tested, but its expression was confirmed by RT-PCR in the peripheral blood mononuclear and polynuclear cells as well as myeloid cell lines, U937, HL-60, and KU-812; the B-cell line Wil-2; and the breast cancer cell line HBL-100 (Fig. 5B). Galectin-12 was weakly expressed in the human T cell line HuT-78, the hepatic cell line HepG2, the human ovarian cancer line SKOV3, and the human breast cancer line T47D. Immunoblot analysis with the anti-peptide antibodies, however, failed to detect galectin-12 protein in these cell lines (data not shown), although this procedure detected the protein in the lysates from galectin-12 transfectants (Fig. 4).

Up-regulation of Galectin-12 Expression by Cell Cycle Synchronization at G1 or G1/S Boundary—Since a partial galectin-12 cDNA clone was isolated from a Jurkat cell library

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**Fig. 3. Protein sequence comparison between galectin-12 and other galectins of known genomic structures.** A, alignment of N-terminal CRD amino acid sequences of galectin-12 with corresponding exons in galectin-6 (68) and -10 (70). B, alignment of C-terminal CRD sequences of galectin-12 with corresponding exons in galectin-1 and -2 (67), -3 (66), and -4 (68). Suffixes in Roman numerals indicate the exon number for each member. The predicted β strands are overlined, and residues corresponding to those shown to interact with lactose in galectin-1 (58) and -2 (59) are marked by an asterisk. C, percentages of protein identities among CRDs of human galectin family members were calculated with Gap in GCG version 10. Interspecies comparisons are shown for rat galectin-6 (71), murine galectin-6 (68), ovine galectin-11 (72), and rat GRIFIN (7). PP13, placental protein 13 (73). D, a phylogenetic tree of galectin family members, constructed with Phylip (74) from alignments performed with ClustalW (75). hu, human; mu, murine; ov, ovine; -N, N-terminal CRD; -C, C-terminal CRD.
25 mM sucrose or lactose. After extensive washing, the bound protein was eluted with SDS-sample buffer and immunoblotted with anti-galectin-12 antibody.

FIG. 4. Lactose binding activity of galectin-12. Lysate from Jurkat E6–1 cells transfected with either control vector (pEF1) or galectin-12-expressing construct (pEF1-Gal12) were incubated with either sucrosl- or lactosyl-Sepharose 4B, in the absence or presence of 25 mM sucrose or lactose. After extensive washing, the bound protein was eluted with SDS-sample buffer and immunoblotted with anti-galectin-12 antibody.

FIG. 5. Analysis of galectin-12 mRNA expression in various tissues. mRNA expression of galectin-12 in human tissues (A) and cell lines (B) was analyzed by RT-PCR. Mononuclear and polymorphonuclear peripheral blood cells were pooled from three normal donors. RNA was isolated, and RT-PCR was performed for galectin-12, β-actin, or glyceraldehyde-3-phosphate dehydrogenase (G3PDH), and the products were analyzed on agarose (A) or polyacrylamide gels (B).

Fig. 6. Up-regulation of galectin-12 mRNA expression by cell cycle synchronization. Jurkat cells were cultured overnight with 1 mM hydroxyurea, 0.1 mM thymidine, 0.2 mM theophylline plus 0.5 mM dibutyryl-cAMP, or 0.1 μg/ml nocodazole. Following each treatment, RNA was isolated, and RT-PCR was performed. Similar results were obtained in four experiments.

arrested at G1, we wished to determine whether the expression of the message is up-regulated under conditions that induce cell stasis. Jurkat cells were treated with hydroxyurea or thymidine to synchronize cells at the G1/S boundary (48), with theophylline plus dibutyryl-cAMP to synchronize cells at G1 (49), or with nocodazole to block cells at mitosis. As shown in Fig. 6, synchronization at G1 or G1/S boundary, but not mitosis, induced galectin-12 expression.

Cell Cycle Arrest by Ectopic Expression of Galectin-12—Changes in galectin-12 expression in response to cell cycle synchronization prompted us to test the possible function of this protein in cell cycle transition. We developed a highly efficient transfection method that can reproducibly transfect the human cervical cancer cell line HeLa with efficiencies between 80 and 90%, regardless of the DNA constructs used in the transfection (Fig. 7A), using a protocol similar to that used for cell cycle studies of the breast cancer cell line MCF-7 (53). This allowed us to perform transient transfection experiments without cotransfection of a selection marker. This is well suited for cell cycle analysis, because the effects of the transfected gene on the cell cycle need only 1–2 days to manifest.

