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Human recombinant galectin-1 as a potential growth modulator

Cristina S. Weinberg

A thesis submitted in fulfilment of
the requirements for the degree of Doctor of Philosophy in Biochemistry
University of Auckland

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ABSTRACT

Human galectin-1 is a soluble form of lectin known to play a role in various cellular processes by mediating recognition events in which glycoconjugates are implicated.

As a number of studies have shown that galectin-1 is a growth inhibitor (Wells and Mallucci, 1991; Manilal et al., 1993), the starting hypothesis for this thesis was that galectin-1 might be a substrate for a growth-related proteinase (GRP). The intention was to identify the mechanism of action responsible for this growth-inhibitory property by looking at the effect of galectin treatment on the expression of the c-fos proto-oncogene.

Galectin-1 was synthesised as a recombinant fusion protein expressed in E.coli. For this purpose the published sequence of galectin-1 (Abbot and Feizi, 1989) was used to construct primers for a polymerase chain reaction. The template in this reaction was cDNA obtained through a reverse transcription reaction from osteosarcoma RNA. Ligation into a pGEX vector yielded a glutathione S-transferase fusion protein, which was purified from the bacterial cell lysate by affinity chromatography on glutathione-agarose. Galectin-1 was released by thrombin cleavage and purified through asialofetuin-agarose affinity chromatography.

The structure of the recombinant galectin-1 molecule was investigated in mass spectrometry determinations. Galectin-1 was further characterised in hemagglutination, cellular growth, cytotoxicity, proteolysis and cellular degradation experiments. Even though the recombinant galectin-1 was not identical with the natural protein because it contained two pGEX-linker amino acid residues, and had an apparently 933 Da bigger molecular weight, it fully retained the carbohydrate binding and mitogenicity properties and was still a biphasic growth modulator.
Repeated DNA sequencing and mass spectrometry determinations of the tryptic peptides have accounted for all the galectin molecule and have not detected an insertion. We concluded that the abnormal size was the result of a calibration error in the mass spectrometer.

Even though the recombinant galectin was proved to be very susceptible to soluble proteinase action, there was no evidence for its active degradation when incubated with cells, and this disproved the original hypothesis.

We showed for the first time that $\alpha_1$-antitrypsin inhibitor (which inhibits the GRP) had a down-regulatory effect on c-fos expression. Galectin-1 treatment of U2OS and HELE cells had a downregulatory effect on c-fos expression, which confirmed the hypothesis that this proto-oncogene is affected by the signal transduction pathway through which galectin-1 inhibits cell growth. C-fos expression is affected in HELE cells even though they do not undergo growth inhibition, indicating that this process is not as simple as we initially believed. Galectin-1 treatment also downregulated galectin-1 gene expression. This meant that feedback inhibition could take place in these tumour cells.
ACKNOWLEDGEMENTS

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<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>Amp</td>
<td>ampicillin</td>
</tr>
<tr>
<td>ATP</td>
<td>adenosine 5'-triphosphate</td>
</tr>
<tr>
<td>α1AT</td>
<td>α1 antitrypsin</td>
</tr>
<tr>
<td>α-CHC</td>
<td>α-cyano-4-hydroxycinnamic acid</td>
</tr>
<tr>
<td>BBI</td>
<td>Bowman-Birk soybean proteinase inhibitor</td>
</tr>
<tr>
<td>BCE</td>
<td>bovine corneal endothelial cells</td>
</tr>
<tr>
<td>BSA</td>
<td>bovine serum albumin</td>
</tr>
<tr>
<td>CAPS</td>
<td>3-[cyclohexylamino]-1-propanesulphonic acid</td>
</tr>
<tr>
<td>cDNA</td>
<td>complementary deoxyribonucleic acid</td>
</tr>
<tr>
<td>CHO</td>
<td>Chinese hamster ovary cells</td>
</tr>
<tr>
<td>CIAP</td>
<td>calf intestinal alkaline phosphatase</td>
</tr>
<tr>
<td>CRD</td>
<td>carbohydrate-recognition domains</td>
</tr>
<tr>
<td>CytC</td>
<td>cytochrome C</td>
</tr>
<tr>
<td>DHB</td>
<td>2,5-dihydroxybenzoic acid</td>
</tr>
<tr>
<td>DMEM</td>
<td>Dulbecco modified Eagle’s medium</td>
</tr>
<tr>
<td>DMEMS</td>
<td>Dulbecco modified Eagle’s medium containing 10% foetal calf serum</td>
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<td>deoxynucleotidetriphosphates</td>
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<tr>
<td>DTT</td>
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<td>EDTA</td>
<td>ethylenediamine-tetraacetic acid</td>
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<td>epidermal growth factor</td>
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<td>foetal calf serum</td>
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<td>FP</td>
<td>fusion protein</td>
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<tr>
<td>GBP</td>
<td>β-galactosyl binding protein</td>
</tr>
<tr>
<td>GRP</td>
<td>growth-related proteinase</td>
</tr>
<tr>
<td>Abbr.</td>
<td>Description</td>
</tr>
<tr>
<td>-------</td>
<td>-------------</td>
</tr>
<tr>
<td>GST</td>
<td>glutathione S-transferase</td>
</tr>
<tr>
<td>Hep2</td>
<td>human hepatoma cell line</td>
</tr>
<tr>
<td>HEla</td>
<td>human epidermal carcinoma cell line</td>
</tr>
<tr>
<td>HPLC</td>
<td>high performance liquid chromatography</td>
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<tr>
<td>IEGs</td>
<td>immediate early genes</td>
</tr>
<tr>
<td>IL-2</td>
<td>interleukine-2</td>
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<tr>
<td>IPTG</td>
<td>isopropyl β-D-thiogalactopyranoside</td>
</tr>
<tr>
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<tr>
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<tr>
<td>LAGs</td>
<td>lactosaminoglycans</td>
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<td>LAMPS</td>
<td>lysosomal associated membrane proteins</td>
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<tr>
<td>LB</td>
<td>Luria Bertani broth</td>
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<tr>
<td>LDH</td>
<td>lactate dehidrogenase</td>
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<tr>
<td>LSIMS</td>
<td>liquid secondary ion mass spectrometry</td>
</tr>
<tr>
<td>M</td>
<td>molar</td>
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<tr>
<td>MALDI-TOF MS</td>
<td>matrix assisted laser desorption/ionisation time-of-flight mass spectrometry</td>
</tr>
<tr>
<td>mGBP</td>
<td>mouse β-galactosil binding protein</td>
</tr>
<tr>
<td>MPS</td>
<td>Malcolm Paslow's skin cells</td>
</tr>
<tr>
<td>MTT</td>
<td>tetrasodium salt</td>
</tr>
<tr>
<td>MW</td>
<td>molecular weight</td>
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<tr>
<td>N-terminal</td>
<td>amino terminal</td>
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<tr>
<td>°C</td>
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<tr>
<td>p.s.i.</td>
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<tr>
<td>PAGE</td>
<td>polyacrylamide gel electrophoresis</td>
</tr>
<tr>
<td>PBS</td>
<td>phosphate-buffered saline</td>
</tr>
<tr>
<td>PCR</td>
<td>polymerase chain reaction</td>
</tr>
<tr>
<td>PEG</td>
<td>polyethylene-glycol</td>
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<tr>
<td>PLAG</td>
<td>polylactosaminoglycan</td>
</tr>
<tr>
<td>PMSF</td>
<td>phenylmethylsulfonly fluoride</td>
</tr>
<tr>
<td>PSTI</td>
<td>pancreatic secretory trypsin inhibitor</td>
</tr>
<tr>
<td>RP</td>
<td>reverse phase</td>
</tr>
<tr>
<td>RNA</td>
<td>ribonucleic acid</td>
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</table>
r.p.m. revolutions per minute
SBTI soybean trypsin inhibitor
SDS sodium dodecyl sulphate
SDS-PAGE sodium dodecyl sulphate-polyacrylamide gel electrophoresis
SMC smooth muscle cells
TAE tris acetate EDTA buffer
TE tris-EDTA buffer
TGFβ transforming growth factor β
TNF tumour necrosis factor
Tris tris (hydroxymethyl) aminomethane
U2OS human osteosarcoma cell line.
UTI urinary trypsin inhibitor
V voltage
v/v volume per volume
w/v weight per volume
w/w weight per weight
X-gal 5-bromo-4-chloro-3-indolyl-β-D-galacto-pyranoside
Chapter 1

Introduction
Chapter 1: Introduction

1.1 PROTEINASES

Proteinases have been the subject of investigation for many years. They are involved in varied cellular processes such as growth control, cell contact inhibition, metastasis, intracellular protein regulation, hormone activation and inactivation, pre-peptide processing and general metabolic control. This introduction discusses some of the properties and classification of proteinases, then reviews some of the more recent evidence for proteinase involvement in growth control and proliferation of animal cells in culture.

1.1.1. Classification

The proteinases are classified by the type of activity they exert as endopeptidases and exopeptidases. The endopeptidases can be further classified by the type of active site as serine proteinases, thiol proteinases (cysteine residue), aspartic proteinases or metalloproteinases (Zn or Ca in the active site; Barett, 1986).

Exogenous and endogenous growth-related proteinases have been identified. Exogenous growth-related proteinases are the proteinases secreted by cells, or the proteinases added in the medium of cells in culture. Trypsin and other proteinases from this category are mitogenic, stimulating DNA synthesis and cell division in quiescent, confluent mouse fibroblasts (Burger, 1970; Sefton and Rubin, 1970).

Thrombin has been shown to be an effective mitogenic proteinase (Chen and Buchanan, 1975). Through proteolytic cleavage of the thrombin receptor which results in conformational change and receptor activation, thrombin stimulates the hydrolysis of phosphatidylinositol-4,5-bisphosphate to produce the intracellular second-messengers inositol triphosphate and diacylglycerol, known to have a growth-promoting role
1. Introduction

(Stiemberg et al., 1983; Pouyssegur et al., 1985). There is some evidence for the existence of other thrombin-mediated signalling systems, like the one using cyclic AMP as a second messenger (Kavanaugh et al., 1988).

1.1.2 Endogenous growth-related proteinases

A correlation between growth-rate and cell surface neutral proteinase activity was shown for several human cell cultures (Hatcher et al., 1976). The activity was also shown to increase prior to mitosis in synchronised cultures. Antibodies to a human cell-surface proteinase, the so-called growth-related proteinase (GRP), inhibited DNA synthesis and cell proliferation in fibroblasts, leucocytes and some human tumour cell cultures (Pitts and Scott, 1983; Fraser and Scott, 1984; Scott and Seow, 1985). The inhibition of the GRP blocks the mitogenic stimulus of different peptide growth factors, but not that of exogenous calcium (Scott et al., 1989).

The plasminogen activators (urokinase and tissue plasminogen activator) are enzymes that seem not to be required for cell growth because antibodies to plasminogen activators did not inhibit the in vitro growth of the cells, but prevented metastasis of the primary tumour in vivo (Ossowski and Reich, 1983). In this view, these enzymes may be part of a proteolytic cascade which results in degradation of extracellular matrices and promotes tumour invasion (He et al., 1989). Urokinase proved to have a growth inhibitory effect on the cells of the promyelocytic leukemia line HL-60 (Howell et al., 1994).

1.1.3 Proteinase inhibitors as growth inhibitors

The growth inhibitory effect that the addition of proteinase inhibitors has on cell cultures is a proof of the implication of proteinases in growth regulation through inhibition of intracellular proteolysis. This may indirectly influence proliferation by inhibition of protein turn-over or of protein processing (Scott, 1987). Specific growth-related intracellular proteinases have been identified (Billings et al., 1987; Wong et al., 1987; Bories et al., 1989). The same conclusion that the inhibition of pericellular proteinases relates to inhibition of cell proliferation resulted from studies with proteinase inhibitors which are proteins of high-molecular-weight (Schnebli, 1975). It has been shown that soybean trypsin inhibitor (SBTI) and human $\alpha_1$– antitrypsin ($\alpha_1$AT) inhibit both GRP and cellular
1. Introduction

growth. There has been interest in using this research in the inhibition of tumour growth (Troll et al., 1970), and there has been some success in demonstrating (in animal systems) the inhibitory effect of the Bowman-Birk soybean proteinase inhibitor on chemically-induced tumour proliferation (Weed et al., 1985), but the mechanism(s) by which proteinase inhibitors suppress carcinogenesis is still unclear.

Yavelow et al. (1987 a, b) have suggested that enzymes similar to GRP act as extracellular membrane receptors for the binding and internalization of proteinase inhibitors. This mechanism has features in common with the autocrine action of some growth factors, but as the proteinase is not secreted in a soluble form, the word "pericrine" could be used to indicate the percellular localisation of the effector enzyme. If this hypothesis is proved to be correct, there is a need to search for intracellular proteinase substrates with signalling function.

Intracellular events following proteinase inhibition have been identified. The Bowman-Birk inhibitor has been shown to inhibit expression of the c-fos oncogene in transformed mouse fibroblasts. This effect is specific, it is not a generalised inhibition of transcription, and indicates that proteinases and their inhibitors can affect genes implicated in the regulation of cell proliferation (Caggana and Kennedy, 1989).

The effects of proteinase inhibitors on proto-oncogene expression brings us back to the consideration of their effects on tumour cell proliferation. The report that anti-GRP antibodies inhibited cell growth of some tumour cells or transformed cells and had no effect on others (Pitts and Scott, 1983) has been supported by the fact that α1-antitrypsin was also shown to inhibit the growth of some human tumour lines, but not others (Scott, 1988; Scott and Tse, 1992). It may be possible to conclude from such experiments that the expression of some oncogenes (but not all) may override the effect of GRP inhibition, for example the U2OS osteosarcoma cell line which expresses the v-sis oncogene (Weich et al., 1986) is still sensitive to GRP inhibition (Scott and Tse, 1992). The loss of sensitivity to growth inhibition by proteinase inhibitors has been investigated in many tumour cells and in these circumstances, proteinase inhibitors produced and secreted by tumour cells may act as autocrine growth stimulators (see Section 1.1.5), or may serve to protect cells from secreted proteinases involved in invasion and metastasis (Scott, 1992).
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1.1.4 Possible mechanisms of action of growth-related proteinases

Except for the effects on proto-oncogenes by a putative intracellular enzyme, and the previously-mentioned stimulations of phosphoinositide and cyclic AMP metabolism by thrombin, there is not much known about the mechanism by which proteinases could stimulate cell growth (Scott, 1987).

Inhibition of GRP blocks the mitogenic action of epidermal growth factor (EGF), but binding and internalisation of EGF are not affected (Scott and Seow, 1985). Proteolytic activation of protein kinase C has been demonstrated, but depends upon cytoplasmic or cytoskeletal proteinases (Tapley and Murray, 1984; Pontremoli et al., 1990). This requirement could be met by re-internalisation of a "pericrine" proteinase (see Section 1.1.3), but it has been shown that the inhibition of GRP has no effect on protein kinase C activity in human fibroblasts (Borich et al., 1994).

The mitogenic action of urokinase on human renal cells may involve protein kinase C because down-regulation or inhibition of this enzyme inhibits the mitogenic effect (He et al., 1991). Dumler et al. (1994) have shown that the binding of urokinase to its receptor induces the rapid and transient expression of the c-fos proto-oncogene in OC-7 ovarian carcinoma cells. This signal generates protein tyrosine kinase activity and through a signal transduction pathway activates nuclear transcription factors. These observations and others regarding the growth inhibitory effect of these proteinases mentioned in Section 1.1.2. show that urokinase can have quite opposite effects on different types of cells. It can either promote growth inhibition or growth stimulation.

A possibility that has not been yet experimentally tested, is that an autocrine growth inhibitor could be continually degraded by a growth-related extracellular proteinase, but would accumulate if the enzyme were inhibited. The galactose-binding protein identified as a labile, negative growth regulator in mouse fibroblasts (Wells and Mallucci, 1991), has been found in humans (galectin-1) (Couraud et al., 1989). The GRP levels decline in confluent fibroblasts (Harper et al., 1984, Manilal et al., 1993); if the galactose-binding protein is a substrate for this enzyme, then it could maybe accumulate in confluent cultures. Manilal et al. (1993) also showed that the treatment of cells with a proteinase inhibitor results in the additional accumulation of galectin-1 in the medium.
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1.1.5 Proteinase inhibitors as mitogens

There are reports that rat, human and ovine pancreatic secretory trypsin inhibitors (PSTIs), UTI, proteinase inhibitors from human hepatoma cells and other proteinase inhibitors could act as mitogens (Fukuoka et al., 1986; McKeenan et al., 1986; Scott and Tse, 1988; Hamilton et al., 1990). Two human endothelial cell growth factors from hepatoma cells are tumour-associated proteinase inhibitors: ECGF-2α and ECGF-2β inhibited bovine pancreatic trypsin at a concentration of 750 ng/ml and the same growth factors stimulated endothelial cells growth at 50, 80 and 130 ng/ml. These were shown to be PSTI and UTI, respectively (McKeenan et al., 1986).

The antimitogenic action of a specific inhibitor is maximal at approximately the same concentration at which GRP inhibition is maximal. In the case of PSTI, the mitogenic effect is seen at much lower concentrations at which inhibition of the GRP is not detectable. However for other proteinase inhibitors the two effects take place in a narrower concentration range. This kind of evidence suggests that when proteinase inhibitors act as mitogens, they bind to receptor(s), which is, or are, distinct from the growth-related proteinases so far identified (Scott, 1992).

1.2 GALECTINS

1.2.1 Introduction

For normal development, cell growth and differentiation, there is a need for various types of cellular interactions amongst cells, and between cells and exogenous soluble and insoluble macromolecules. One class of molecules that plays a role in these processes by mediating recognition events are the cell surface glycoconjugates. These are present predominantly in membranes (Paulson, 1989; Sharon and Lis, 1989). They act as receptors for growth factors, enzymes and hormones and are recognised as markers of cell differentiation, development, pathological states and as antigenic determinants (Paulson, 1989; Rutishauser and Jessel, 1988; Sharon and Lis, 1989).
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As opposed to the peptides and oligonucleotides where the information content is based only on the number of monomeric units and their sequence, in carbohydrates information is also encoded in the position and anomeric configuration of the glycosidic units and in the occurrence of branch points. This suggests that the major function of the carbohydrates in glycoconjugates is to encode biological information in the form of three-dimensional structures (Drickamer, 1988).

One way in which information in oligosaccharide structures can be recognised or "decoded" is through the binding of lectins, each of which interacts with a selected subset of these structures (Sharon and Lis, 1989). The list of lectin-mediated processes already includes diverse biological phenomena, from intracellular routing of glycoproteins to cell-cell adhesion and phagocytosis (Drickamer, 1994).

1.2.2 Definition and History

Lectins are carbohydrate-binding proteins that were originally identified in plant extracts more than 40 years ago. Lectins were originally defined as multivalent, nonenzymatic and nonimmune carbohydrate-binding proteins operationally defined by their ability to agglutinate cells bearing appropriate saccharide structures (Sharon and Lis, 1972).

A more recent definition includes proteins with a single carbohydrate-binding site (Barondes, 1988). The presence of a lectin-like protein in vertebrate liver was described by Ashwell and Morell more than 20 years ago. This was an integral membrane protein having a physiological role in binding and endocytosis of circulating asialo-glycoproteins by liver hepatocytes. Another lectin was identified in the electric organ of the electric eel by Teichberg et al (1975). Since then, lectins have been found to constitute a family of proteins that are widely distributed in different species starting with plants and ending with humans.

1.2.3 Classification of animal lectins

Animal lectins can be grouped into classes based on a number of properties: the nature of their saccharide ligands, the type of biological processes in which they are involved,
their subcellular localisation, their physical properties such as solubility in water, or their dependence on metal co-factors. The primary structures of fifty animal lectins are known and this makes possible a classification based on shared sequence characteristics. While the overall structures of the lectins vary, the carbohydrate-binding activity can be confined to just a limited portion of a lectin. This segment is designated the carbohydrate-recognition domain (CRD) (Drickamer, 1988).

Using the comparison of the sequences of the CRDs, lectins can be classified into three groups: P, C and S. The C-type lectins owe their name to the fact that they require calcium ions for activity. They bind various ligands. The S-type lectins are named this way because they are often dependent on reducing agents for full activity. Because they share the ability to bind β-galactosides, they have been alternatively designated S-Lac lectins. They are soluble proteins since they can be isolated from cells and tissues without the use of detergents. It has been recently agreed that the S-type lectins should be called galectins (Barondes et al., 1994). The P-type lectins are called also pentraxins, and they bind mannose 6-phosphate as their primary ligand.

1.2.4 Further classification of galectins

The family of galectins contains galectin-1, galectin-2, galectin-3 and galectin-4. In addition to these four well characterised mammalian galectins, four related mammalian proteins have been identified and named tentatively galectin-5,-6,-7 and -8 (Barondes et al., 1994).

Galectin-1 (or L-14-I) is of similar structure with galectin-1. Galectin-1 was found in human placenta, bovine spleen, rat intestine, mouse skeletal muscle, chicken cardiac muscle, smooth muscle, lung, liver, kidney skin, brain, motor neurones, sensory neurones, thymus, bone marrow, fibroblasts (Barondes et al., 1994).

Galectin-2 (or L-14-II) is a noncovalent homodimer of 2 subunits of approximately 130 amino acids of CRD folded as one compact globular domain. Galectin-2 was found in the gastro-intestinal tract and is abundant in the lower small intestine (Barondes et al., 1994).

Galectin-3 (or L-29) has at the C-terminal a subunit of 130 amino acids of CRD and at the N-terminal a Pro/Gly/Tyr-rich repeating domain. Galectin-3 was found in the human
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lung, in dog brain, in rat fibroblasts, and in mouse macrophages, lymphocytes, basophils and mast cells (Barondes et al., 1994).

Galectin-4 (or L-36) has two carbohydrate-binding domains in the same peptide chain. Each domain is about 40% identical to the other and to L-29. Galectin-4, like galectin-2 is restricted to the gastrointestinal tract (Barondes et al., 1994).

Fig. 1.1. Schematic of the overall structures of galectin-1,-2,-3, and -4 (reproduced from Barondes et al., 1994). The proteins are shown schematically as linear diagrams corresponding to single peptide chains (top) and as assembled proteins (bottom).

The amino acid identity in the carbohydrate-binding domains amongst different known galectins from one mammalian species ranges from about 20 to 40 %, while the identity of the same galectin from different mammalian species is 80-90 % (Barondes et al., 1994).

1.3 GALECTIN-1

The crystal structure of bovine galectin-1 isolated from spleen has been determined in a complex with N-acetyllactosamine (LacNAc) at 1.9 Å resolution (Liao et al., 1994). Galectin-1 has been shown to have a similar molecular structure to legume lectins, a structure consisting of a β-sandwich fold with a "jelly-roll" topology (Bourne et al., 1994). The molecular weight of galectin-1 is 14 584 Da. The galectin-1 dimer forms a 22-strand
anti-parallel β-sandwich, with the N and C termini of each monomer at the dimer interface. The structure reveals that there is one carbohydrate-binding site per monomer. The integrity of the dimer is maintained by the β-sheet interactions across the monomers and by formation of a hydrophobic core common to both (Liao et al., 1994).

### 1.3.1 Galectin-1 gene. Galectin-1 amino acid sequence

Couraud et al. (1989) have revised the amino acid sequence for a 14 kDa lectin they have isolated from human placenta which is identical (with the exception of a Leu/Tyr polymorphism at positions 104 and 119) with the sequence reported by Abbot and Feizi in HepG2 cells and in HL60 cells in 1989. The conclusion of these studies was that there is a single gene coding for galectin-1.

Gitt and Barondes (1991) reported the nucleotide sequence of the human galectin-1 gene including all exons and introns and 250 base pairs of the upstream region. This sequence is the one from the genomic DNA encoding the human galectin-1 (Fig. 1.2, p 11).

The gene for galectin-1 consists of four exons splitting the regions of the coding sequence into several domains. The genomic sequence shows no evidence of a secretory signal peptide. The conserved residues mentioned in Section 1.3.2. are encoded by a single exon (exon III) (Gitt and Barondes, 1991). Alu sequences in the introns surrounding exon 3 and their known role in in vivo recombination suggest the possible use of the exon 3 domain as a functional unit for glycoconjugate binding (Gitt and Barondes, 1991).

The upstream region of the galectin-1 gene suggests that it may be regulated in many ways. At position -130 there is a putative heat shock element diverging from the consensus (CNNGAANNNTCNNG) by one nucleotide, but the presence of a heat-shock consensus sequence may not be sufficient for heat-shock regulated expression. Many heat-shock regulated genes display also developmental expression independent of heat-shock stimuli (Gitt and Barondes, 1991).

A putative steroid-binding site is located at position -210 and has the consensus sequence (T/C GG T A/T C A/A/T N T G T T/C C T). There is evidence for glucocorticoid regulation of synthesis of rat galectin-1 (Clerch et al., 1988). There is also a sequence related to the Y box of histocompatibility genes overlapping the putative steroid-binding site. It has the consensus sequence TTCTGATTGTTAC of which the galectin-1 gene
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shares 12 of 14 nucleotides. The possible relation of the galectin-1 with the histocompatibility system is of interest since lectins have been implicated in a primitive defence system by binding surface glycoconjugates of invading organisms, facilitating phagocytosis.

The presence of intronic Alu sequences and the possibility of downstream Z-DNA suggested by a G/T cluster downstream of the polyadenylation signal, may also add other levels of regulation (Gitt and Barondes, 1991). Intron I includes some small direct repeats which may be targets for binding cellular factors. The same intron contains several Spl binding site consensus sequences that can play a role in regulation even downstream of the transcriptional initiation site. Sodium butyrate (2 mM) was the only differentiation inducing agent that could induce both differentiation and expression of galectin-1 in the KM12c human colon carcinoma (HCC) cells (Ohannesian et al., 1994). It is possible that sodium butyrate affects galectin-1 expression through a different regulatory element than the one mentioned before.

These mechanisms of regulation are consistent with observations that the role of this lectin may differ in developing and mature tissues and in the many cell types in which it is expressed (Gitt and Barondes, 1991).
Figure 1.2. Nucleotide and derived amino acid sequence of the cDNA of human β-galactoside-binding lectin from HepG2 cells (reproduced from Abbot and Feizi, 1989)

The nucleotide sequence is numbered with the adenine of the initiating methionine designated as "1". The derived amino acid sequence is numbered with the first alanine residue designated "1". The general designation of the genes encoding galectins is LGALS (lectin, galactoside-binding, soluble), and gene numbering is kept consistent with the numbering of the proteins. The gene encoding galectin-1 is designated in the Genome Data Base as LGALS1 (Barondes et al., 1994). GeneBank/EMBL accession numbers for the sequence of the human galectin-1 are J04456, J0503, X14829 (Barondes et al., 1994). In humans LGALS1 has been mapped to the q12-q13 region of chromosome 22 (Mehraban et al., 1993).

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GGGC
-40
TGACAGCCTGCTGGCCCTGCCCCGCAGAATCACCTCTCTGGGACTCAATC
45
ATGGCCTTCTGGCTGGCTGCCAGCAACCTGAATCTTAAACCTGGA
MACGLVNASNLLNKLPC
90
CAGTCCTCTCAGGGAGGGAGGTGGCTCTGACGCTAACAGG
ECLREVGRGEPADKS
135
TTGCTGCTGAACCTGGCAAGACAGCAACAACCTGTGGCTGCAC
FVLNLGKDSDNNLCLR
30
180
TTCAACCCCTGGCTTTCAACGGGCCCACGGGACGCAACCAACCATGCTG
FNPFRFNAHGDANTIV
225
TGCAACACGGAAGGACGCCGCGGCTGGGCGGCCAGGAGGCGCCGGAG
CNSKDKGGAWGEQRE
60
270
GCTGCTTTCTTCCTCCAGCTGGAATGTTGGCAAGGGGTGTGCATC
AVFPFQPGSVAEVC
315
ACCTGGCAAGGCAAGGGCCACCTGACCTGAAGCTGGCCAGTTGATAC
TFDQANLTVKLPDGY
90
360
GAATTCAGGCTCCACGGCTGCAACCTGGGACGGCCATCAACTAC
EFKFPNRLNLEAINY
405
ATGCGAGCTGACGGTGACTCAAGATCAAAAGTTGGCCTCTCAAC
MAADGFKIKKCVAFFD
L20
TGAAATCGCAGCCCATGGCCCCAAATTAAAGGCACGTGCCCTTG
***
C6C6CG
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1.3.2 Carbohydrate-Binding-Site Interactions

Lectins bind carbohydrates specifically and noncovalently. Most amino acid residues involved in sugar binding are invariant in all sequences of S-lectins. The binding site depression is shaped to complement the galactose moiety with extensive van der Waals contacts and a network of electrostatic interactions.

An average of fifteen water molecules surround each saccharide and amongst them, ten mediate interactions between the sugar and galectin. The axial 4-OH of galactose forms two key electrostatic interactions; one with the N atom of Arg-48 and the other with the N atom of His 44 (both are invariant residues in the sequences of S-lectins). A network of three salt bridges formed between Arg 48, Asp 54, Arg 73 and Glu 71 contributes to the optimal side-chain conformations (Liao et al., 1994). Other hydrogen bonds, that are proved by site-directed mutagenesis to be essential for binding, are Asn 46, Glu 71 and Arg 73 (Hirabayashi and Kasai, 1991).

There seems to be a direct correlation between the degree of conservation of amino acid residues in galectins from different species and their contribution to carbohydrate-binding. In the galectin family the amino acid residues 3, 5, 14, 21, 30, 33, 35, 41, 43, 44, 46-49, 51-54, 59, 61, 68, 69, 74, 77-79, 82, 100-103, 108-110 are conserved. These include the invariant HFNPRF (44-49) and WGTEQRE (68-74) residues involved in galactoside-binding.

The substitution of the highly conserved Trp 68 to tyrosine slightly reduced lactose binding ability, but the mutant human galectin could still be separated on asialo-fetuin-agarose (Hirabayashi and Kasai, 1991). The substitution of Trp 68 with phenylalanine resulted in a considerable reduction of sugar binding activity and substitution with leucine almost abolished binding in the mutant bovine galectin-1 analysed by Abbot and Feizi (1991).

The conclusion from these mutagenesis experiments might be that Trp 68 residue of human galectin-1 contributes to the stabilisation of the sugar binding through van der Waals interactions because the aromatic side chain of Trp 68 stacks adjacent to the galactose ring. Other mutant galectins in which the conserved hydrophilic amino acids Asn 46, Glu 71 and Arg 73 were substituted to respectively Asp, Gln and His, failed to bind to asialofetuin agarose. These hydrophilic residues are important for the sugar binding site because they can form hydrogen bonds with hydroxyl groups of specific
carbohydrate chains, directly or indirectly through a water molecule (Hirabayashi and Kasai, 1991).

The conserved hydrophilic residues proved to be more important in carbohydrate recognition than the cysteine and tryptophan residues. This is contrary to the widely accepted concept that the latter residues are essential (Hirabayashi and Kasai, 1991). Abbot and Feizi (1991) have expressed several mutants of the bovine galectin-1 in order to gather information about the functional importance of some of its highly conserved amino acids and about the polypeptide length required to form the carbohydrate recognition domain. Six mutants have been created deleting the following amino acids: 1-9, 1-23, 88-122, 88-134, 107-134 or 124-134. Also a frameshift mutant has been made in which the 23 amino acids at the C-terminal were completely changed. Only mutant 1-9 retained carbohydrate-binding, although this was impaired. The results of these experiments suggested that almost the complete polypeptide chain of the galectin-1 was necessary for the correct folding of the carbohydrate recognition domain.

1.3.3 Environment of thiol groups and their role in the oxidative inactivation of galectin-1

Cysteine residues have been considered important because galectins usually lose their sugar-binding activity under nonreducing conditions and addition of thiol reagent protects this activity. The cysteine residues are not highly conserved, except for Cys 60, and this might be also an indication that they have no significance in the sugar binding function (Hirabayashi and Kasai, 1991). None of the existing thiol groups are involved in sugar binding, and all thiol groups bind heavy atoms without any impairment of sugar binding.

The only cysteine close to the binding site is Cys 60 and its main-chain atoms are involved in the formation of the active-site depression. It seems that the thiol group oxidation is less likely to directly damage the carbohydrate-binding site, but the formation of unfavourable intra- or intersubunit disulfide bonds might result in the alteration of the native lectin conformation. Thus the role of the thiol-reducing agent, which is added in order to stabilise the lectins, seems to be not for maintaining the key thiol group in a reduced state, but for protection against the destruction of the native conformation of the protein by random formation of disulfide bonds (Hirabayashi and Kasai, 1991). This hypothesis was confirmed by the analyses of the reduced and oxidised forms of the
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purified bovine lectin by electro-spray ionisation-mass spectrometry (ESI-MS) and by liquid secondary ion mass spectrometry (LSIMS) done by Tracey et al. (1992). The results of LSIMS analyses of tryptic and peptic peptides in conjunction with Edman sequencing indicate that disulfide bonding occurs predominantly between Cys 2 and Cys 130, Cys 16 and Cys 88, and Cys 42 and Cys 60.

1.3.4 Physicochemical properties

Galectins-1 are soluble proteins and they can be solubilised in the presence of detergents when they bind to the carbohydrate portions of tightly bound membrane components (glycoproteins, glycolipids or proteoglycans) (Drickamer, 1988).

Studies of galectin-1 purified from different sources indicate that these proteins can be divided into two or more molecular species by isoelectric focusing. Their pl values range from 4.6 to 5.8 (Briles et al., 1979; Clerch et al., 1988; Sparrow et al., 1987). As there is no evidence for post-translational modifications of these galectins (other than N-terminal methionine cleavage and acetylation; Barondes et al., 1994), the reason for the existence of molecules with different isoelectric points remains unknown.

The majority of the galectins-1 are dimeric under non-denaturing conditions (Briles et al., 1979; Clerch et al., 1988; Levi and Teichberg, 1981; Whitney et al., 1986), but monomeric lectins have been found in chicken intestine (Beyer et al., 1980), and 3T3 fibroblasts (Roff and Wang, 1983).

1.3.5 Carbohydrate-binding specificity

The binding activity of the galectins was investigated in most cases by agglutination of trypsinised rabbit erythrocytes, or by binding of radiolabelled galectin to erythrocytes or to asialofetuin. These analyses revealed that: 1) galectin has a combined site at least as large as a disaccharide, 2) the disaccharides having non-reducing terminal β-galactosyl residues linked (1,3),(1,4) and (1,6) to Glc or GlcNAc are better inhibitors of galectin binding than free Gal (Ahmed et al., 1990).

Galectins have overlapping saccharide specificities. They can all be isolated from cells lysates by affinity chromatography on immobilised lactose or asialofetuin-Sepharose and
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their hemagglutination activity is inhibitable by lactose. Galectin-1 binds preferentially disaccharides (lactose and its derivatives) and oligosaccharides that contain terminal non-reducing β-galactose residues rather than monosaccharides (galactose and its glycosides) (DeWaard et al., 1976). Investigations of carbohydrate recognition by human lung lectin (Sparrow et al., 1987) showed that lactose interacts with galectin-1 through the hydroxyl groups at positions C4 and C6 of Gal and position 3 of Glc. The major interaction is with the galactose residue in lactose, but the 100-fold higher activity for lactose compared with galactose shows that the interaction with the glucose residue in lactose is also important (Leffler and Barondes, 1986).

Although the galectin did not recognise N-acetylgalactosamine as a monosaccharide, the presence of this sugar in Gal β1-4- and Gal β1-3-linked disaccharides increased the binding affinity 500-fold. Galectins tolerate substitution at the 3’ position of LacNAc (position 3 on Gal), so the poly(LacNAc) type glycans are binding tighter than LacNAc itself (Sparrow et al., 1987; Merkle and Cummings, 1988; Leffler and Barondes, 1986). Galectins are multivalent by their capacity to form dimers (Drickamer, 1988).

The monomers of galectin-1 associate to form an extended β-sandwich and such an extended site might accommodate consecutive N-acetyllactosamine residues as found in poly(LacNAc) glycans (Barondes et al., 1994) which are the major components of polyactosaminoglycan chains, PLAG or LAGs (Merkle and Cummings, 1988). Laminin and lysosomal associated membrane proteins (LAMPS) are the major carriers of LAGs and bind specifically galectin-1 (Zhou and Cummings, 1990; Do et al., 1990). Laminin contains an epidermal growth factor-like motif and can stimulate DNA synthesis and cell growth in fibroblasts possessing the EGF receptor (Lin and Bertics, 1995).

1.3.6 Localisation of galectin-1

Wilson et al. (1989) have found that the lectin mRNA is translated on free ribosomes and not on endoplasmic reticulum-bound ribosomes (the site of synthesis of membrane-associated and secreted molecules). Immunocytochemical studies have not localised the lectin to the Golgi apparatus or in cytoplasmic vesicles. There is evidence that these galectins are externalised by non-classical secretory mechanisms (Cooper and Barondes, 1990). Non-classical secretion of galectin-1 has been studied in skeletal muscle, where the protein moves from a diffusely intracellular location to an extracellular
location during \textit{in vivo} development (Harrison, 1991; Cooper and Barondes, 1990). In
cultured myoblasts, galectin remains in the cytosol until it is externalised during
differentiation by membrane evagination (ectocytosis) (Cooper and Barondes, 1990).

This process has however not been shown to occur \textit{in vivo}. In other cell types galectin-
1 is also distributed diffusely throughout the cytoplasm and it is notable that galectin-1
shares many molecular characteristics with other secretory proteins lacking N-terminal
hydrophobic sequences (Muesch \textit{et al.}, 1990). These proteins are also not glycosylated
and they appear to be present at high concentrations in the cytosol, not secreted by all
cell types and they are probably released slowly. In this family are cytokines, interleukin-
1, the fibroblast growth-factors and the lipocortins, all of which exert profound effects
extracellularly (Harrison, 1991).

The presence of the galectin-1 in the extracellular medium has proved controversial
because it has been suggested that this protein might be inactivated under the oxidising
conditions outside the cell. Cho and Cummings (1995) showed that 50\% of the galectin-1
synthesised by the chinese hamster ovary cells was on the cell surface with the
remainder intracellular. The cell surface form of galectin-1 in CHO cells was active and
bound to surface glycoconjugates, but the lectin accumulating in the culture medium was
inactive. Lectin synthesised by mutant Lec8 CHO cells (which are unable to galactosylate
glycoproteins) was not found on the surface and quantitatively accumulated in the
medium in an inactive form.

Another possible reason for the galectins to be secreted by non-classical pathways is
that they need to be segregated from the complementary glycoconjugate ligands that are
externalised by the classical pathway (Barondes \textit{et al.}, 1994).
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Figure 1.3. A scheme showing both hypothetical and factual cellular localisation of the 14- and 34 kDa galectins and their interactions with complementary glycoconjugates (by protein-carbohydrate recognition), or with proteins (by protein-protein recognition) (illustration reproduced from Lotan, 1992).

1.3.7 Biological functions of galectin-1

The conservation, distribution, developmentally-regulated expression and abundance of galectins led to the hypothesis that they play important functions in biological processes. Galectins are involved in embryonic development, connective tissue regulation, organisation of the nervous system, tumour development and immune regulation (Harrison, 1991).

a. Interactions of cells with the extracellular matrix

The presence of lectins in the extracellular matrix has been demonstrated in several normal tissues (Gitt and Barondes, 1986; Harrison and Catt, 1986) and tumour biopsy specimens (Gabius et al., 1986). The extracellular matrix contains several glycoprotein constituents that could serve as ligands for the galectins. A major component of the basement membrane is laminin (see Section 1.3.5).
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In skeletal muscle, galectin-1 [secreted during differentiation and binding to laminin (Cooper et al., 1991)] inhibits cell-matrix interactions. This might be a result of the binding of galectin-1 to polylactosamine chains of laminin and interference with laminin recognition by the major laminin receptor on myoblasts, integrin α7β1 (Gu et al., 1994). Cooper et al. (1995) have found that galectin-1 is expressed at extremely high levels in vascular smooth muscle cells (SMC). When galectin-1 was added to SMCs in culture it had little effect on substrate adhesion, but almost eliminated migration on laminin, fibronectin or collagen. It was postulated that this inhibition resulted from modulation of integrin receptor function, integrins (including α5β1) being the predominant glycoconjugate ligands for galectin-1 on the SMC surface. Expression of galectin-1 was greatly decreased in migratory SMC involved in vascular remodelling during closure of the ductus arteriosus or intimal thickening in response to angioplastic injury.