The effect of galectin-12 on the cell cycle distribution was compared with another two-CRD galectin, galectin-9. Thus, the cells were transfected with cDNA constructs designed in such a way that the expressed proteins would be tagged with an epitope of the influenza hemagglutinin (HA). The expression levels of the two proteins after transfection could thus be compared by immunoblotting using the same antibody, mouse anti-HA clone C12A5. As shown in Fig. 7B, cells transfected with HA-galectin-9 and HA-galectin-12 expressed comparable levels of the two galectins. However, there were significant differences in cell cycle distribution in the resultant transfectants (Fig. 7C). Transfection with both 15 and 30 μg of the HA-galectin-9 construct barely altered the percentages of cells in G1, S, and G2/M. In significant contrast, transfection with 15 μg of the HA-galectin-12 construct resulted in a considerably higher percentage of cells in G1, with compensatory decreases in cells in S and G2/M. A more pronounced effect was seen when the cells were transfected with 30 μg of the HA-galectin-12 construct.

In order to demonstrate that the accumulation of cells at G1 was a result of G1 arrest rather than accelerated M to G1 transition, we employed a protocol commonly used in studies of G1 cell cycle arrest (e.g. see Ref. 53), in which nocodazole is added to the cells after transfection. This drug disrupts the formation of spindle fibers and results in a block at mitosis, thus preventing cells from recycling back to G1. Thus, 1 day after transfection with different doses of the constructs, HeLa cells were cultured in the presence of nocodazole. Under these conditions, an even more dramatic effect of galectin-12 expression on the cell cycle distribution was seen, as demonstrated by the substantial increases in the percentages of cells in G1 (Fig. 7C). There was also a minor effect at the higher dose of HA-galectin-9, but the effects of HA-galectin-12 were substantially greater.

Growth Suppression by Ectopic Expression of Galectin-12—Cell cycle arrest invariably results in cell growth suppression; therefore, the above results suggested that transfection with galectin-12 would result in retarded cell growth. To test this, we compared the growth rates of cells transfected with galectin-9 and galectin-12, respectively. In transient transfection, cells gradually lose expression of the transfected gene; thus, the procedure is not suited for long term analysis. Therefore, we cotransfected HeLa cells with a control vector, the HA-galectin-9 construct, or the HA-galectin-12 construct, together with a puromycin resistance gene, so that cells expressing the transfected genes could be selected by culturing in the presence of puromycin. The advantage of this system is that it requires only 3–5 days to eliminate all the cells that do not express the puromycin resistance gene, in contrast to another more commonly employed system, using G418 as the selection marker, which usually takes 1–2 weeks to achieve the selection. As
shown in Fig. 8, while cells transfected with the vector or HA-galectin-9 continued to grow during 5 days of culturing, those transfected with HA-galectin-12 failed to proliferate. After 5 days of selection, the number of HA-galectin-12 transfectants was about one-sixth of that of HA-galectin-9 transfectants or control vector transfectants.

**DISCUSSION**

A number of structural features of galectin-12 support the possibility that this protein is evolutionarily related to galectins. First, it contains a basic two-CRD type of structure characteristic of a subfamily of galectins, with two homologous domains separated by a linker. Second, the exon organization and the size of each exon correspond well to those of other galectins. Third, many residues of the consensus sequences found in other galectins are present in this protein, particularly in the N-terminal domain. These structural similarities, plus the observation that the full-length protein binds lactose, make galectin-12 a bona fide galectin. However, significant divergence from galectins is also evident. While the N-terminal domain of galectin-12 contains all of the elements to form a CRD, the C-terminal domain strays significantly from the consensus sequences.

Several features of galectin-12 mRNA suggest that the expression of the protein is tightly regulated. The start codon of full-length galectin-12 is predicted to be a weak initiator for translation under normal conditions. Significantly, galectin-12 mRNA carries AU-rich motifs in the 3′-untranslated region, which confer instability to mRNA (56, 57). Northern blot anal-
A summary of the document content is as follows:

**Cell Cycle Regulation by Galectin-12**

Ysis indeed showed that galectin-12 mRNA is barely detectable in many tissues tested. The results were not due to the quality of the galectin-12 cDNA probe, since strong signals were observed when mRNA from cells transfected with galectin-12 cDNA were analyzed using the same probe (data not shown). Also, we have not been able to detect galectin-12 protein in a number of cell lines by immunoblotting with an anti-peptide antibody. However, we cannot determine quantitatively the levels of the protein in these cells, because we do not know the detection limits of this antibody in immunoblot analysis.