Considering this, one physiological function of galectin-1 may be to modulate integrin activity so as to restrain vascular SMC migration in stable mature tissues (Cooper et al., 1995). Adhesion of A-121 human ovarian carcinoma cells to extracellular matrix is partly mediated through interaction between galectins and specific cell surface carbohydrate receptors identified as being the lysosomal associated membrane proteins, LAMP-1 and LAMP-2 (Skrinkosky et al., 1993, Section 1.3.5). It was demonstrated in in vitro assays that recombinant galectin-1 increased melanoma cell attachment to laminin in a dose-dependent manner. This effect was abolished by lactose. Antigalactin-1 antibodies also inhibited adhesion of melanoma cells to laminin (van den Brûle et al., 1995). The endogenous galectins and their surface receptors play a role in tumour cell adhesion and perhaps metastasis (Woynarowska et al., 1994).

Because of its bivalent structure resulting from its dimerisation, galectin-1 can also promote cell-matrix adhesion for other cell types by cross-linking cell surface glycoconjugates (Zhou and Cummings, 1993; Mahanthappa et al., 1994).

Ozeki et al. (1995) have discovered that placental and amniotic fibronectins are ligands for galectin-1 as well. This indicates that galectin-1 might play a role also in the assembly of the extracellular matrix.

b. Cell-cell recognition and adhesion

Human peripheral blood leucocytes were agglutinated by human lectin isolated from pancreatic and colonic mucosa. Both types of cells contain cell-surface blood group
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active lactosaminoglycans (LAGs) which could serve as ligands for the lectins (Allen et al., 1987b). Cultured lung alveolar fibroblasts and endothelial cells were found to synthesise galectin-1 and to bind exogenous lectin, whereas alveolar macrophages did not synthesise this lectin but had the binding site for the lectin. This suggested that galectin could mediate cell-cell interactions.

Lectins might function by binding complementary glycoconjugates on the surface of tumour cells to mediate homotypic aggregation, or on the surface of host cells to mediate heterotypic aggregation (attachment to endothelial cells or extracellular matrix; Sharon and Lis, 1989).

c. Mitogenic activity and immunological modulation

Mammals contain galectins in the spleen and thymus (Allen et al., 1987a; Monsigny et al., 1988) and it was suggested that the endogenous lectins may play a role in the stimulation of specific sub-populations of lymphoid cells in vivo (Lipsick et al., 1980). Kajikawa et al., (1986) have discovered that human placental lectin could trigger the production of the tumour necrosis factor (TNF) by primed macrophages.

Purified placental galectin-1 induced the production of cytotoxic factors in the cultured mouse macrophage-like cell line J774.1 and in human peripheral blood monocytes. The importance of cell-surface N-linked oligosaccharides in the interactions of cytotoxic T cells and NK cells with target tumour cells and the discovery of lectins on the surface of different lymphoid cells suggest that lectins may also mediate effector cell-target cell adhesion (Monsigny et al., 1988).

The release of the lectins in the vicinity of lung macrophages during lung remodelling could also stimulate them to phagocytose tissue remains (Whitney et al., 1985).

Another way in which lectins may be involved in the process of phagocytosis is when the maturing erythroblast undergoes enucleation: the extruded nuclei become surrounded by immunoreactive lectin and this leads to subsequent phagocytosis of the nucleus by macrophages (Harrison and Catt, 1986).

The mitogenic activity of the lectin from chick-embryo was studied on lymphocytes from mouse lymph (Pitts and Yang, 1981). It has been proposed that the stimulation of cell proliferation by lectins may not be restricted to lymphoid cells, but could also include cells in embryonal tissues. This is because endogenous lectins have been found to be
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associated with the surface of embryonic cells and lectin levels increase during development when active cell proliferation occurs (Lipsick et al., 1980).

Galectin-1 purified from conditioned medium and human urine has a mitogenic effect at low concentrations (under 1 µg/ml) on bovine corneal endothelial cells (BCE) in cultures (Manilal et al., 1993). Purified TGFγ2 (identical to galectin-1, see p. 21) had a potent mitogenic activity for BALB 3T3 cells but does not have any sugar-binding activity. When purified TGFγ2 was treated with reducing agents, the mitogenic activity diminished but the sugar-binding activity contrarily appeared (Yamaoka et al., 1996).

β-Galactoside-specific lectins from peanut and mushroom have been shown to stimulate growth of cultures of smooth muscle cells and pulmonary arterial cells (Sanford and Harris-Hooker, 1990). Peanut and soybean lectins have been shown to be mitogenic towards lymphocytes (Novgorodsky et al., 1977).

Concanavalin A initiates lymphocyte proliferation by inducing the secretion of interleukin 2 (IL-2), which acts as lymphocyte mitogenic factor in the medium of culture. Leukoagglutinating phytohemagglutinin, concanavalin A and wheatgerm agglutinins, all being considered mitogenic lectins, bind to the antigen receptor complex (TcR) on both the normal and lymphoblastoid T lymphocytes (Stadler et al., 1983).

d. Growth inhibitory and apoptotic activity

There is a possibility that cell-cell and cell-extracellular matrix lectin-mediated adhesion might lead to growth inhibition in certain cell types because it has been shown that the amount of cell surface galactosyl residues increases when murine fibroblasts reach confluence and become "contact-inhibited" (Nicolson and Lacorbiere, 1973). Immobilised N-linked plasma membrane glycopeptides, terminating in galactoside residues inhibited the growth of human diploid fibroblasts (Wieser and Oesch, 1986), but addition of recombinant galectin-1 to smooth muscle cells in culture had no effect on proliferation (Cooper et al., 1995).

There is evidence that galectin-1 participates in regulating cell proliferation (Wells and Mallucci, 1991; Sanford and Harris-Hooker, 1990). The murine galectin-1, identified by Wells and Mallucci, was shown to inhibit cell growth in vitro acting both as a cytostatic factor arresting the cells in G0 and as an inhibitor of cell replication, preventing the traversing of cells from G2 into cell division. In these experiments galectin-1 was isolated
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from conditioned medium and was the secreted form of the protein lacking the sensitivity to oxidation.

Wells and Mallucci (1992) showed that the same murine galectin-1 investigated earlier did not have a saccharide binding capacity in a tetrameric form, but the ability of this protein to inhibit proliferation was unaffected. This also implies that the exclusion of the saccharide binding region of the molecule by competing sugars does not affect the growth inhibitory effect of galectin (Wells and Mallucci, 1991).

Human galectin-1 from conditioned medium and human urine had been shown to inhibit the growth of MPS fibroblasts and U2 OS osteosarcoma cells at concentrations higher than 1 µg/ml (Manilik et al., 1993).

Galectin-1 was found to induce apoptosis of activated human T-cells and human T leukaemia cell lines. Resting T cells also bound galectin-1, but did not undergo apoptosis. Human endothelial cells that expressed galectin-1 induced apoptosis of bound T cells. This induction of apoptosis by an endogenous mammalian lectin represents a new mechanism for regulating the immune response (Perillo et al., 1995). Galectin-1 cDNA was 10-fold overexpressed during the induction of apoptosis in the glucocorticoid-sensitive human leukaemia cell line CEM C7 (Goldstone and Lavin, 1991). Mistletoe (Viscum album) lectins which are specific for galactosides have been shown to induce apoptosis in human lymphocytes (Büssing et al., 1996).

e. Development, differentiation, malignant transformation and lectin expression

Malignant transformation can also result in changes in carbohydrate structure (Fukuda, 1985). Transformation enhances the expression of galectins (Raz et al., 1987). The analysis of the galectin-1 gene expression in two normal thyroid cell lines (FRTL-5 and PC 113), and the same cells transfected by several oncogenes that induce different degrees of malignancy and differentiation, has shown that galectin-1 mRNA levels correlate with the expression of the malignant phenotype (Chiarotti et al., 1992).

On the other hand over-expression of a rat galectin-1 in mouse BALB3T3 fibroblast cells by stable induction of a galectin-1 cDNA expression plasmid resulted in the acquisition of transformed phenotype which included loss of anchorage dependence, reduced contact inhibition, colony formation in soft agar and tumour formation in nude
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mice (Yamaoka et al., 1991). This novel transforming factor (TGFβ2) from an avian sarcoma virus-transformed rat cells proved to be identical to galectin-1.

A monoclonal antibody against galectin-1 was used for quantitative analyses of cell-surface galectin on non-tumorigenic, tumorigenic and metastatic cells of different histological types and origins. Galectins were either absent or present in a very low density on the surface of normal cells, while on the neoplastic cells galectins were present in high density.

Among related tumour cell variants of the K-1735 melanoma and UV-2237 fibrosarcoma tumour systems, the cells exhibiting a higher lung colonising potential also expressed higher levels of cell surface galectin (Raz et al., 1986).

Recent studies have established that galectin-1 expression correlates with human thyroid tumour histotypes and that galectin-1 expression, at both mRNA and protein levels, is increased in several thyroid carcinoma-derived cell lines compared with normal and adenoma thyroid cells. In follicular carcinomas galectin expression was unchanged but variably increased in most papillary carcinomas and greatly increased in almost all anaplastic carcinomas (Chiariotti et al., 1995). Xu et al. (1995) have shown that all thyroid malignancies of epithelial origin expressed high levels of both galectin-1 and galectin-3. The medullary thyroid carcinomas showed a weaker and variable expression of these galectins, but neither benign thyroid adenomas nor adjacent normal thyroid tissue expressed either of the galectins.

There is data suggesting that galectin-1 might participate in melanoma cell adhesion to laminin and in this way could modulate invasion and metastasis (van den Brûle et al., 1995).

The ability to grow without anchorage to a solid substrate is one of the hallmarks of the malignant transformation. It has been suggested that the ability of tumour cells to grow in semisolid agarose is due to the expression of cell surface lectins because a monoclonal antibody, which recognised both lectins, was capable of inhibiting the growth of several tumour cell lines in agarose, just by binding to the cell surface-associated lectins (Lotan et al., 1985).

It has been suggested (Raz et al., 1986) that the increased expression of lectins by malignant and metastatic cells might play a role in the metastatic process by mediating cellular recognition and adhesion in organ implantation. In this view galectin-1 might act both in a lectin manner, favouring cell adhesion, and as negative growth factor on
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responsive adjacent normal cells to facilitate tumour invasion. Chiariotti et al. (1992) proposed the following chain of events leading to the galectin-1 overexpression:

1) Escape from the cell cycle regulation induces the expression of a negative growth factor (galectin-1).

2) The protein cannot exert its function on tumour cells because of loss of receptor-mediated signalling.

3) These events could lead in turn to overexpression of galectin-1 due to loss of putative feedback regulatory mechanism.

Galectin-1 expression was transiently induced by thyrotropin stimulation of normal quiescent FRTL-5 rat thyroid cells. Retinoic acid treatment of transformed neural cells lead to acquisition of a differentiated phenotype accompanied by a 30-fold decrease of galectin-1 mRNA levels. These results indicate that growth stimulation and induction of cell differentiation are accompanied by strong modulation of galectin-1 gene expression (Chiariotti et al., 1994). Treatment of the KM12 HCC colon carcinoma cells, the only tumour cell line that expresses galectin-3 but not galectin-1, with sodium butyrate (differentiation inducing agent) resulted in the induction of galectin-1.

This result seems to indicate that the induction of galectin-1 in these cells represents an aspect of their acquisition of a normal phenotype associated with a more differentiated state. This early increase in galectin expression might be responsible for the subsequent growth inhibition of K12 cells (Ohannesian et al., 1994).

The overall hypothesis that was formulated from these observations was that if the galectins highly expressed in tumour cells are growth inhibitors they might serve the purpose of inhibiting the growth of adjacent normal cells and mediate in this way the onset of metastasis.

Because galectin was shown to stimulate the growth of vascular cells (Sanford and Harris-Hooker, 1990), it might be possible that it facilitates angiogenesis in tumours in which cells have lost sensitivity to galectin inhibition.

It seems that low levels of galectin are associated with normal growth and development.

f. Functions of lectins in the nucleus

Galectins have been detected in cell nuclei by indirect methods including immunofluorescent staining with anti-galectin-1 antibodies (Childs and Feizi, 1980). It has
been suggested that the nuclear lectins may be involved in transport of glycoproteins or modulation of the activity of glycosylated nuclear enzymes, such as DNA polymerase (Seve et al., 1985).

The presence of neoglycoprotein-binding material in nuclear regions where RNA concentration was high suggested that the lectins may be involved in the synthesis, processing and transport of RNA (Seve et al., 1986). Galectin-1 and galectin-3 have been localised in the nuclei of mouse 3T3 fibroblasts and HE LA cells and are found in HE LA splicing extracts (Dagher et al., 1995).

Addition of galectin-specific saccharides to HE LA nuclear extracts inhibits pre-mRNA splicing while addition of saccharides with no affinity for galectins has no effect on RNA processing. Depletion of nuclear galectins by adsorption to a lactose-agarose matrix abolished both pre-mRNA splicing and spliceosomal complex formation. Both activities were reconstituted by the addition of purified recombinant galectin-1 and/or galectin-3.

The conclusion of these studies was that nuclear galectins are involved in nuclear RNA biogenesis, but their splicing activity is functionally redundant. The ability of galectin-1 to restore splicing activity to galectin-depleted extracts suggests that only domains containing the carbohydrate recognition site of galectins are required for splicing function (Dagher et al., 1995).

g. Other galectin functions

Cultured endothelial cells (human aortic and umbilical vein endothelial cells) expressed galectin-1. Activation of the cultured endothelial cells increased the level of galectin-1 expression.

These results suggest that galectin-1 expressed by endothelial cells may bind and affect the traffic of cells migrating from blood cells into tissues (Baum et al., 1995).

Galectin-1 participates in the thymocyte-thymic epithelial cell interactions which may be regulated by expression of relevant oligosaccharide ligands on the thymocyte cell surface. The degree of galectin-1 binding to thymocytes is correlated with the maturation stage of the cells: immature thymocytes bound more galectin-1 than the mature thymocytes (Baum et al., 1995).

Mice lacking galectin-1 have been genetically engineered and so far no phenotype has been detected (Poirier and Robertson, 1993). Absence of phenotype is frequently seen
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with gene knockout experiments and is attributed to alternative proteins and biological mechanisms that compensate for the missing protein.

1.4 THE C-FOS PROTO-ONCOGENE, SIGNAL TRANSDUCTION AND GROWTH STIMULATION

The way in which cells respond to their environment is by binding external ligands to specific receptors. This generates second messengers which lead to signals resulting in DNA synthesis. It is believed that there is more than a second messenger system for all stimulators of DNA synthesis (Marshall et al., 1988).

Generally most of the receptors are localised in the plasma membrane and they transmit the information through changes in protein phosphorylation. Another class of receptors are intracellular and respond to hydrophobic ligands which enter the cell through passive diffusion and act primarily through changes in gene expression (Rayter et al., 1989).

An intimate involvement in the growth-stimulatory process had been suggested for the oncogene-encoded proteins. Several oncogenes (sis, hst, int-2) have been shown to encode growth-stimulating hormones (Waterfield et al., 1983; Sakamoto et al., 1986; Bovi et al., 1987 and Moore et al., 1986). In many transformed cells oncogenes activate the expression of normally quiescent growth factor genes which subsequently stimulate tumour cell growth (Sporn and Todaro, 1980). Other oncogenes (erbB and fms) have been found to encode protein-tyrosine kinases (Downward et al., 1984; Sherr et al., 1985). A fourth category includes oncogenes (ras) that encode proteins that show homology to G-proteins (Gilman, 1987). Finally the human proto-oncogenes c-myc, c-jun and c-fos encode transcription factors that mediate gene expression in response to activation of protein kinases (Bohmann et al., 1987).

The c-fos proto-oncogene is considered to be part of a subset of mitogen-inducible genes called immediate early genes (IEGs) because of their rapid and transient transcriptional induction profile. This gene is stimulated in response to signals which induce proliferation and/or differentiation, as well as to the various cellular stresses (Cohen et al., 1989) (Fig. 1.4, p. 26).

Proteinase inhibitors like BBI and antipain were shown to reduce the levels of c-fos mRNA in treated cells (Caggana and Kennedy, 1989; Kennedy, 1993). This aspect of the
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Proteinase inhibitor effect on some oncogenes is important because the development of malignancy involves phenomena that can be regulated in normal cells, but escape regulatory control in cancer cells. There is a general correlation between the capacity of a proteinase inhibitor to affect c-fos gene expression and its ability to suppress radiation-induced transformation in vitro (Kennedy and Little, 1981). This does not correlate perfectly with the experiments on the GRP because the proteinase inhibited by BBI and antipain is intracellular. However, it is likely that inhibition of c-fos could be a consequence of GRP inhibition and could contribute to the growth inhibitory effect. If galectin-1 is a mediator of GRP action, then its effects on c-fos expression will also be of interest.

1.5 AIMS OF THESIS

The aim of this thesis was to synthesise and purify a recombinant human galectin-1. This was needed for experiments in which we intended to verify the hypotheses that galectin-1 a mediator of GRP action, and to assess the effect of galectin-1 treatment of cells in cultures on the expression of the c-fos proto-oncogene.
1. Introduction

Figure 1.4. Schematic of various signal transduction systems investigated in relation with inhibition of cell proliferation by proteinase inhibitors (reproduced from Borich et al., 1994).

The pairs of vertical lines represent the plasma and nuclear membranes. Agonists listed in the column at the left initiate intracellular signalling pathways, represented schematically, by way of a specific plasma membrane receptor in most cases. The area below the bold broken horizontal line represents agonists and messengers known to be unaffected by inhibition of the GRP, whilst those above this line are inhibited in their growth-promoting action when the GRP is inhibited.

**Abbreviations:** TK-tyrosine kinase; serum Gfs-growth factors in foetal calf serum, (mainly PDGF and IGF-1); MSA-multiplication-stimulating activity (IGF); FOS, MYC-nuclear proto-oncogenes; FGF-fibroblast growth factor; EGF-epidermal growth factor, PKC-protein kinase C; DAG-diaclylglycerol; PIP$_2$-phosphatidylinositol 4,5 bisphosphate; IP$_3$-inositol trisphosphate.

Figure 1.4. summarises the aspects of GRP inhibition which have been already elucidated and its effect on signal transduction pathways. The nuclear effects leading to inhibition of DNA synthesis have not yet been investigated, but are inferred from experiments on the effects of proteinase inhibitors on c-fos expression. Caggana and Kennedy (1989) showed that the c-fos expression is transcriptionally regulated via a de-repression mechanism.
Chapter 2

Synthesis and purification of a recombinant human galectin-1
Chapter 2: Synthesis and purification of a recombinant human galectin-1

2.1 INTRODUCTION

The first task for this project was the synthesis of a recombinant human galectin-1 for use in subsequent experiments.

Galectin-1 is a protein expressed constitutively in all cell types, but the task of purifying this protein from animal tissues proved very difficult. In previous work undertaken in this laboratory, small quantities of a β-galactoside-binding protein were isolated using serum-free conditioned medium from human fibroblasts (and from human urine) with an apparent molecular weight of 14 kDa (Manilal et al., 1993). A lectin was also purified from soluble extracts of human lung (Sparrow et al., 1987). Even more successful were the attempts to synthesise a recombinant human galectin in bacterial systems (Hirabayashi et al., 1989; Couraud et al., 1989; Lobsanov et al., 1993).

Initially we tried to obtain the recombinant galectin using a yeast expression system. A manipulation vector kindly donated by Dr Allan Donald was used for this purpose. Difficulties experienced in this process determined us to try and express the recombinant galectin in a bacterial system. The pGEX-4T-1 expression vector kindly donated by Dr Christie made this option possible.

This chapter describes the synthesis, amplification and cloning of the human galectin-1 gene, the expression of galectin-1 as a fusion protein and the final purification of this lectin.
2.2 MATERIALS AND METHODS

MATERIALS

Unless otherwise specified, all chemicals used were of analytical grade and were obtained from Sigma Chemical Company, U.S.A. and Bio-Rad, Richmond, U.S.A. Foetal calf serum was purchased from Life Technologies Limited (Gibco-BRL). Oxygen and carbon dioxide used for cell growth were supplied by New Zealand Industrial Gases, Auckland. All glassware and pipette tips used for cell culture and molecular biology work were autoclaved at 15 p.s.i. for 20 minute. Dulbecco's modified Eagle's medium (DMEM), trypsin, ampicilin, IPTG and other reagents were filter-sterilised in a Class II biological safety cabinet using 0.2 μm Acrocap filters obtained from Gelman Sciences, Michigan, U.S.A.. Primers were synthesised by the Centre for Gene Technology, University of Auckland. DNA-modifying enzymes and restriction enzymes were purchased from Life Technologies Limited (Gibco-BRL). Wizard miniprep DNA purification system was purchased from Promega. pBluescript and pCNInv vectors were kindly donated by Dr Allan Donald (plasmid map for pCNInv in Appendix 1). pGEX 4T-1 expression vector and E. coli DH5α bacterial cells were kindly donated by Dr David Christie.

S. cerevisiae DBY 746 α with the genotype his3-51, leu2-3, leu2-112, ura3-52, trp1-289, gal (up)s was kindly donated by Dr Allan Donald. 1 kb DNA mass ladder was purchased from Life Technologies Limited (Gibco-BRL). The RiboClone cDNA Synthesis System with AMV reverse transcriptase was purchased from Promega. AmpliTaq DNA Polymerase was purchased from Perkin Elmer. 20 mM dNTPs were purchased from Pharmacia Biotek. GeneClean II kits were purchased from Bresatech. LB medium was purchased from Life Technologies. Glutathione-agarose was firstly purchased from Sigma and then prepared in our laboratory. Fetuin was kindly donated by Dr. Christie.

Cyanogen-bromide-activated Sepharose was purchased from Sigma. DNA was visualised by fluorescence using a transilluminator at 302 nm from Ultra Violet products Inc. All water used during this study was supplied by a MilliQ water purification System (Millipore).
2.2 Materials and Methods

METHODS

2.2.1 Cell culture

The tumor cell line mainly used as the source of RNA was U2OS (human osteosarcoma cell line, Scott, 1988). HELA (human epidermal carcinoma cells) and MPS (Malcolm Pasiow’s skin cells) were also used as sources of RNA (Scott, 1988). Cells were maintained in Coming culture flasks at 37°C in a humidified atmosphere of 5 % CO₂/95 % air in a Nuaire incubator. Cells were maintained in culture medium consisting of 90 % Dulbecco Modified Eagle’s Medium (DMEM from Sigma Chemical Co.) supplemented with 0.25 % sodium bicarbonate (w/v), 0.029 % glutamine (w/v), 10 % foetal calf serum (v/v), 100 U/ml penicillin and 100 μg/ml streptomycin.

2.2.2 Subculturing

The culture medium and trypsin (0.25 %) were pre-warmed to 37°C. Medium from confluent cells was removed by vacuum suction in sterile conditions (Class II Biological safety laminar flow cabinet). Detachment (trypsinisation) of cells was achieved by washing the cells twice with 1 ml of 0.1 % trypsin. This was partially removed and the flasks were incubated at 37°C for 5 minutes. Cells were re-suspended in approximately 6-8 ml of DMEM containing 10 % foetal calf serum and dispensed equally in two other flasks to which an appropriate volume of DMEMS medium was added. The newly established cultures were placed in the incubator until the cultures had almost reached confluence. Cultures of cells nearly confluent were used as a source of RNA. A sterilised cell scraper was used to detach cells from the surface of the flask.

2.2.3 RNA extraction using the rapid method of Gough (1988)

Detached cells were re-suspended in approximately 2 ml of ice-cold phosphate-buffered saline (PBS) (pH 7.4). The cells were kept on ice while being processed. The cell suspension was centrifuged using a bench centrifuge at 3 000 r.p.m. for 5 minutes. The supernatant was removed by aspiration with a sterile Pasteur pipette and the cells were re-suspended in 1 ml cold PBS and placed in a sterile microfuge tube. Cells were spun in a microfuge at 3 200 r.p.m. for 5 minutes. The supernatant was removed and the pellet was re-suspended in 200 μl solution A (ice cold lysis buffer: 10 mM Tris-HCl, pH
2.2 Materials and Methods

7.5, 0.15 M NaCl, 1.5 mM MgCl₂ and 0.65 % NP 40). The mixture was vortexed vigorously and incubated on ice for 5 minutes. The cell nuclei were removed by a spin at 15 000 r.p.m. for 1 minute in a Costar microfuge. The supernatant was transferred to a new microfuge tube containing 200 μl solution B (7 M urea, 1 % SDS, 0.35 M NaCl, 10 mM EDTA, 10 mM Tris-HCl (pH 7.5) and 400 μl phenol/chloroform/isoamyl-alcohol (50/50/1) which ensured the extraction of protein from the post nuclear cytoplasmic lysate). The mixture was vortexed and the deproteinised cytoplasmic extract was separated from the phenol phase by microfugation at 15 000 r.p.m. for 1 minute in a Costar microfuge. The phenol-chloroform precipitation was repeated once to remove any traces of proteins.

The RNA was precipitated from the aqueous phase using 40 μl 3 M sodium acetate solution and 1 ml ice cold ethanol absolute for 30 minutes. Incubation on dry ice was followed by a 20 minutes spin at 4°C and 15 000 r.p.m. in a Costar microfuge. The RNA pellet was re-suspended in 50 μl filter-sterilised water and stored at -70°C.

2.2.4 RNA and DNA estimation

RNA was quantified by measuring the absorbance at 260 and 280 nm and using the absorption coefficient of 1 A 260 unit = 40 μg/ml. DNA was quantified by measuring the absorbance at 260 and 280 nm and using the absorption coefficients of 1 A 260 unit = 50 μg/ml. An Ultrospec III spectrophotometer was used for this purpose.

2.2.5 First strand cDNA synthesis

Preparation of the first strand cDNA using 2.6 μg total RNA as template and the RiboClone cDNA Synthesis System with AMV reverse transcriptase was performed according to the manufacturer's protocol (Promega Technical Manual).

2.2.6 Polymerase Chain Reaction

The polymerase chain reaction was carried out using primer oligonucleotides synthesised at the Centre for Gene Technology in the University of Auckland.
2.2 Materials and Methods

Primers

To design the oligonucleotides we referred to the sequence of the cDNA of the human β-galactoside-binding lectin from Hep G2 cells (Abbot and Feizi, 1989).

For cloning in the Bluescript sequencing vector the forward primer was 5'-TGGCAGATCTCAGTCAAAGGCCACACA-3' and the reverse primer was 5'-CTGGGATCTTTCATGGCTTGTGGTCTG-3' (GBP1 and 2 respectively).

Both the forward and the reverse primer included a Bgl II site for subsequent cloning in the pCNInv vector. For cloning in the pGEX vector the forward primer was 5'-CTGGGATCCATGGCTTGCTGTC-3' and the reverse primer was 5'-ACGCGTGCACACTCAAAAGCCACACA-3' (GBP 3 and 4 respectively).

The forward primer included a Bam H I site and the reverse one included a Sal I site to make possible the cloning of the DNA fragment into the pGEX-4-T1 expression vector.

Polymerase Chain Reaction

The polymerase chain reaction was performed under the following conditions: 50 mM KCl, 1.5 mM MgCl₂, 10 mM Tris-HCl, pH 8.8, 0.25 mM dNTPs, 20 pmol of forward primer and 20 pmol of reverse primer, 5 µl cDNA, 1.25 units AmpliTaq DNA polymerase and water to 50 µl. The amplification was performed for 30 cycles, each of them consisting in denaturation at 95°C for 1 minute, annealing at 55°C for 90 sec, extension at 72°C for 90 sec, followed by a final extension step at 72°C for 5 minutes, using a programmable thermal controller (MJ Research).

2.2.7. DNA electrophoresis in agarose gels

The DNA fragments were electrophoretically separated on 0.8-1.5 % agarose horizontal gels containing 0.5 mg/ml ethidium bromide for approximately 1 h and 30 minutes at 60 V and variable current intensity. The buffer used for analytical agarose gels was TAE (40 mM Tris acetate, 1 mM EDTA, pH 8).

We used 1 µl of 1 Kb DNA ladder as a size marker. DNA bands of interest visualised by fluorescence were excised from low melting point agarose gels and eluted using a
2.2 Materials and Methods

GeneClean II kit and following the manufacturer's recommendations. The DNA was eluted in TE buffer (10 mM Tris-HCl and 1 mM EDTA, pH 8) or Milli-Q water.

2.2.8. Phenol:chloroform and ethanol precipitation of DNA

To a volume of product or vector, half that volume of phenol and chloroform (1:1) was added. The mixture was then centrifuged at 10 000 r.p.m. for 1 minute. The upper phase was transferred to a new microfuge tube and mixed with the same volume of chloroform as the initial volume of DNA solution. Then the 2 phases were separated by centrifugation and the aqueous phase (upper phase) was added to a mixture of half the initial volume of 3M sodium acetate, 1 ml ice cold ethanol absolute and 1 μl of tRNA or 2 μl glycogen (carriers). This mixture was incubated for 20 minutes on dry ice and then centrifuged at 10 000 r.p.m. for 20 minutes. The pellet obtained was re-suspended in 10 μl TE buffer or 10 μl sterile water.

2.2.9 Cloning the galectin-1 PCR product in the pBluescript vector

pBluescript vector (1 μl of 1.2 μg/μl concentration) was digested with 1 μl of the enzyme Sma I (10 U/μl) in 2 μl restriction enzyme buffer supplied by the manufacturer, in a final volume of 20 μl for 1 h at 37°C. Because Sma I is a restriction enzyme which gives blunt ends, the vector was dephosphorylated by treatment with calf intestinal alkaline phosphatase (CIAP) to avoid re-ligation. CIAP (2 μl) was added to the vector digestion mixture and re-incubated for 1h at 50°C. PCR products (1.4 μg) were treated with 2 μl Klenow fragment (6 U/μl) in 2 μl (10 x) Klenow buffer and 0.5 μl 1 mM dNTPs in a total volume of 20 μl at 37°C for 1 h to backfill any T overhangs on the PCR product. To this reaction mixture 3 μl T4 polymerase buffer, 0.2 μl T4 kinase (10 U/μl), 3 μl 10 mM ATP and water to 30 μl was added. The mixture was incubated at 37°C for a further 15 minutes to phosphorylate ends of the PCR fragment. In the case of the ligation (blunt ligation) of galectin-1 gene in pBluescript vector, we used only 10 ng dephosphorylated pBluescript vector, 140 ng Klenow polymerase-treated phosphorylated PCR product and 0.5 μl T4 ligase (1 U/μl). The mixture was incubated overnight at 14°C and half of it was used to transform competent bacterial cells (protocols for preparation of competent bacterial cells and transformation are in the Appendix 4). Cultures of 3 ml of the bacterial transformed colonies were grown overnight and the plasmid DNA was isolated using a
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Miniprep DNA Purification Kit (Promega) according to the manufacturer’s protocols. The DH5α bacterial strain allowed blue/white colour selection for pBluescript vectors containing inserts when plated on LB plates with 100 μg/ml ampicillin, 40 μg/ml X-gal, 5 mM IPTG. pBluescript plasmids with inserts grew as white colonies, since the inserts interrupt the coding region of the LacZ gene fragment, while pBluescript plasmids with no inserts in the polylinker grew as blue colonies (Stratagene data sheet). The presence of insert was re-checked by restricting the plasmid DNA obtained from transformed colonies with the Sma I enzyme and visualising the products on a 1.5 % agarose gel.

2.2.10 Cloning the galectin-1 PCR product in the pCNNinv yeast expression vector

The 1.36 μg of pBluescript plasmid which contained the 450 bp fragment was digested with 0.5 μl Bgl II (10 U/μl) restriction enzyme in 15 μl restriction enzyme buffer (React 3 buffer), in 25 μl final volume at 37°C for 1 h, and the 450 bp product was separated on a 1.5 % agarose gel. 5 μg of plasmid pCNNinv (an E.coli-yeast shuttle vector; plasmid map is in the Appendix 1) was digested at 37°C for 1 h with 0.5 μl Bgl II (10 U/μl) restriction enzyme in 25 μl React 3 buffer, added for a final volume of 35 μl. For the cloning of the galectin-1 gene in pCNNinv a cohesive termini ligation was performed. The reaction mixture contained 0.4 μg plasmid and 0.4 μg of the PCR product, 0.5 μl T4 ligase, 2 μl ligase buffer and water to 20 μl. The reaction mixture was incubated at room temperature for 3 h and half of it was used to transform competent yeast cells (protocols for preparation of competent yeast cells and transformation are in the Appendix 4) while the other half was used to transform bacterial cells because pCNNinv vector does not allow blue/white screening.

The fact that pCNNinv contained the right size insert was verified by digesting the plasmid obtained from bacterial minipreps with the Bgl II restriction enzyme and visualising the restriction products on a 1.5 % agarose gel. The right size insert was present and I continued by plating the yeast transformants and growing them on a selective medium. I used for transformation a S. cerevisiae strain (DBY 746 α), an auxotrophic mutant that has a non-functional leu-gene (a LEU2 marker for the selection of transformants). The transformed yeast cells were plated on minimal growth medium lacking the relevant nutrient: [ SD medium (0.67 % yeast nitrogen base without amino acids, 2 % glucose as a source of carbon and 1.5 % agar) supplemented with 20 μg/ml L-histidine, 20 μg/ml uracil and 40 μg/ml L-tryptophan]. Selection was possible because
2.2 Materials and Methods

transformants contained a plasmid-borne copy of the leu2 gene, so that they could grow in the absence of the leu amino acid, unlike the non-transformed cells.

2.2.11 Cloning the galectin-1 PCR product in the pGEX bacterial expression vector

DNA was digested in reactions containing 1-10 μg DNA, the restriction enzyme buffer supplied by the manufacturer and enzyme (1 U per 1 μg DNA) in a final volume of 20 μl. Both plasmid and PCR product were cut with the same restriction enzymes (0.7 μl Bam H I and 0.7 μl Sal I, each at a concentration of 10 U/μl) in the same reaction buffer (Reaction Buffer 3). The reaction mixture was incubated at 37°C for 30 minutes and then DNA was purified by phenol:chloroform and ethanol precipitation (see Section 2.2.8).

Digestion products were separated electrophoretically on 1% agarose gels and visualised with ethidium bromide. In the case of the ligation of galectin-1 gene in the pGEX vector, the only variations from the general protocol were the use of 1.5 μg of pGEX vector instead of 50 ng and a molar ratio of 1:4 (vector to insert) instead of 1:1 molar ratio. The mixture was incubated for 3 hours at 14 °C and half of it was used to transform competent bacterial cells.

Because pGEX-4T-1 vector does not allow blue/white screening, the selection of the transformants was done by digesting the plasmid DNA obtained from the minipreps with the same restriction enzymes used for cloning and visualising the restriction products on a 1.5 % agarose gel.

2.2.12 Isolation of plasmid DNA for sequencing

pBluescript plasmid is present in a high number of copies in cells so a miniprep contained enough plasmid DNA for sequencing. Because pGEX vector is present in low number of copies in cells, plasmid DNA was prepared for sequencing by equilibrium centrifugation in CsCl-ethidium bromide gradients by the method of Sambrook et al. (1989). This method yielded a volume of 50 μl at a concentration of 1.42 μg/μl which was sufficient for sequencing.
2.2 Materials and Methods

2.2.13 DNA sequencing

pBluescript and pGEX-4T-1 plasmids containing the right size insert were sequenced on the automatic sequencer using the dideoxy chain termination method (Sanger et al., 1977). Sequencing was carried out by the Centre for Gene Technology, University of Auckland. Database searches were performed using software from the University of Wisconsin Computer group (Devereux et al., 1984) on a vax computer system.

2.2.14 Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE)

SDS-PAGE of proteins was carried out according to the method of Laemmli (1970) using 10% and 15% vertical acrylamide mini gel slabs (10 cm x 8 cm x 0.5 mm) with a constant ratio of bisacrylamide concentration. Samples were loaded into wells (protocol for the preparation of samples for SDS-PAGE is in Appendix 4) and electrophoresis was carried out at a constant current of 10 mA through the stacking gel and 20 mA as samples entered the resolving gel. The gel was stained using a solution of 0.125% Coomassie Brilliant Blue R-250, 50% methanol and 10% glacial acetic acid), then destained, washed and vacuum-dried using a Hoeffer slab gel drier (Model SE 1160).

2.2.15 Preparation of galectin-1 using transformant yeast cultures

Aliquots (10 μl) of stored transformed yeast culture were used to inoculate 1L of YPD medium (2% fructose as a source of carbon, 2% Bacto-peatone, 1% yeast extract) and were grown at 30°C for 3 days. The cells were pelleted by centrifugation at 3 000 r.p.m. for 30 minutes and the supernatant was applied to an ε-amino-caproyl-galactosamine-agarose (2 ml) affinity column. The column was washed with 30 mM Tris-HCl buffer, pH 7.5 and eluted with 100 mM lactose in Tris buffer.

Fractions of 1.5 ml were collected. The first 10 samples were pooled, freeze-dried and re-suspended in 1 ml MilliQ water then mixed with bromthymol blue and added to a Sephadex G-25 column (25x4 cm; Pharmacia) desalting column using MilliQ water as an eluent. Protein peaks from this column were pooled and the samples concentrated by freeze drying. SDS-polyacrylamide gel electrophoresis was carried out in the same manner as in Methods Section 2.2.21.

Another culture of 1 L YPD medium in which cells were grown at 30°C for 3 days was centrifuged at 3 000 r.p.m.in a GSA rotor for 30 minutes and the supernatant was applied
to an asialofetuin affinity column using the same protocol as in Section 2.2.19. Fractions of 1.5 ml were collected and pooled, freeze-dried and re-suspended in a small volume of water and desalted using the same protocol mentioned before in this section. Concentrated samples were visualised by SDS polyacrylamide gel-electrophoresis.

2.2.16 Preparation of fusion protein

2.2.16a Small scale preparation of fusion protein

Screening for the presence of a fusion protein, the product of the pGEX vector with an included insert, was done by growing small scale cultures (3 ml) of the transformant colonies and visualising fusion proteins on a SDS-PAGE.

2.2.16b Expression and purification of glutathione-S-transferase fusion proteins

For the large scale expression of the fusion protein, 3 litre cultures of a transformant colony were grown and induced for 3 hours with 1 mM IPTG. The cultures were then centrifuged in a Sorval GSA rotor at 5 500 r.p.m. for 10 minutes and the pellet was re-suspended in 30 ml ice-cold sodium phosphate-buffered isotonic saline (PBS) containing 0.1 mM PMSF to prevent proteolysis. The cells were lysed in a French Cell Press at 1,400 p.s.i. and Triton X-100 was added to a concentration of 1 %. The cell lysate was then centrifuged using a Sorvall SS-34 rotor at 11 000 r.p.m. for 15 minutes to remove insoluble material and cell debris. The supernatant was collected and passed through 20 ml of glutathione-agarose affinity resin (protocol for the coupling of glutathione (reduced) to epoxy-activated Sepharose in Appendix 4). The column was washed with PBS. The unbound fraction was also collected and analysed by SDS polyacrylamide electrophoresis. The fusion protein was eluted with 50 mM Tris-HCl buffer (pH 8.0), containing 5 mM reduced glutathione according to the method of Smith and Johnson (1988). The unbound fraction proved to still contain some fusion proteins. As a consequence, it was spun at 11 000 r.p.m. in an SS-34 rotor and the supernatant was applied once more through the column in order to enhance fusion protein recovery. The samples containing the concentrated fusion protein were pooled and dialysed overnight in thrombin cleavage buffer (50 mM Tris-HCl pH 7.5, 150 mM NaCl and 2.5 mM CaCl₂).
2.2 Materials and Methods

The less concentrated fractions were pooled and concentrated in Macrosep concentrators with a molecular weight cut-off of 3 KDa at 7000 r.p.m. in a Sorvall centrifuge using an SS-34 rotor, then the elution buffer was replaced with cleavage buffer. At the end all concentrated samples were pooled and the amount of protein present was estimated using the Bradford protein quantitation method (Bradford, 1976).