AU-rich motifs are found in the 3′-untranslated regions of many messenger RNAs of proto-oncogenes and genes coding for nuclear transcription factors and cytokines. They represent the most common determinant of RNA instability in mammalian cells (56, 57). Messenger RNA degradation directed by these motifs is influenced by many exogenous factors, including phorbolesters, calcium ionophores, cytokines, and transcription inhibitors. The protein products of many messenger RNAs carrying these motifs have critical roles in cell growth and differentiation (56, 57). Consistent with this, several lines of evidence strongly point to the role of galectin-12 in cell cycle regulation. First, its cDNA is cloned from cells arrested at the G1 phase. Second, its expression is increased in cells undergoing cell cycle arrest. Most importantly, its ectopic expression induces cell cycle arrest as clearly demonstrated by transfection experiments (Fig. 7). The ability of galectin-12 to induce cell cycle arrest is also supported by the finding that the cells ectopically expressing galectin-12 fail to proliferate (Fig. 8).

Determination of how galectin-12 affects cell cycle progression awaits further investigation, but it may involve interactions with other intracellular proteins important for cell growth control. Preliminary data showed down-regulation of cyclin A levels and retinoblastoma protein (Rb) phosphorylation by galectin-12 by immunoblotting from lysates of galectin-12 transfectants using antibodies specific for cyclin A, Rb, and phosphorylated Rb (data not shown). Previously, we found that galectin-3 shares sequence similarity with Bcl-2 and binds Bcl-2 (19). Galectin-3-Bcl-2 interaction is inhibitory by lactose, although Bcl-2 is not glycosylated. The results suggest that galectin-3 may employ its carbohydrate-binding site to interact with intracellular proteins, by recognizing peptide sequences that are structurally similar to the carbohydrate ligands of the lectin. In the case of galectin-12, however, whether the carbohydrate-binding site is involved in its cell growth-regulatory function remains to be determined.

Galectin-12 may play an important role in host response to adverse situations through its up-regulated expression, leading to cell cycle arrest. The possible role of this protein in tumorogenesis is particularly attractive. The galectin-12 gene is found in several human chromosome 11q13 clones by BLAST search. This is a region frequently altered in many human cancers (see the Cancer Genome Anatomy Project site on the World Wide Web). Within this region lies the **CCND1** gene encoding cyclin D1, ectopic expression of which is implicated in several types of leukemia. Suppressor gene(s) in this region, however, have yet to be identified. The ability of galectin-12 to arrest cell cycle and inhibit cell proliferation of several cancer cell lines makes it a candidate tumor suppressor in chromosome 11q13. Moreover, the findings suggest that gene therapy using galectin-12 cDNA may prove to be a valuable approach for the treatment of cancers.

Recently, other investigators reported that galectin-3 regulates cell cycling (63). They found that while parental BT549 cells undergo apoptosis (anoiokis) when they are grown as suspension cultures, galectin-3-overexpressing transfectants undergo G1 arrest, without detectable cell death, in response to the loss of adhesion. The effect of galectin-3 appears to be mediated by down-regulation of G1-S cyclin levels and up-regulation of their inhibitory protein levels. Galectin-1 has also been shown to induce cell cycle arrest when added to human mammary cancer cells (64) and alter the cell cycle when added to human T lymphocytes (65). In these cases, the lectin appears to act through extracellular mechanisms by binding to cell surface proteins, which then transmit signals to the intracellular machinery, culminating in cell cycle arrest. Therefore, regulation of the cell cycle may be a common and important function of members of the galectin family. Although galectin-9 was found not to have significant cell cycle regulation activity in the cell line tested (Figs. 7 and 8), we cannot exclude the possibility that it regulates cell cycle in other cell types.

Our findings shed light on the understanding of the functions of galectins. An emerging picture is that they may play important roles in regulation of cellular homeostasis. As mentioned in the Introduction, four galectins, galectin-1, -3, -7, and -9, have been shown to either induce or inhibit apoptosis, although by different mechanisms. Also, the intracellular sites may be the primary location in which the galectins normally function. These proteins have been shown to localize intracellularly, consistent with the absence of a classical signal sequence, and some of them (galectin-1, -3, and -7) have been ascribed with important intracellular functions, such as pre-mRNA splicing and regulation of apoptosis. Interestingly, these functions may not be dependent on the carbohydrate recognition properties. It is conceivable that the ancient galectin CRD is utilized as a template in evolution to generate many galectin-related proteins in which the carbohydrate recognition motif is adapted for protein-protein interactions.

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