Variations from the protocol used for the growth of large scale cultures consisted of inoculating fresh LB/Amp plates, then using single colonies from this to start 3 ml liquid cultures that were grown for 8 h and one colony was used to inoculate a 300 ml overnight culture. This was in its turn used to inoculate a 3 litre culture. Ampicillin was added not only at the time of the inoculation, but also after growing the large scale culture for 2 h.

2.2.17 Removal of GST carrier by proteinase cleavage

A recognition site for the proteinase thrombin was incorporated in the fusion protein to facilitate recovery of the wanted protein (Guan and Dickson, 1991).

The cleavage reaction was conducted in solution: thrombin was added to the fusion protein (0.2 %, 0.6 % or 1 % w/w fusion protein) and the mixture was incubated for 90 minutes at 25°C.

2.2.18 Attempted cleaved protein purification on glutathione agarose

After incubation the mixture was added to the glutathione-agarose affinity column and the released protein was recovered by washing the column with 1 column volume of 50 mM Tris-HCl buffer (pH 7.5), containing 150 mM NaCl: 2.5 ml fractions were collected. The released protein was concentrated using a Macrosep centrifugal concentrator with a molecular weight cut-off of 1 kDa at 7000 r.p.m. using a Sorvall centrifuge and a SS-34 rotor.

2.2.19 Further purification of the cleaved protein on Sephadex G-75

A 50 ml of Sephadex G-75 gel filtration column was used to further purify the cleaved protein. The buffer used was 30 mM Tris-HCl, pH 7.5 and 2.5 ml fractions were collected.
2.2 Materials and Methods

2.2.20 Cleaved protein purification on an asialofetuin-Sepharose affinity column

Asialofetuin-Sepharose (10 ml) was used to separate the cleaved protein from the glutathione-S-transferase and the uncleaved fusion protein. After the reaction mixture was applied to the column, the column was washed with 30 mM Tris-HCl, pH 7.5 and eluted with 100 mM lactose in the same buffer. Fractions eluted this way were desalted and concentrated using a Microsep concentrator with a cut off of 1 kDa (the protocol for coupling of asialofetuin to CNBr Sepharose is in Appendix 4).

2.2.21 Protein estimation

The Bradford protein assay (Bradford, 1976) was used for protein estimation using a solution of BSA of 100 μg/ml as a standard.

2.2.22 Electroblotting of samples for protein sequencing

Samples for sequencing were resolved on SDS-PAGE under reducing conditions. The gel was then soaked for 5 minutes in CAPS electroblot buffer (0.01 M CAPS, pH 11, 10 % HPLC grade methanol) before being sandwiched in an electroblot cassette. The electrophoresed proteins were transferred to proBlott (Applied Biosystems) membranes by electroblotting at 90 V for 20 minutes (Matsudaira, 1987). Protein bands were detected by Coomassie Blue staining.

2.2.23 Protein sequence analysis

N-terminal amino acid sequence analysis was performed by Ms Catriona Knight using a gas-phase Sequencer (model 470A). Both chemicals and programme used were supplied by the manufacturer (Applied Biosystems).

2.2.24 Reverse Phase HPLC (RP-HPLC)

Asialofetuin-purified recombinant galectin (100 μl of a 0.1 μg/μl solution) was injected onto an Aquapore RP-300 column (Applied Biosystems, 0.2x22cm, 7 μm bead diameter) connected to a HPLC with a 140A solvent delivery system and a 1000S diode array detector (Applied Biosystems). The solvent system consisted of solvent A, 0.1%
2.2 Materials and Methods

trifluoroacetic acid in water (purified using Milli-Q system) and solvent B, 0.085% tr trifluoroacetic acid in acetonitrile/ water, (80:20, v/v). The column was equilibrated with 90 % A, 10 % solvent B at a flow rate of 200 µl/min. The column then received a 10 minute wash in equilibration buffer before injecting the sample. A 45min gradient of acetonitrile (from 10-70 % B) was used for elution. Peaks were detected by absorbance at 214 and 280 nm.

2.2.25 Matrix-Assisted Laser Desorption /Ionisation Time-of-Flight Mass Spectrometry (MALDI/TOF MS)

This method is an analytical tool that can accurately determine the molecular weight of proteins (Zaluzec et al., 1995). The intact galectin-1 as well as its enzymatic degradation products were analysed by matrix-assisted laser desorption/ionisation time-of-flight mass spectrometry (MALDI-TOF) using a HP G2025A MALDI-TOF System (Hewlett Packard) equipped with a N₂ laser (337 nm, 3 ns pulsewidth). The laser energy used was 4.2 µJ. The matrices used for different determinations were:100 mM solution of 3,5-dimethoxy-4-hydroxycinnamic acid in acetonitrile/methanol/water (sinapinic acid matrix), 100 mM solution of 2,5-dihydroxybenzoic acid in methanol/water (DHB matrix) and 33 mM solution of α-cyano-4-hydroxycinnamic acid in acetonitrile/methanol (α-CHC matrix). The spectra represent the accumulation of 30 single laser shots. They were calibrated externally but also internally (in the case of the intact galectin) by a standard sample (bovine insulin, 5 813.5 Da, equine cytochrome C, 12 360 Da and bovine serum albumin 6 6430 Da) that was prepared on the same target (2 µl of each standard to which 6 µl matrix was added). Sample mixture was analysed in a 1:1 (v/v) mixture with matrix (sample concentration between 0.1-1 µg/µl).

For the production of tryptic peptides we incubated recombinant galectin-1 with bovine pancreatic trypsin (Sigma) in a substrate-to-enzyme ratio of 20-1 (w/w), for 18 h at 37°C. The reaction was terminated by freezing the sample at -80°C. Prior to use the sample was vacuum-dried and redisolved in MilliQ water.

For the production of tryptic peptides in reducing conditions galectin-1 was treated before tryptic digestion with dithiothreitol (molar ratio of 1:50) for 4 h at 50°C.
2.3 RESULTS

2.3.1 DNA Cloning

a. First strand cDNA synthesis

All the RNAs extracted from U2OS, HELA and MPS fibroblast cells were used for the synthesis of cDNA and PCR amplification. Only the transformants containing the PCR product of the U2OS cells were used for the production of galectin-1.

b. Galectin-1 gene synthesis

The product of PCR amplification of cDNA from U2OS cells was 450 base pairs in length irrespective of whether the GRP1 and 2 or GRP 3 and 4 primers were used. A similar DNA fragment was generated from fibroblast cDNA. In the case of Hela cDNA two fragments of 450 and 250 bp were sometimes seen. The smaller fragment was not further characterised (Appendix 5).

c. DNA sequencing

Because the product of the PCR amplification was re-cloned in pGEX we needed to sequence this gene again. The sequencing confirmed that the insert both in the pBluescript vector (sequencing done both with forward and reverse primers) and in the pGEX vector (sequencing done in one direction only), was identical with the published sequence for the human galectin-1 (sequencing results showed in Appendix 1).
2.3 Results

Figure 2.1. Screening of transformant bacterial cultures for the presence of a 450 bp fragment.

Minipreps from different colonies were subjected to digestion with the appropriate restriction enzymes, the products of this digestion were separated on 0.8% agarose gels, stained with ethidium bromide and visualised under UV light.

A: 1-kb DNA ladder.
   2-10 µl p bluescript (5 µg) digested with Bgl II restriction enzyme.
B: 1-kb DNA ladder.
   2-10 µl pCNNinv (6.2 µg) digested with Bgl II restriction enzyme.
C: 1-10 µl pGEX (1.4 µg) digested with Sal I and Bam H I restriction enzymes.
   2-1 kb DNA ladder.

The colonies that proved to have the 450 bp insert were used further on for sequencing (in the case of pBluescript vector), the production of galectin-1 (in the case of pCNNinv) and for the production of fusion protein (in the case of pGEX).
2.3 Results

2.3.2 Expression and purification of galectin-1 from yeast

Figure 2.2 Isolation of galectin from yeast growth medium.

1 liter culture medium in which the transformant yeast was grown for 3 days was applied to an ε-aminocaproyl-galactosamine affinity column pre-equilibrated with 30 mM Tris-HCl, pH 7.5. The size of the collected fraction was 1.5 ml.

![Absorbance vs Fraction Number](image)

The proteins bound to the column were eluted as a single peak with 30 mM Tris-HCl/100 mM lactose, pH 7.5, pooled, freeze-dried and desalted. Only the elution stage illustrated.

Figure 2.3 Desalting of freeze-dried samples purified on ε-aminocaproyl-galactosamine

Freeze-dried samples were redissolved in 1 ml MilliQ water and bromthymol blue was added before sample was applied on the desalting column. The first 15 fractions eluted from the column were pooled, freeze-dried and re-suspended in 1 ml MilliQ water. Fraction size was 2.5 ml.

![Absorbance vs Fraction Number](image)
2.3 Results

Figure 2.4. SDS-PAGE of proteins recovered from the G-25 desalting column. Samples were freeze-dried and resuspended in MilliQ water, then 10µl aliquots were analysed by SDS-PAGE under non-reducing conditions on a 15% polyacrylamide gel. The electrophoresed proteins were visualised following staining with Coomassie Brilliant Blue.

Lane 1 : 10µl sample eluted from the asialofetuin column.
Lane 2 : 10µl sample eluted from the ε-aminocaprooyl galactosamine column.
Lane 3 : 10µl low molecular weight markers.
Lanes 4, 5 : 10µl non transformed yeast unpurified media.
Lanes 6, 7 : 10µl transformed yeast unpurified media.

In the case of the proteins purified on ε-aminocaprooyl galactosamine, 4 main protein bands were present, migrating at 6 000 Da, 20 000 Da, 32 500 Da and 45 000 Da. No protein bands were present in the sample eluted from the asialofetuin column (lane1). In neither case was there a band corresponding to the expected molecular weight (14 500 Da) of galectin-1. As a result of these findings, it was decided to use a bacterial fusion protein system to express the recombinant galectin-1.
2.3 Results

2.3.3 Expression and purification of the fusion protein from bacterial systems

2.3.3.a Small scale expression of the fusion protein

Figure 2.5. SDS-PAGE of bacterial proteins from a small scale preparation

Two liquid cultures of 5ml were inoculated with 50μl glycerol-stock cultures of colonies 1 and 5, grown until the optical density value was 0.8 at A600, then induced for 3 h with IPTG (final concentration of 1mM). The cultures were spun and the pellet was resuspended in 100μl water and 10μl of this was boiled in loading buffer and loaded on a 10% polyacrylamide gel.

Lanes 3,4,7,8: 10μl samples of colonies induced with IPTG (1mM final concentration).
Lanes 5,6: 10μl high molecular weight markers.
Lanes 1,2: 10μl samples of uninduced colonies.

The role of a small-scale preparation of the fusion protein was to screen for the presence of this protein in the bacterial cells before being engaged in the growth of large scale cultures. A 43 kDa protein was detected as being overexpressed in the small scale cultures induced with IPTG.
2.3 Results

2.3.3.b Large scale expression of the fusion protein

Figure 2.6. SDS-PAGE of bacterial proteins.

The proteins were analysed on a 10% SDS-polyacrylamide gel under non-reducing conditions and visualised following staining with Coomassie Brilliant Blue.

Lanes 1, 7: 3 μl of high molecular weight markers.
Lane 2: 10 μl Glutathione-S-transferase (1 mg/ml).
Lanes 3, 4: 3 μl and 10 μl respectively of fusion protein (3.4 mg/ml) purified on glutathione-agarose.
Lane 5: Supernatant after cell lysis and before fusion protein purification.
Lane 6: Unbound fraction washed off from glutathione-agarose.

Samples were kept from the 3 liter culture, from the uninduced stage, from the pellet and from the supernatant that resulted from the harvesting and centrifugation of the large scale cultures.
Samples were kept also from the pellet and the supernatant of the cell lysate fraction. The fusion protein was present in the supernatant but also in large amounts in the pellet (data not shown).
2.3 Results

2.3.3. c Purification of fusion protein

Figure 2.7 Purification of fusion proteins from bacterial cells lysate using a glutathione-agarose affinity chromatography column.

Bacterial lysate was centrifuged and approximately 30 ml of supernatant were applied to a glutathione-agarose affinity column. The column was washed with PBS then eluted with 50 ml Tris-HCl buffer (pH 8.0) containing 5 mM reduced glutathione. The samples were then pooled and dialysed against cleavage buffer. The fusion protein was eluted in 2.5 ml fractions from the glutathione-agarose matrix, in 50 mM Tris-HCl buffer (pH 8.0) containing 5 mM reduced glutathione. Only the elution stage is illustrated.

Fractions giving readings over 0.100 at A280 were pooled and dialysed against cleavage buffer over night. The unbound fraction was applied again on the glutathione-agarose column because it was still containing some fusion protein. Following protein estimation, the lowest quantity of fusion protein obtained from a 3 L culture was 20 mg and the highest quantity was 155 mg, but generally an average of 50 mg of fusion protein was obtained.
2.3 Results

2.3.3.d Removal of GST carrier by proteinase cleavage

In the first attempts to cleave the fusion protein 0.2 % (w/w) thrombin was used. This proved to be insufficient. A range of 0.6, 0.8 and 1 % thrombin was used in the following experiments.

2.3.3.e Attempted galectin-1 purification on glutathione agarose and analysis by SDS-PAGE

Figure 2.8 Purification of galectin-1 from the cleavage mixture using a glutathione-agarose affinity column.

15 ml of sample was applied on the column. The unbound proteins were washed from the column in 1 column volume of 50 mM Tris-HCl buffer (pH 7.5), containing 150 mM NaCl as a single peak. The size of the collected fraction was 1.5 ml.

Fractions 2 -13 were pooled as they constituted the peak, and concentrated with a Macrosep centrifugal concentrator with a molecular weight cut-off limit of 1 kDa before further analysis on a SDS-polyacrylamide gel.
2.3 Results

Figure 2.9. SDS-page analysis of proteins eluted from the glutathione agarose affinity column.

10µl eluted samples were analysed by SDS-PAGE under non-reducing conditions on a 15% polyacrylamide gel. The electrophoresed proteins were visualised following staining with Coomassie Brilliant Blue.

Lanes 1, 3: 10 µl cleaved protein (0.2 mg/ml) purified on a glutathione-agarose affinity column.
Lane 2: 3 µl low molecular weight markers.
Lane 4: 10 µl fusion protein (1 mg/ml).

The glutathione-agarose purified sample proved to be a mixture of 3 proteins with molecular weights of 39 000 Da, 29 000 Da and 14 000 Da respectively. As I was not satisfied with the purity of the galectin sample, I decided to use a gel filtration method to further separate the 3 components.
2.3 Results

2.3.3.1 Further purification of the cleaved protein on Sephadex G-75 and analysis by SDS-PAGE.

Figure 2.10 Purification of the cleaved protein from the mixture of proteins eluted from the glutathione agarose column, through gel filtration on a Sephadex G-75 column. The 2.5 ml of sample applied to the Sephadex G-75 (50 ml) consisted of the pooled and concentrated unbound fractions collected from the glutathione agarose column when cleavage mixture was applied to it (Fig 2.8). The elution buffer was 30 mM Tris HCl (pH 7.5) and fractions of 1.5 ml were collected. Only the elution stage is illustrated.

Proteins were eluted from this column as 2 peaks. Fractions 7-20 constituted peak (PK1) and fractions 21-24 constituted peak (PK2). The fractions PK1 were pooled and concentrated using a Microsep centrifugal concentrator with a Molecular weight cut-off of 1 kDa. The fractions for PK2 were processed the same way.
2.3 Results

Figure 2.11. SDS-PAGE of proteins separated on a Sephadex G-75 gel filtration column.

Proteins were analysed by SDS-PAGE on a 15% polyacrylamide gel under non-reducing conditions and visualised by staining with Coomassie Brilliant Blue.

Lane 1: 5 µl of PK1 sample (2.7 mg/ml).
Lane 2: 20 µl of PK2 sample (<5 µl).
Lane 3: 3 µl of low molecular weight markers.
Lane 4: 10 µl of PK1 sample.
Lane 5: 5 µl of GST sample (4 mg/ml).
Lane 6: 5 µl of FP sample (3.1 mg/ml).
Lane 7: 5 µl of PK1.

This revealed that the peak 1 (PK1) contained three bands corresponding to 25 000 Da, which was overloaded, a very faint band running at 39 000 Da and a band running at 14 000 Da. The second peak (PK2) contained only a 29 000 Da faint band.
2.3 Results

2.3.3.g Protein Sequencing

Figure 2.12. Protein sequencing of the 14 kDa band of the first peak eluted from Sephadex G-75 column.

The N-terminal amino acid sequencing of the 14kDa band revealed the existence of 2 components: a major and a minor fraction. The major fraction was identical to the first 8 amino acids of galectin-1 including the pGEX linker, apart from the non assigned residue at position 5. The minor fraction had the first amino acids identical with the 8 amino acids from positions 52-59 of the recombinant galectin. Residues detected by sequencing in plain text format; published galectin-1 sequence in bold text (Abbot and Feizi, 1989).

```
1  5  10  15  20
G S M A C G L V A S N L N L K P G E C L R
 I I I I I I I I
G S M A ( ) G L V
  25  30  35  40
V R G E V A P D A K S F V L N L G K D S N
  45  50  55  60
N L C L H F N P R F N A H G D A N T I V C
 I I I I I I I I
F N A H G D A N
  65  70  75  80
N S K D G G A W G T E Q R E A V F P F Q P
  85  90  95  100  105
G S V A E V C I T F D Q A N L T V K L P D
  110  115  120  125
G Y E F K F P N R L N L E A I N Y M A A D
  130  135
G D F K I K C V A F D
```

In the original galectin-1 sequence the first Ala is counted as residue no.1 (see section 1.3.1). In our case galectin-1 obtained from cleavage of the GST-fusion protein includes two amino acids of the pGEX linker (Gly and Ser) and Met so the first Ala residue becomes residue no 4. The Arg-48 residue at which the second thrombin cleavage occured becomes residue no 51.
2.3 Results

2.3.3 Galectin-1 purification on asialofetuin-Sepharose and analysis by SDS-PAGE

Figure 2.13 Purification of galectin-1 from a cleavage mixture using an asialofetuin Sepharose affinity chromatography column.
27 ml of cleavage mixture was applied to the column and then washed with 30 mM Tris-HCl, pH 7.5. Galectin-1 was eluted with the same Tris buffer containing 100 mM lactose and 1.5 ml fractions were collected. The size of the collected fraction was 1.5 ml. Only the elution stage of the chromatography is illustrated.

Fractons 14-19 were pooled, desalted and concentrated using a Microsep centrifugal concentrator with a cut-off limit of 1 kDa. Following protein estimation the amount of galectin-1 obtained as a result of the cleavage of the fusion protein with thrombin varied between 1.125 mg and 5.45 mg from 3 liter cultures. Initially the retarded sample did not look very pure (Fig. 2.14,A) therefore we proceeded to a second purification using the same type of column, the results of which are shown in Fig.2.14B. The unbound fraction was also re-chromatographed in order to recover the rest of the unretarded 14 kDa band.

2.3.3.i Protein sequencing

A N-terminal amino acid sequence analysis was performed on both the retarded and the unretarded fractions of the asialo-fetuin column (Fig. 2.14, A). The 25 kDa protein from the unretarded fraction had a slightly jagged N-terminus: MXPILGY or: PILGY that was identical with the published protein sequence of GST (Smith et al., 1986). The protein from the retarded fraction had the N-terminal sequence: GSMACGL (See Fig. 2.12).
2.3 Results

Figure 2.14. SDS-PAGE of proteins purified on a asialofetuin-Sepharose affinity chromatography column.

Samples were desalted and concentrated after being eluted from the affinity column in a Microsepr concentrator with a molecular weight cut-off of 1 KDa, then analysed by SDS-PAGE on a 15 % polyacrylamide gel under non-reducing conditions and visualised using Coomassie Brilliant Blue stain.

A: Samples recovered after being subjected to affinity chromatography.
Lane 1: 10 µl retarded fraction (6.9 mg/ml)
Lanes 2,3: 10 µl unretarded fraction.
Lane 4: 3 µl low molecular weight marker.

B: Samples recovered after rechromatography of the retarded fraction (lane 1, photo A) on the same affinity column.
Lane 1: 10 µl unretarded fraction from the re-chromatography of the first unretarded fraction.
Lane 2: 10 µl FP after long time storage in the freezer.
Lane 3: 10 µl retarded fraction (3 mg/ml).
Lane 4: 3 µl low molecular weight markers.
2.3 Results

2.3.3.j Reverse-phase HPLC

Figure 2.15 RP-HPLC using intact recombinant galectin-1 purified on an asialofetuin affinity column.

The first peak was eluted at 29 minutes from the start of the run (46% gradient) the second peak at 32 min (50% gradient) and the last peak (minor) after 38 min (59% gradient). There was no evidence for the presence of a mixture of proteins in the electrophoretic assay (see figure 2.14 photo B, lane 3) but the HPLC analysis revealed the existence of 2 major and 1 minor peaks. This result made us consider subjecting our sample to a mass spectrometry assay.
2.3 Results

2.3.3. Matrix-Assisted Laser Desorption/ Ionisation Time-of-Flight Mass Spectrometry (MALDI-/TOF MS)

Figure 2.16 MALDI-positive ion mass spectra of galectin.
The m/z regions containing the clusters of multiply charged peaks are shown here. Their respective average molecular masses were calculated. The mass scales were calibrated using multiply charged peaks derived from a standard of BSA, cytC and insulin.
A-The standards were used for an external calibration
B-Internal calibration.

In the first case 3 peaks were detected: 15 818 Da peak representing the specie \((M+H)^+\), a 7 918 Da peak representing the species \((M+2H)^+\) and a 31 839.9 Da peak representing the species \((2M+H)^+\) the galectin-1 dimer. In the second case a 15 786 Da \((M+H)^+\) peak was detected. In an attempt to identify the cause of the abnormal size of our recombinant galectin-1 (expected size was 14853.5 Da and 933 Da was the difference between the detected and the expected molecular weight), we proceeded to use recombinant galectin-1 tryptic peptides for a mass spectrometry determination.
2.3 Results

Figure 2.17. Amino acid sequence of 14-KDa galectin from HepG2 cells to which we added the 2 amino acid residues for the pGEX linker. The tryptic peptides are designated as T1 to T13. We started the counting of amino acid residues from the first linker residue, but we maintained the original position numbers when referring to Cys residues in order to be consistent with the literature (Abbott and Feizi, 1989).

Trypsin cleaves proteins at the C terminal side of Lys and Arg unless they are followed by a proline. As the sequence of the human galectin-1 contains 8 Lys and 5 Arg residues, 13 tryptic peptides were expected to result after trypsin treatment of galectin-1. Galectin-1 contains 6 Cys located in T1, T5, T6, T8 and T13. T1 contains 2 Cys residues (Cys 2 and Cys 16), T5 contains Cys 42, T6 contains Cys 60, T8 contains Cys 88 and T13 contains Cys 130.
2.3 Results

The MALDI-TOF spectra of the tryptic digests of native and DTT-treated galectin are presented in the Appendix 3.

Table 1. Average molecular mass [(M+H) ions] in MALDI-TOF spectra of trypsin digested recombinant human galectin-1 untreated (native) or DTT-treated.

For the theoretical calculations of the peptides, all Cys groups have been assumed to contain free sulphydryl groups. The matrix used for these determinations was sinapinic acid. Signals of 207.2, 225.2 and 448.7 Da representing the species (M-H2O)+H, (M+H) and (2M+H) were given by the matrix.

<table>
<thead>
<tr>
<th>Fragment sequence</th>
<th>Theoretical calculation (M+H) + (Da)</th>
<th>Observed peaks</th>
<th>Peptide assignment</th>
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<tr>
<td></td>
<td>Native (Da)</td>
<td>% error</td>
<td>DTTtreated (Da)</td>
</tr>
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<td>1-21</td>
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<td>22-23</td>
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<tr>
<td>24-31</td>
<td>785.85</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>32-39</td>
<td>877.05</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>40-51</td>
<td>1428.56</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>52-66</td>
<td>1589.72</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>67-76</td>
<td>1076.09</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>77-102</td>
<td>2810.19</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>103-110</td>
<td>968.07</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>111-114</td>
<td>532.60</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>115-130</td>
<td>1785.00</td>
<td>1796.0</td>
<td>0.61</td>
</tr>
<tr>
<td>131-132</td>
<td>259.35</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>133-137</td>
<td>552.62</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

0.5 % is the maximum error accepted between the detected value and the theoretical calculation.

The T5 fragment appeared only in the DTT-treated sample and this suggested the existence of a disulfide bond between T5 and T6. T6 though was supressed in the DTT-treated sample.
2.3 Results

Table 2. Assignment of the tryptic peptides obtained from recombinant galectin-1 to the fragments obtained from the theoretical cleavage of galectin-1 when a maximum of 3 miscleavages occurred.

The matrix used for these determinations was sinapinic acid.

<table>
<thead>
<tr>
<th>Fragment sequence</th>
<th>Theoretical calculation</th>
<th>Observed peaks</th>
<th>% error</th>
<th>Peptide assignment</th>
</tr>
</thead>
<tbody>
<tr>
<td>1-31</td>
<td>3154.6</td>
<td>3163.7</td>
<td>0.28</td>
<td>T1+T2+T3</td>
</tr>
<tr>
<td>67-110</td>
<td>4818.3</td>
<td>4828.5</td>
<td>0.20</td>
<td>T7+T8+T9</td>
</tr>
<tr>
<td>103-114</td>
<td>1482.6</td>
<td>1494.2</td>
<td>0.78</td>
<td>T9+T10</td>
</tr>
<tr>
<td>111-137</td>
<td>3075.5</td>
<td>3081.4</td>
<td>0.19</td>
<td>T11+T12+T13</td>
</tr>
</tbody>
</table>

Apart from the peaks contained in Tables 1 and 2, 6 peaks (1088, 1155, 1834, 2182, 3011 and 4584 Da) were detected in the "native" sample and 6 peaks (238, 248, 471, 487, 710 and 2423.6 Da) were detected in the DTT-treated sample. All but peak 3011 remained unassigned. T4 was the only tryptic peptide unaccounted for when we used sinapinic acid as a matrix.

The results of liquid secondary ion mass spectrometry (LSIMS) and Edman sequencing done by Tracey et al. (1992) on bovine galectin-1 have indicated that disulfide bonding occurs predominantly between Cys-2 and Cys-130, Cys-16 and Cys-88 and between Cys-42 and Cys-60. I started by trying to find peaks in the "native" sample that would coincide with the molecular weight of the fragments that could result if these disulfide bonds were present.

A 3 000.2 Da peak was expected in case there was a disulfide bond and a peptide link between fragments T5 and T6 (Cys-42 - Cys-60). A 3 018.2 Da peak was expected if there was only a disulfide bond that linked the two peptides and this was the closest to the the 3 011.0 Da peak detected.

A 2 684.1 Da peak was expected in case there was a disulfide bond between T1 and T13 (Cys-2 - Cys-130) and a 4941.5 Da peak was expected if there was a disulfide bond between T1 and T8 (Cys-16 - Cys-88). A 5 494.3 Da peak was expected if both disulfide bonds mentioned above were present at the same time. No peaks of this size or of the sizes corresponding to any other possible combination of disulfide bonds (Appendix 3) were present in the "native" sample.
### 2.3 Results

**Table 3. Average molecular mass [ (M+H) ions ] in MALDI-TOF spectra of trypsin digested recombinant human galectin-1 untreated (native) or DTT-treated detected using a α-CHC matrix and (values in brackets) a DHB matrix.**

Signals of 190.2 and 379.2 Da representing the \((M+H)^+\) respectively the \((2M+H)^+\) species were emitted by the α-CHC matrix.

Signals of 137.1 and 155.1 Da representing the \((M-H_2O+H)^+\) respectively the \((M+H)^+\) species were emitted by the DHB matrix.

<table>
<thead>
<tr>
<th>Fragment sequence</th>
<th>Theoretical calculation (M+H)^+ (Da)</th>
<th>Native (Da)</th>
<th>% error</th>
<th>DTT treated (Da)</th>
<th>% error</th>
<th>Peptide assignment</th>
</tr>
</thead>
<tbody>
<tr>
<td>1-21</td>
<td>2131.50</td>
<td>2131.9</td>
<td>0.01</td>
<td>2131.9</td>
<td>0.01</td>
<td>T1</td>
</tr>
<tr>
<td></td>
<td>(2134.1)</td>
<td>(2134.1)</td>
<td>0.12</td>
<td>(2131.7)</td>
<td>0.00</td>
<td></td>
</tr>
<tr>
<td>22-23</td>
<td>273.33</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>T2</td>
</tr>
<tr>
<td>24-31</td>
<td>785.85</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>T3</td>
</tr>
<tr>
<td>32-39</td>
<td>877.05</td>
<td>876.8</td>
<td>0.02</td>
<td>-</td>
<td>-</td>
<td>T4</td>
</tr>
<tr>
<td></td>
<td>(876.0)</td>
<td>(876.0)</td>
<td>0.11</td>
<td>-</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>40-51</td>
<td>1428.56</td>
<td>1429.5</td>
<td>0.01</td>
<td>-</td>
<td>-</td>
<td>T5</td>
</tr>
<tr>
<td></td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>T6</td>
</tr>
<tr>
<td>52-66</td>
<td>1589.72</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>T7</td>
</tr>
<tr>
<td>67-76</td>
<td>1076.09</td>
<td>1076.1</td>
<td>0.00</td>
<td>1077.0</td>
<td>0.08</td>
<td>T8</td>
</tr>
<tr>
<td></td>
<td>(1077.1)</td>
<td>(1077.1)</td>
<td>0.10</td>
<td>(1075.0)</td>
<td>0.13</td>
<td></td>
</tr>
<tr>
<td>77-102</td>
<td>2810.19</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>T9</td>
</tr>
<tr>
<td>103-110</td>
<td>968.07</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>T10</td>
</tr>
<tr>
<td>111-114</td>
<td>532.60</td>
<td>532.9</td>
<td>0.05</td>
<td>-</td>
<td>-</td>
<td>T11</td>
</tr>
<tr>
<td>115-130</td>
<td>1785.00</td>
<td>1785.4</td>
<td>0.02</td>
<td>1787.1</td>
<td>0.00</td>
<td>T12</td>
</tr>
<tr>
<td></td>
<td>(1787.0)</td>
<td>(1787.0)</td>
<td>0.1</td>
<td>-</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>131-132</td>
<td>259.35</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>T13</td>
</tr>
<tr>
<td>133-137</td>
<td>552.62</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td></td>
</tr>
</tbody>
</table>

Apart from the peaks listed in Tables 3 and 4, peaks of 914, 1 982, 1 734, 3 019 and 5 507 Da were detected in the "native" sample and 441, 504, 1 563, 2 424, 2 785 and 1 787 Da were detected in the DTT-treated sample when we used the α-CHC matrix. Peaks of 1 564 and 1 726 were detected in the "native" sample and 2 784 and 2 424 Da were detected in the DTT-treated sample when we used the DHB matrix. None of these peaks, except the 3 019 Da and the 5 507 Da ones, corresponded to any of the fragments that could have resulted from miscleavages or to any of the possible disulfide-linked peptide combinations (Appendix 3). The 3 019 Da peak represented peptides T5 and T6 linked only through a disulfide bond (3 020 Da expected size, 0.1 % error) while the 5 507 was a minor peak that could correspond to the disulfide -linked peptides T13-T1-T8 (5 494 expected size, 0.2 % error).
### 2.3 Results

Table 4. Assignment of the tryptic peptides obtained from recombinant galectin-1 to the fragments obtained from the theoretical cleavage of galectin-1 when a maximum of 3 miscleavages occurred: A) when a α-CHC matrix was used and B) when a DHB matrix was used.

<table>
<thead>
<tr>
<th>Fragment sequence</th>
<th>Theoretical calculation</th>
<th>Observed peaks</th>
<th>% error</th>
<th>Peptide assignment</th>
</tr>
</thead>
<tbody>
<tr>
<td>1-31</td>
<td>3154.6</td>
<td>3156.7</td>
<td>0.13</td>
<td>T1+T2+T3</td>
</tr>
<tr>
<td>22-31</td>
<td>1041.2</td>
<td>1042.1</td>
<td>0.01</td>
<td>T2+T3</td>
</tr>
<tr>
<td>67-102</td>
<td>3868.2</td>
<td>3876.4</td>
<td>0.21</td>
<td>T7+T8</td>
</tr>
<tr>
<td>77-114</td>
<td>4274.8</td>
<td>4278.8</td>
<td>0.09</td>
<td>T8+T9+T10</td>
</tr>
<tr>
<td>103-114</td>
<td>1482.6</td>
<td>1482.9</td>
<td>0.02</td>
<td>T9+T10</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Fragment sequence</th>
<th>Theoretical calculation</th>
<th>Observed peaks</th>
<th>% error</th>
<th>Peptide assignment</th>
</tr>
</thead>
<tbody>
<tr>
<td>22-31</td>
<td>1041.1</td>
<td>1041.2</td>
<td>0.00</td>
<td>T2+T3</td>
</tr>
<tr>
<td>22-39</td>
<td>1900.2</td>
<td>1899.4</td>
<td>-0.04</td>
<td>T2+T3+T4</td>
</tr>
<tr>
<td>67-102</td>
<td>3868.2</td>
<td>3879.0</td>
<td>0.27</td>
<td>T7+T8</td>
</tr>
<tr>
<td>103-114</td>
<td>1482.6</td>
<td>1484.6</td>
<td>0.13</td>
<td>T9+T10</td>
</tr>
<tr>
<td>115-132</td>
<td>2026.3</td>
<td>2028.6</td>
<td>0.11</td>
<td>T11+T12</td>
</tr>
</tbody>
</table>

In the case of the DHB matrix, in the DTT-treated sample we detected the T2+T3, and T11+T12 fragments resulted from miscleavages.

The purpose of the mass spectrometry analysis of tryptic peptides was to detect a peptide of an abnormal size that could have explained the unexpected size of the whole galectin-1 molecule. We accounted for all the tryptic peptides and none of them seem to have suffered any change from the predicted size.
2.4 DISCUSSION

For first strand cDNA synthesis, 2 flasks of nearly confluent U2OS cells were necessary, or 2 flasks of nearly confluent fibroblasts, but only one flask of nearly confluent HEla cells. The synthesis of cDNA was performed the very same day after the extraction of RNA from cells because previously when I used a 1 day old RNA, I was unsuccessful in synthesising cDNA. I thought that this was evidence that the RNA is subject to degradation.

The use of a "hot start" in the protocol for PCR was aiming to minimise non-specific annealing and primer elongation events prior to the start of the amplification of the cDNA template. This involved the addition of the antisense primer and AmpliTaq DNA polymerase once the reaction mixture had reached 95°C.

2.4.1 Synthesis of recombinant galectin-1 in yeast

A number of yeasts have become important host organisms for foreign gene expression because of advantages in promoter strength, secretion efficiency, or ease of growth to high cell density, but insertion of a foreign gene into an expression vector does not guarantee a high level of the foreign protein because gene expression is a complex multi-step process and problems can arise from the stages of transcription through to protein stability (Romanos et al., 1992). There seems to be a frequent wide variation in the productivity of different transformants when 2 μ vectors are used to express foreign genes in yeast. This phenomenon is due to a variation in the plasmid copy number between different transformants (Romanos et al., 1992).

The transformation of E. coli with the modified pCNNinv vector was performed in order to verify the presence of the insert in the plasmid. This would have been very hard once the vector was introduced in yeast cells because they are much harder to lyse. Once I was certain that an insert was present in the plasmid I proceeded to transformation of yeast cells for recombinant galectin-1 production. None of the proteins eluted from the affinity columns, used in order to separate galectin-1, had the molecular weight of galectin-1 (Fig. 2.4), and the yields obtained were very low.

As mentioned in Section 1.3.6, galectin-1 has no typical signal peptide but it is abundant both in the cytosol and extracellularly. The fact that galectin was not recovered as expected from the growth medium of the yeast cells might be due to the fact that it
became insoluble when expressed in the yeast cytoplasm as do many other foreign secreted proteins, but there are also examples of secreted proteins which are soluble and biologically active in the same environment.

For the usually secreted proteins there are a number of stages in the secretory process in which problems might occur. They could be malfolded, retained in the ER and degraded, retained in the Golgi apparatus and degraded, or retained in the cell wall (for large proteins). I could not be certain that this applies to our protein, as galectin-1 does not seem to be secreted by the classical secretory mechanisms anyway. There are other undesired events such as aberrant processing or hyperglycosylation, that might affect the secretion of foreign proteins. Binding of galectin to β-galactosides during secretion might also be an explanation of the absence of galectin from the medium.

Because galectin-1 clearly does not have a signal sequence, I decided to express it using a plasmid that would equip this protein with a yeast invertase (SUC2) signal sequence. The signal peptide is usually removed in the endoplasmic reticulum and the protein is extensively glycosylated even at sites which are normally not glycosylated (Romanos et al., 1992).

Proteins like interleukin (IL)-1 α and 1β, which are naturally secreted proteins but are not externalised through the normal secretory pathway in mammalian cells and thus are not glycosylated, are glycosylated when secreted from yeast. This might be the case with galectin-1 as well and this might explain why I obtained bands of different sizes than expected when I analysed these samples on SDS-polyacrylamide gels. Because the yields were low and the molecular weights of the bands obtained did not correspond to the molecular weight of galectin-1 I decided to use a bacterial expression system as an alternative.

2.4.2 Synthesis of recombinant galectin in bacteria

For the induction of the synthesis of the fusion protein on a small scale, a final concentration of 0.2 mM IPTG (Guan and Dixon, 1991) was tested but no induction was seen. As a result of this, a 1 mM IPTG final concentration was tested and proved successful. The variations from the initial protocol for the growth of large scale cultures aimed at improving the yield of fusion protein obtained.

The pGEX vector confers resistance to ampicillin for the E.coli cells because it contains the β-lactamase gene. Some of the β-lactamase protein is secreted into the growth
medium where it destroys ampicillin. Once all the ampicillin is destroyed, cells that have lost the expression plasmid may grow (Glover and Hames, 1995). This is why I chose to add more ampicillin during the incubation of cultures and that is why fresh plates were inoculated with freezer stored colonies and used as an inoculum for large scale cultures.

The presence of the fusion protein in the pellet resulting from the centrifugation of the cell lysate of the large scale preparations indicated that a fraction of this protein was precipitating out of solution despite the fact that Triton X-100 was added to the cell lysate to keep the protein soluble during processing of samples. Immediate application of supernatant from the cell lysate on a glutathione-agarose column was recommended because more of the fusion protein precipitated if supernatant was stored in the freezer and then thawed. It was observed that truncated GST-galectin fusion proteins seemed to be even less soluble than the original fusion proteins (Cameron, 1995). The mobility of the fusion protein in an SDS-PAGE corresponded to 37 kDa while a value of 43 kDa would be expected from the known molecular weights of the components: 14 kDa for galectin-1 and 25 kDa for glutathione S-transferase.

The best results for the removal of the GST carrier were when 1 % w/w thrombin was used for cleavage. However (see below) the yields were low. The separation of the cleaved protein from the glutathione S-transferase was apparently not successful because the uncleaved fusion protein and the GST were not retained by the column as expected. On the other hand, the sequencing of samples eluted from an asialofetuin column showed that the 25 kDa band contained only the galectin dimer.

This could explain the presence of a 25 kDa band washed from the glutathione-agarose column together with the monomeric galectin of 14 kDa, but cannot explain why the uncleaved fusion protein was not retained on this column. The anomalous mobility and the difficulty in separating the GST and galectin-1 by glutathione-agarose chromatography, suggest that some form of interaction might occur between the two proteins. I was almost certain that the presence of a 14 kDa band in the unretarded fraction (Fig. 2.14, A lanes 2 and 3) is due to the fact that the asialofetuin-Sepharose column had reached saturation before it could bind all the amount of galectin present in the sample.

I calculated that about 50 mg of fusion protein should yield 18 mg of galectin. Comparing this result with the amount of protein actually obtained (5.45 mg) I noticed that the efficiency of the cleavage reaction was about 30 %. An explanation for the fact that
2.4 Discussion

The cleavage reaction did not have the expected efficiency is that there was some proteolytic degradation of the galectin itself. Another explanation could be the fact that the purified fusion protein was slightly precipitating when thawed and prepared for cleavage. Other research groups succeeded in obtaining 6 mg of recombinant galectin-1 from 3 L cultures using a pH14GAL expression plasmid (Hirabayashi et al., 1989).

The sequencing of the 14 kDa fraction (Fig. 2.12) provided some evidence for thrombin degradation of the recombinant galectin during fusion protein cleavage (the minor fraction). Although this component was apparently 48 amino acids shorter than galectin-1, they both had the same mobility on a SDS-PAGE gel.

This suggests that the N-terminal peptide generated by thrombin cleavage at Arg-48 (Arg 51 with the linker) remains attached to the rest of the protein, probably through a disulfide bond.

When sequenced, the 25 kDa band from the retarded fraction (Fig. 2.14 A, lane1), proved to be the galectin dimer. That is why the retarded fractions containing both 14 kDa and 25 kDa, obtained later on, were considered to be pure galectin. When sequenced, the 25 kDa band from the unretarded fraction (Fig. 2.14 A, lanes 2 and 3) proved to be GST.

The HPLC analysis of the recombinant galectin revealed the existence of a doublet peak which could have represented the 2 fractions (major and minor) observed in the sequencing of the 14 kDa band. The second peak (a minor one) could have represented the galectin dimer. In order to get more information about the purity of our galectin preparation we decided to subject it to a mass spectrometry analysis.

The MALDI-TOF analysis of the intact galectin gave a molecular weight of 15 786.2 Da for the intact recombinant galectin-1, 933 Da bigger than the theoretically calculated molecular weight for galectin-1 (accounting for the pGEX linker), 14 853.7 Da. Obviously, the measured masses did not agree with the theoretically calculated molecular weights. I thought that there might be an insert of some kind in our protein, so I repeated the DNA sequencing (see Appendix 1) and proceeded to do a mass spectrometry analysis of the tryptic peptides of galectin-1.

The DNA sequencing showed no evidence of any mutation or extension of the galectin-1 sequence. Using 3 different kinds of matrices for MALDI-TOF analysis I accounted for all the tryptic peptides of galectin-1.

Initially I assumed that the “minor” fraction that was detected during the protein sequencing consisted of 86 amino acid residues and therefore had a molecular weight of
2.4 Discussion

9 447 Da. I did not detect any peak of this size in the mass spectrometry determinations so it might have been that this fragment was much shorter and corresponded to one of the tryptic peptides or miscleavage fragments obtained.

Another hypothesis was that the transcription of the galectin-1 gene failed to end at the first stop codon but continued and stopped 30 nucleotides (10 amino acids) down-stream from that, where a second stop codon was present, in phase with the rest of the sequence (see Appendix 1).

From the DNA sequence I identified the 10 amino acids contained in a likely extra peptide at the C-terminal of the recombinant galectin as being VDSSGRIVTD, and I calculated the size of this peptide as being 1 029.2 Da and not 932.5 Da bigger, as resulted from the comparison of the MALDI-TOF determination with the predicted size of the galectin-1.

There is an Arg residue in the theoretical extra peptide which, if it was subjected to cleavage, would have yielded an 428 Da fragment and a T13 + X = 552.6 Da + 601.2 Da = 1 153.8 Da fragment. In one of the MALDI-TOF determinations a 1,155 Da fragment was detected (% error 0.1 Da, Appendix 3). A 428 Da fragment was not detected.

It is worth mentioning that we would have expected to detect both species: the normal size and the larger size protein in case this hypothesis was true.

I think that the anomalous size of galectin-1 might be due to detection errors as both Ms Christina Buchanan and I experienced difficulties in identifying one of the molecular weight markers (Cyt C, see Fig. 2.16 B).

As for the disulfide bonds in the galectin-1 molecule there was clear evidence for the existence of a disulfide bond between Cys 42 and Cys 60 but no evidence for the existence of any of the other possible disulfide bonds. Despite the presence of this disulfide bond galectin-1 had retained its carbohydrate-binding properties. This confirms the conclusion of Abbot and Feizi (1991) that only the oxidation of the Cys 2 and Cys 130 residues are critical for the carbohydrate-binding function of galectin-1. The formation of the disulfide bonds at these Cys residues was probably affecting the invariant HFNPREF (47-52) residues which are involved in galactoside-binding while the other invariant residues (WGTEQRE, 71-77) should be still functional. This might mean that as long as the last of these two conserved sequences is available, galectin-1 should still have a functional carbohydrate-binding site.
Chapter 3

Functional studies of human recombinant galectin-1
Chapter 3. Functional studies of human recombinant galectin-1

3.1 Introduction

Cell numbers in the human body are regulated by a balance between proliferation and programmed cell death (apoptosis). These are active processes regulated by mitogenic growth factors and negative growth factors, as well as by survival factors (Shaun and Thomas, 1996). In contrast to the cell lines, the primary cells in the human body are either not dividing or are in the process of renewing or expanding (blood cells, epithelial cells from epidermis and intestinal epithelium). Once a quiescent cell has been stimulated to proliferate by a mitogenic growth factor, it enters the cell cycle. This can be arrested during either G₁ or G₂ if DNA needs to be repaired or the cell requires nutrients, but arrest can occur also when a cell is exposed to negative growth factors, or if the level of the positive growth factors is decreased (Shaun and Thomas, 1996). Growth factor deprivation can also lead to apoptosis in certain cells. One relevant question is: does treatment with galectin (a negative growth factor) actually induce apoptosis in cultured cells?

A negative growth regulator which was first identified in mouse fibroblasts was shown to be a galectin (mGBP, Wells and Mallucci, 1983).

A β-galactoside-binding protein was also isolated from conditioned medium from human fibroblasts and this protein acted both as a cellular growth inhibitor and as a lectin (Manilal et al., 1993). Most of the published work regarding these proteins was discussed in Chapter 1, p.20.

The lability of the mGBP suggested the possibility that it might be a substrate for a proteinase (the mouse homologue of the growth-related proteinase, GRP). Some
3.1 Introduction

circumstantial evidence to support this hypothesis was obtained when the yields of the human GBP increased in cultures treated with GRP inhibitors. This purified protein acted as a cellular growth inhibitor on human fibroblasts and on U2OS osteosarcoma cells, but not on HELA carcinoma cells (Manilal et al., 1993).

The recombinant galectin was needed in order to extend this work. This chapter describes the hemagglutination, growth inhibition, degradation and apoptosis experiments in which the recombinant galectin was used.

3.2 Materials and Methods

Materials

Unless otherwise specified, all chemicals used were of analytical grade and were obtained from the Sigma Chemical Company, U.S.A.
Rabbit erythrocytes were obtained from the bleeding of a New Zealand White rabbit.
Human fibroblasts (MPS), human epithelial carcinoma (HELA) cells, hepatoma cells (Hep2) and human U2OS osteosarcoma cells were used in these experiments.
The materials that have not been listed here but have been used in these experiments have been already specified in Section 2.2.

Methods

Cell culture and subculturing techniques employed in this chapter are outlined in Sections 2.2.1 and 2.2.2.

3.2.1 Hemagglutination assays

The method used for hemagglutination experiments was developed by Lis and Sharon (1972). Erythrocytes, separated from the venous whole rabbit blood by centrifugation at 2 000 r.p.m. for 5 min in a bench top centrifuge, were used to make a stock blood cell suspension in Alsever's solution. This was made of a mixture of 100 ml of a solution of 0.1 M glucose, 0.027 M sodium citrate and 0.07 M of NaCl in water brought to pH 6.1 by the addition of solid citric acid, to which 1/30 volume of anticoagulant (0.27 M sodium
3.2 Materials and Methods

citrate and 54 ml of 37 % formaldehyde in 100 ml saline) was added. A standard trypsinised erythrocyte suspension was prepared on the day of the assay. Erythrocytes were collected from the stock blood cell suspension by centrifugation using a bench centrifuge at 2 000 r.p.m. for 5 minutes (as before) and washed 3 times with 5 ml saline solution (0.9 % solution of NaCl in water) for each ml of erythrocytes. One part of 1 % trypsin solution was added to 10 parts of erythrocyte suspension and the mixture was incubated at 37°C for 1 hour. The trypsinised erythrocytes were then washed with saline to remove trypsin and the cells were re-suspended as a 4 % suspension in 0.1 M glycine in PBS. The hemagglutination reaction mixture was set up in a ceramic tile and the contents of each well were:

100 µl 0.15 M NaCl
100 µl 1% BSA in 0.15 M NaCl
100 µl recombinant galectin dissolved in PBS or MEPBS (1 mg/ml serial three-fold dilutions in the same buffer).
100 µl of trypsinised erythrocytes

The mixture was mixed gently and then incubated for 1 minute. The blank contained exactly the same volumes of reagents except for the absence of the lectin.

Hemagglutination experiments were carried out in the presence and absence of β-mercaptoethanol (10 mM) and of lactose (100 mM). The identification of the endpoint of the titration was done by visually comparing the samples with the blank.

3.2.2 Cell proliferation assays

Confluent flasks of cells were trypsinised and cells were re-suspended in an aliquot of Dulbecco's Modified Eagle's Medium containing 10 % foetal calf serum. A fraction of this was transferred to a sterile vial and serial dilutions were carried out in the same medium. 100 µl of diluted cells were dispensed into 96 well plates (Nunc). The cells were allowed to grow for 24 hours and the wells which were 20 % confluent were selected for growth studies. The medium from these wells was replaced with medium containing the appropriate effectors in serial dilutions (100 µl). Wells that had a change of medium without effectors were used as controls. The effectors were either fusion protein (FP), glutathione-S-transferase (GST), galectin-1, galectin-1 in conjunction with lactose (100
3.2 Materials and Methods

mM) or lactose only. GST, FP and galectin-1 were each separately diafiltered against DMEM in a 1 kDa Microsep concentrator to a concentration of 0.44 mg/ml and then filter-sterilised. In experiments where lactose was used in conjunction with these effectors or by itself lactose was added to the medium to a concentration of 100 mM before filter sterilising. Sterile foetal calf serum was added to this mixture to a concentration of 10 %. Serial dilutions (3-fold) of the effectors were achieved using sterile DMEMS and then the effector solutions were added to cells.

The 96 well plates were incubated at 37°C until the control wells became confluent. At this stage the medium was removed and the cells were washed twice with PBS and then fixed with buffered neutral formalin (22.2 mM Na₂HPO₄, 54.2 mM NaH₂PO₄, 4 % formalin), which was followed by three rinses with MilliQ water. The wells were afterwards incubated at room temperature with Giemsa stain (100 µl, 0.1 % in PBS) for 16 hours. After the stain was removed the wells were washed with PBS, and dried before measuring the absorbance in each well at 540 nm using a Titertek Uniscan spectrophotometer. The spectrophotometer was calibrated using an empty well. Because of the costs of producing the recombinant galectin, cell growth was often measured only in duplicate microwells. Average values are expressed as a percentage, relative to control wells (the wells that had a change of medium), arbitrarily set at 100 %.

Experimental variation was determined as previously described (Scott and Tse, 1988).

For studies of DNA synthesis, quiescent cells in 24-well culture plates were washed several times in serum-free Dulbecco's modified Eagle's medium and incubated in this medium for 24 h. Treatment with effectors was performed for another 24 h. A positive control was provided by adding 10 % foetal calf serum to duplicate wells at this stage. After 24 h, [³H] thymidine (Amersham; 80 Ci/mmol; 20 µCi/well) was added for 2 h, after which the cells were washed four times in serum-free medium, and solubilised in 1% Triton X-100 for 2h. Trichloroacetic acid-insoluble radioactivity was measured as described by Hamilton et al. (1990).

3.2.3 Cytotoxicity studies

The method developed by Ponsoda et al. (1991) and modified by Mr J. Bai (personal communication) measures reduction of a tetrazolium salt (MTT) by the NADH generated by the LDH (from viable cells), which results in the formation of a violet formazan dye.
3.2 Materials and Methods

U2OS cells were plated out in 96-well plates and grown in DMEMS. When the cells had reached 90% confluence, galectin (diafiltered against DMEM to which sterile foetal calf serum was added to 10%) replaced the initial growth medium. Galectin concentrations were 0.5 mg/ml, 0.25 mg/ml, and 0.125 mg/ml in 200 µl. The experiment was done in triplicates and galectin was incubated on cells for 20 hours. A fourth triplicate in which the initial medium was replaced with DMEMS and the cells were also incubated for 20 hours was kept as a control. When treatment of the cells was finished, the 96-well plate was washed with PBS. MTT (5 mg/ml) was diluted in DMEMS (1:10) then added to the cells and incubated for further 4 hours. The medium was replaced with a lysis buffer containing 20% SDS/50% DMF (100 µl/well). Plates were incubated at 37°C overnight and read the next day using an ELISA plate reader at 570 nm. The experiment was repeated with plates in which cells were left to reach 60% confluence. These studies were done with much-appreciated help from Mr. J Bai and the data from his studies of the effect of amylin on rat insulinoma cells were used as a positive control.

3.2.4 Experiments of galectin proteolysis and degradation by cells

For the proteolysis experiments, 3 aliquots of 10 µl galectin solution (3.5 mg/ml) were mixed with 10 µl 1% trypsin solution and incubated for 1, 3, and 6 hours at 37°C. PMSF was added at the end of the incubation in order to stop the proteolysis reaction. Proteolysis experiments were performed also in the presence of lactose. In this case the trypsin stock solution contained also 100 mM lactose. Samples were electrophoresed on a 15% SDS polyacrylamide gel after proteolysis.

For degradation experiments galectin was diafiltered in a 1kDa Microsep concentrator against DMEM in the absence of foetal calf serum, and filter-sterilised. Sterile foetal calf serum was added to the aliquot to a concentration of 10% and then 100 µl aliquots of this solution were incubated on cultures of U2OS or MPS confluent cells in 96 well plates for 1, 4, 6 and 24 hours and then the reaction was stopped by adding 10 µl 0.1 mM PMSF. The medium was removed from cultures and 10 µl of each were analysed on 15% or 20% SDS polyacrylamide gels. In other experiments galectin was incubated with cells for 4, 6, 16 hours, 2 days and 3 days and then the reaction was stopped with PMSF. As before, 10 µl of medium was electrophoresed on 15% SDS polyacrylamide gels.
3.3. Results

3.3.1 Hemagg.

Figure 3.1. Hemagg.

Initial concentration of the fusion protein was an infective dose, as seen in the subsequent one in the supernatant.

Well 1A: 100 μl of sample
Well 1B: 100 μl of dilution
Well 1C: 100 μl of dilution
Well 1D: 100 μl of dilution
Well 2A: 100 μl of dilution
Well 2B: 100 μl of dilution
Well 2C: 100 μl of dilution
Well 2D: 100 μl of dilution
Well 3A: 100 μl of dilution
Well 3D: 100 μl of dilution

The fusion protein, FP, agglutinated to 1 μg/ml and this was demonstrated. An identical result was obtained (not shown).
3.3. Results

3.3.1 Hemagglutination experiments

Figure 3.1. Hemagglutination experiment using fusion protein (FP) as an effector. Initial concentration of FP was 1 mg/ml in PBS and this was subsequently diluted in three-fold serial dilutions to be used for the rest of the wells. All the wells contained the usual reaction mixture described in Section 3.2.

Well 1A: 100 µl of FP (1 mg/ml).
Well 1B: 100 µl of FP (0.33 mg/ml).
Well 1C: 100 µl of FP (0.11 mg/ml).
Well 1D: 100 µl of FP (0.036 mg/ml).
Well 2A: 100 µl of FP (0.012 mg/ml).
Well 2B: 100 µl of FP (0.004 mg/ml).
Well 2C: 100 µl of FP (0.0013 mg/ml).
Well 2D: 100 µl of FP (0.0004 mg/ml).
Well 3A: 100 µl of FP (0.0001 mg/ml)-agglutination end point.
Well 3D: 100 µl of PBS (blank).

The fusion protein was still a lectin, as seen in this experiment. FP agglutinated the trypsinised rabbit erythrocytes down to a concentration of 0.1 µg/ml and this was considered to be the agglutination end point. An identical result was obtained in the presence of β-mercaptoethanol (results not shown).
3.3 Results

Figure 3.2 Hemagglutination experiment using fusion protein and cleaved protein as effectors, in the presence of lactose.

Initial concentration of FP was 1 mg/ml in PBS and this was subsequently used for three-fold serial dilutions for the rest of the wells. For one of the wells, undiluted cleaved protein (100 μl) purified on a glutathione agarose column was used.

All the wells contained the usual reaction mixture described in Methods (Chapter 3) except for the fact that the 100 μl of 0.15 M NaCl also contained lactose to a concentration of 100 mM. The wells contained also 100 μl of different concentrations of effector.

Well 1A: 100 μl FP (1 mg/ml).
Well 1B: 100 μl FP (0.33 mg/ml).
Well 1C: 100 μl FP (0.11 mg/ml).
Well 1D: 100 μl FP (0.036 mg/ml).
Well 2A: 100 μl FP (0.012 mg/ml).
Well 2B: 100 μl FP (0.004 mg/ml).
Well 2C: 100 μl FP (0.0013 mg/ml).
Well 2D: 100 μl FP (0.0004 mg/ml).
Well 3A: 100 μl FP (0.0001 mg/ml).
Well 3C: 100 μl PK1 (<50 mg/ml).
Well 3D: 100 μl PBS (blank).

Lactose, as expected, inhibited the hemagglutination reaction, independent of the effectors used.
3.3 Results

Figure 3.3 Hemagglutination experiment using glutathione-S-transferase (GST) as effector.

Initial concentration of GST was 1 mg/ml in PBS and this was subsequently used for the three-fold serial dilutions for the rest of the wells. All the wells contained the usual reaction mixture described in section 3.2. The wells contained also 100 µl of different concentrations of effector.

Well 1A: 100 µl GST (1 mg/ml).
Well 1B: 100 µl GST (0.33 mg/ml)-agglutination end-point.
Well 1C: 100 µl GST (0.11 mg/ml).
Well 1D: 100 µl GST (0.036 mg/ml).
Well 2A: 100 µl GST (0.012 mg/ml).
Well 2B: 100 µl GST (0.004 mg/ml).
Well 3D: 100 µl PBS (blank)

Glutathione-S-transferase (GST) had agglutinating activity, but much weaker then all the other effectors. GST agglutinated trypsinised rabbit erythrocytes down to a concentration of 0.33 mg/ml (agglutination end-point).
3.3 Results

Figure 3.4 Hemagglutination experiment using peak1 (PK1) and peak2 (PK2) as effectors.

Initial concentration of PK1 was 1mg/ml in PBS. Initial concentration of PK2 was 0.1 mg/ml in PBS. These samples were subsequently used for the three-fold serial dilutions for the rest of the wells. All the wells contained the usual reaction mixture described in Section 3.2.

The wells also contained 100 µl of different concentrations of effectors.

Well 1A: 100 µl PK1 (1 mg/ml).
Well 1B: 100 µl PK1 (0.33 mg/ml).
Well 1C: 100 µl PK1 (0.11 mg/ml).
Well 1D: 100 µl PK1 (0.036 mg/ml).
Well 2A: 100 µl PK1 (0.012 mg/ml).
Well 2B: 100 µl PK1 (0.004 mg/ml) end point of agglutination.
Well 3A: 100 µl PK2 (0.1 mg/ml).
Well 3B: 100 µl PK2 (0.033 mg/ml) end point of agglutination.
Well 3C: 100 µl PK2 (0.011 mg/ml).
Well 3D: 100 µl PBS (blank).

PK1 and PK2 were the 2 peaks obtained when we attempted to purify the mixture of proteins eluted from the glutathione agarose affinity column (after FP cleavage was performed), on a gel filtration (G75) column (Section 2.3.3.f). PK1 had agglutinated erythrocytes down to a concentration of 0.004 mg/ml (agglutination end point), while PK2 had an agglutination end point at a concentration of 0.033 mg/ml. In conclusion, it is clear that purified recombinant galectin-1, as well as the GST-galectin fusion protein, were effective in agglutinating rabbit erythrocytes.
3.3 Results

3.3.2. Cell proliferation assays

Figure 3.5 Inhibition of Hep2 human tumour cell growth by recombinant galectin1.

Sparse cultures of human Hep 2 cells were treated with the GST-galectin fusion protein (FP) (rows A), with a "galectin-1 preparation" (rows B) and with recombinant GST (rows C).

Wells 1A: Initial FP concentration was 0.5 mg/ml.
Wells 2, 3, 4, 5, 6A: serial 3-fold dilutions of FP.
Wells 1B: The concentration of the 14KDa protein from the "galectin-1 preparation" was approximately 0.5 mg/ml (the sample was a mixture of 3 proteins (see Fig 2.9) in a ratio of approximately 1:1:1) The initial total protein concentration was 1.5 mg/ml.
Wells 2, 3, 4, 5, 6B: serial 3-fold dilutions of "galectin-1 preparation".
Wells 1C: Initial GST concentration was 0.5 mg/ml.
Wells 2, 3, 4, 5, 6 C: serial 3-fold dilutions of GST.

Growth inhibition was seen only when cells were treated with the mixture of FP, galectin-1 and GST (rows B), a mixture eluted from the glutathione-agarose affinity column. When treated with pure FP (rows A) and GST (rows B) separately there was no growth inhibitory effect.
3.3 Results

In the first set of experiments fusion protein and glutathione-S-transferase were used as effectors in parallel with galectin-1. These precautions were taken because the galectin-1 sample was not pure (see Section 2.3.3.e). Once the purity of the galectin-1 sample was satisfactory these controls were not necessary.

Figure 3.6 Cell proliferation assay using galectin-1 on HEP 2, HELA and U2OS cells and also FP on HEP 2 cells.
Starting concentration of galectin-1 was 0.44 mg/ml.
Starting concentration of FP was 0.44 mg/ml. 4 fold dilutions of the effector were used.
Each point represents the mean of at least four determinations (+S.E.M). Error bars are included only for one data set for clarity purposes.

No inhibitory effect was seen when HELA cells were treated with galectin-1 and when HEP 2 cells were treated with fusion protein (FP). A strong inhibitory effect was seen when HEP 2 and U2OS cells were treated with galectin-1. Fusion protein and glutathione-S transferase were also used as effectors in control experiments because the initial galectin sample was not pure, and still contained traces of the other two earlier mentioned proteins.
3.3 Results

Figure 3.7. Cell proliferation assay using fusion protein, galectin-1 and galectin-1 in conjunction with lactose (100 mM) on MPS fibroblasts.
Starting concentration of FP was 0.98 mg/ml.
Starting concentration of galectin was 0.44 mg/ml. 4 fold dilutions of the effectors were used. Each point represents the mean of at least four determinations (± S.E.M). Error bars are included only for one data set for clarity purposes.

![Graph showing cell growth vs protein concentration.]

Galectin-1 treatment and galectin-1-lactose treatment were both having an inhibitory effect upon MPS cells growth. Unfortunately we could not repeat this experiment to collect data for the higher concentration range for galectin and to assess the effect of lactose only, because the MPS became senescent.

Fibroblasts, from amniotic fluid cultures have been used in similar experiments but showed no reactivity to galectin-1 or any other effectors.
3.3 Results

Figure 3.8 Cell proliferation assay using lactose, galectin-1 and galectin-1 in conjunction with lactose (100mM) as effectors on cultures of U2OS cells.

Starting concentration for galectin-1 was 0.5 mg/ml.

2 fold dilutions of the effector were used.

Each point represents the mean of at least four determinations (+S.E.M). Error bars are included only for one data set for clarity purposes.

These U2OS cells had been passaged several times since the earlier experiments summarised by Figure 3.6.

A phenomenon similar to tumour progression affected our U2OS and HELA cells. Because of this, we could not detect any growth inhibitory effects when we repeated experiments with galectin-1 and galectin-1 in conjunction with lactose.
3.3 Results

Figure 3.9 Mitogenicity of recombinant human galectin-1.

Incorporation of [H\(^3\)] thymidine into a TCA-insoluble form was measured in confluent MPS human fibroblast cultures in 24-well culture plates. Incorporation (the mean of four experimental determinations, ± SEM) is expressed as a ratio with respect to the corresponding mean in serum-free controls, or is normalised relative to the maximal incorporation seen with galectin-1. Foetal calf serum was used as a positive control.

![Graph showing thymidine incorporation](image)

Studies of [\(^3\)H]-thymidine incorporation on MPS cells treated with recombinant galectin-1 only, (effectuated by Ms Linda Adams) revealed a mitogenic effect in the concentration range of 0.2-1\(\mu\)g/\(\mu\)l. The maximum mitogenic effect of galectin was only 50% compared with the one obtained with foetal calf serum. In the same experiments done in the presence of lactose there was little or no mitogenic effect.
3.3 Results

3.3.3 Cytotoxicity experiments

Figure 3.10 Cytotoxicity assay using galectin-1 as effector added to 90% confluent and 60% confluent U2OS cells.

The starting concentration of galectin was 0.5 mg/ml. A 2-fold dilution series of the effector was used. The % of MTT reduction was obtained using the mean of 3 experimental determinations. Amylin was used as a positive control: for rat insulinoma (RINm5F) cells treated with 10 μmol amylin the MTT reduction was 50%.

![Graph](image)

MTT reduction is a measure of LDH activity in viable cells. A slightly lower level of LDH activity was detected when galectin was added to the 60% confluent cells, but was far from being significant when compared to the values for the positive control. These were U2OS cell cultures freshly started from frozen stocks.
3.3. Results

3.3.4. Experiments of galectin proteolysis and degradation by cells

Figure 3.11. SDS-PAGE of galectin-1 samples incubated with trypsin (0.1%) stock solution at 37°C for 1, 3 and 6 hours.

Samples were electrophoresed on a 15% SDS-polyacrylamide gel using non-reducing conditions and visualised by staining with Coomassie Brilliant Blue. Lanes 1-4 have the same concentration of galectin-1 (1.7 mg/ml), lanes 2-4 have galectin-1 treated with 0.1% trypsin.

Lane 1: 10 μl galectin-1 sample.
Lane 2: 10 μl galectin-1 sample incubated for an hour with trypsin.
Lane 3: 10 μl galectin-1 sample incubated for 2 hours with trypsin.
Lane 4: 10 μl galectin-1 sample incubated for 3 hours with trypsin.
Lane 5: 3 μl low molecular markers.

Galectin-1 samples treated with trypsin (lanes 2-4) have been subjected to proteolysis. I proceeded to treating other galectin-1 samples with trypsin and lactose in order to investigate the occurrence of protection from proteolysis in these conditions.
3.3 Results

Figure 3.12 SDS-PAGE of galectin-1 samples incubated with trypsin (0.1%) stock solution and 100 mM lactose at 37°C for 1, 3 and 6 hours.

Samples were electrophoresed on a 15 % SDS-polyacrylamide gel under non-reducing conditions and visualised by staining with Coomassie Brilliant Blue. Lanes 2-4 have the same concentration of galectin-1 (1.7 mg/ml)

Lane 1: 10 µl galectin-1 sample (3.5 mg/ml).
Lane 2: 10 µl galectin-1 sample (1.7 mg/ml).
Lane 3: 10 µl galectin-1 sample incubated for 3 hours with trypsin and lactose.
Lane 4: 10 µl galectin-1 sample incubated for 6 hours with trypsin and lactose.
Lane 5: 3 µl low molecular markers.

The presence of lactose in the reaction mixture had an obvious protective effect on galectin-1 (lanes 3 and 4). These experiments indicated that galectin-1 was susceptible to proteolysis by trypsin, and was protected from this by ligand binding. It was then necessary to find out if galectin-1 was sensitive to proteolytic degradation by cell-associated proteinases.
3.3 Results

Figure 3.13 SDS-PAGE of 10μl of DMEMs containing 1mg/ml galectin-1 incubated from 4 hours to 3 days on U2OS cells.
Samples were electrophoresed on a 15% SDS-polyacrylamide gel in non-reducing conditions and visualised by staining with Coomassie Brilliant Blue. For lanes 5-9 photo A, initial galectin-1 concentration was (0.5mg/ml) for lanes 1-4 and 6, photo B initial galectin-1 concentration was (0.7mg/ml).

A: Lanes 1,2,3: different galectin samples of unknown concentrations.
   Lane 4: low molecular weight markers.
   Lane 5: DMEMs and galectin-1 after a overnight incubation.
   Lane 6: DMEMs and galectin-1 after 2 days incubation.
   Lane 7: DMEMs and galectin-1 after 3 days incubation.
   Lane 8, 9: DMEMs and galectin non-incubated (blank).

B: Lane 1: DMEMs and galectin-1 after 6h incubation.
   Lane 2: DMEMs and galectin-1 after 1 day incubation.
   Lane 3: DMEMs and galectin-1 after 2 days incubation.
   Lane 4: DMEMs and galectin-1 after 3 days incubation.
   Lane 5: low molecular weight marker.
   Lane 6: DMEMs and galectin-1, non-incubated

Galectin-1 incubated on U2OS cells at relatively low passage numbers showed signs of degradation. There was no evidence though of a fast, active uptake of galectin-1 in these cells.
3.3 Results

Figure 3.13a. Further analysis of the lanes 5, 6, 7 from the photo A, figure 3.13 using a NIH Image Analysis program.

Lanes 8 and 9 containing the non-incubated galectin could not be analysed because the bands were distorted and too close. This made it impossible to select them separately using this program. The comparison here is only between the galectin-1 samples that have been incubated over-night (lane 5), for 2 days (lane 6) and for 3 days (lane 7).

Lane 5
Peak area = 156 square pixels

Lane 6
Peak area = 155 square pixels

Lane 7
Peak area = 150 square pixels

The image analysis confirmed the fact that there was a reduction in the level of galectin-1 in the sample that was incubated for 3 days.
3.3 Results

Figure 3.13b Further analysis of the lanes 1-4 and 6 from photo B, figure 3.13 using a NIH Image Analysis program.

The comparison here is only between the galectin-1 samples that have been incubated for 6 hours (lane 1), for 1 day (lane 2), for 2 days (lane 3), for 3 days (lane 4) and non-incubated galectin-1 (lane 6).

Lane 1

Peak area = 63 square pixels

Lane 2

Peak area = 70 square pixels

Lane 3

Peak area = 69 square pixels

Lane 4

Peak area = 62 square pixels

Lane 6

Peak area = 69 square pixels

The size of the peak from lane 1 was obviously affected by the fact that the gel was broken. The image analysis confirmed the fact that there was a reduction in the level of the sample that was incubated for 3 days.
3.3 Results

Figure 3.14 SDS-PAGE of 10 μl of DMEM containing 0.5 mg/ml galectin-1 incubated for 4 days or 6 days on U2OS and fibroblast cells. Samples were electrophoresed on a 15% SDS-polyacrylamide gel in non-reducing conditions and visualised by staining with Coomassie Brilliant Blue.

A: Lanes 1 and 2: DMEM and galectin-1 after 4 days incubation on fibroblasts.
   Lane 3: galectin-1 non-incubated on cells (blank).
   Lanes 4 and 6: DMEM and galectin-1 after 4 days incubation on U2OS cells.
   Lane 5: low molecular weight markers.
   For lanes 1-4 and 6 initial galectin-1 concentration was (0.5 mg/ml)

B: Lane 1: galectin-1 unincubated on cells (blank).
   Lane 2: galectin-1 incubated for 6 days but not on cells.
   Lane 3: galectin-1 incubated for 6 days on U2OS cells.
   Lane 4: galectin-1 and lactose (100 mM) incubated for 6 days on U2OS cells.
   Lane 5: low molecular weight markers. For lanes 1-4 initial galectin-1 concentration was (0.5 mg/ml)

Galectin-1 incubated on U2OS cells at high passage number did not show any signs of degradation. There was no evidence of a fast uptake of galectin-1 by the cells.
3.4 Discussion

The recombinant protein is a lectin as has been demonstrated by the hemagglutination experiments. Lactose was found to inhibit this reaction, and this confirms the specificity of galectin for lactose. The recombinant galectin maintained full lectin properties even as a fusion protein with glutathione S-transferase. The concentrations of FP and PK\textsubscript{1} at the end point of the hemagglutination seem to show that the fusion protein is a more powerful lectin than galectin-1 itself. The fact that PK\textsubscript{2} agglutinated erythrocytes at a concentration of 0.03 mg/ml even though it had a 25 kDa mobility on a SDS-polyacrylamide gel confirmed the hypothesis that it might represent the homodimer of the human recombinant galectin and not the glutathione-S-transferase. GST agglutinated cells at a minimum concentration of 0.3 mg/ml, agglutination which was considered to be due to non-specific binding of GST to erythrocytes. This result encouraged us to proceed with the sequencing of the 25 kDa band present in the galectin sample purified using the asialofetuin affinity column and this confirmed our hypothesis (Section 2.3.3.i).

The fusion protein and GST itself did not have a growth inhibitory effect on U2OS cells. This data and the ones referring to the lectin properties of the fusion protein suggest that growth inhibition does not depend on the carbohydrate-binding site.

The combination of data obtained from cell growth experiments and \textsuperscript{3}H thymidine incorporation revealed the fact that galectin-1 has a biphasic effect on the cell growth of MPS fibroblast cells.

The mitogenic activity of galectin-1 was seen at a low concentration range and seemed to be dependent upon the \(\beta\)-galactoside-binding site because it is inhibited in the presence of lactose.

Any discrepancy between the data obtained using the two methods could be explained by the fact that the inhibition of thymidine incorporation is measured relative to baseline incorporation in serum-free medium in the absence of a mitogenic stimulus, while the inhibition of cell growth is measured against a background of normal growth in complete medium.

If growth stimulation is due to galectin binding and cross-linking of mitogenic receptors that possess galactoside residues, then it might be possible that lactose blocks this effect and renders more galectin available to bind to a putative growth-inhibitory site.
3.4 Discussion

Recombinant galectin-1 had growth inhibitory effects on Hep2 and U2OS cells but no effects on the growth of HELA cells. Initial experiments done with the natural galectin (Manilal et al., 1993), have shown that this lectin also inhibited only the growth of MPS and U2OS cells and not the growth of HELA cells. The recombinant galectin was 5-10 times less effective as a growth inhibitor than the natural one. The abnormal size of galectin detected in mass spectrometry determinations might be connected with this.

On the basis of the evidence obtained with the recombinant galectin we could not assess the physiological significance of the biphasic cellular response because the concentration response range may be different from the one obtained with natural galectin.

The cytotoxicity experiments showed that when recombinant galectin was added to 90% confluent U2OS cells there was no apoptotic effect. When galectin-1 was added to 60% confluent cells there was a slight decrease in the level of MTT reduction which was proportional with the increase in galectin-1 concentration. However, the apoptotic effect of the recombinant galectin-1 was very far from being significant when compared with the values obtained with the positive control (amylin, on rat insulinoma cells). In conclusion the cytotoxicity experiments have shown no evidence that galectin has an apoptotic effect.

The proteolysis experiments have revealed the fact that galectin-1 is susceptible to proteolytic degradation. This was considered to be a cause for a lower yield than expected of galectin from bacterial cultures. As reported by Abbot and Feizi (1991) with bovine galectin and observed by us (Fig. 3.12), the presence of lactose in the assay protects galectin from proteolytic degradation. It seems that the presence of the ligand does not alter the protein folding, but it contributes to a general tightening of the protein.

The proteinases need to attack the galectin at one or more surface residues before rendering buried sites accessible by unfolding of the protein (Abbot and Feizi, 1991). For the cellular degradation experiments, the starting hypothesis was that the degradation of galectin-1 is necessary for the growth of normal cells because galectin-1 is a growth inhibitor. The GRP was the candidate for this degradative function.

The fact that as seen in the experiments of Manilal et al. 1993, the natural galectin did not inhibit cell growth of HELA cells allowed us to speculate that galectin might be somehow processed differently in these cells. It became obvious from these experiments that the inhibition of GRP by proteinase inhibitors and subsequent accumulation of galectin (if galectin is a substrate for this proteinase) did not have as a consequence the
inhibition of the growth of HELEA cells. The accumulation of the natural galectin in the presence of proteinase inhibitors was observed in Hep2 cells which are subject to growth inhibition by this lectin.

Even through in our cellular degradation experiments there were some signs of reduction of recombinant galectin when incubated with U2OS cells (5% difference between an overnight incubation and a 3 days incubation and 4% difference between a non-incubated sample and a 3 days incubated one), subsequent densitometric measurements indicated that the changes were small and not uniform over a period of time. It was concluded that there was no significant degradation, cellular binding or uptake of galectin-1.

Experiments were performed with early-passage and late-passage U2OS cells and indicated that the resistance of late-passage cells to galectin-1 treatment was not due to increased degradation of the lectin. More significantly, the original hypothesis that continuous degradation of galectin-1 by GRP was necessary for cell growth was not supported by these results.

Because we could see little or no degradation of galectin-1 by cellular proteinases, there was no point in going on to experiments with GRP inhibitors.

The phenomenon of the change of the response of cells to agonist or antagonist treatment (Fig. 3.8) had been observed before by Scott and Tse (1994) in colon carcinoma cells and consisted in changes in sensitivity of these cells to treatment with proteinase inhibitors. The effect was reproducible and was analogous to the process of tumour progression. As the U2OS and HELEA cells had been subcultured for more than 100 times before we acquired them, we were not expecting this type of change therefore we did not keep an accurate record of the passage numbers.
Chapter 4

Study of the effect of galectin-1 on the expression of the c-fos proto-oncogene
Chapter 4. Study of the effect of galectin-1 on the expression of the \textit{c-fos} proto-oncogene

4.1 Introduction

In order to elucidate the mechanisms through which galectin-1 inhibits cell growth I decided to investigate the effect of galectin treatment of normal and tumour cells in cultures on the expression of a "nuclear" proto-oncogene: \textit{c-fos}.

The \textit{c-fos} proto-oncogene encodes a transcription factor which is believed to play a critical role in proliferation and differentiation as well as in the physiological response of mature cells to their environment (Field \textit{et al.}, 1992).

Knowing that expression of antisense \textit{c-fos} appears to block cell proliferation (Holt \textit{et al.}, 1986) and that galectin-1 was shown to inhibit the same process, we were hoping to demonstrate that galectin-1 inhibits cell proliferation by down-regulating the levels of the \textit{c-fos} proto-oncogene expression.

Panayotou \textit{et al.}, (1989) have demonstrated that laminin stimulates thymidine incorporation in cultured cells possessing EGF receptors. Kubota \textit{et al.} (1995) found that another site on laminin is active in promoting cell growth and induces a rapid and transient expression of \textit{c-fos} and \textit{c-jun} proto-oncogenes. Galectin-1 was shown to inhibit cell matrix interactions in skeletal muscle by binding to polylactosamine chains of laminin and interfering with laminin recognition by the laminin receptor integrin \(\alpha 7\beta 1\) (Gu \textit{et al.}, 1994). This might be the mechanism through which galectin-1 inhibits growth \textit{in vivo}. We were aware that Marshall \textit{et al.} (1988) have studied the differential effect of human recombinant IFN-\(\alpha 2\) (a known inhibitor of proliferation of many types of cells) on the serum-induced modulation of nuclear proto-oncogene m-RNA levels and have concluded
that growth inhibition is not necessarily coupled with inhibition of proto-oncogene transcription.

C-fos is normally expressed at very low basal levels in most growing cells. The higher levels of c-fos produced in some embryonic and bone marrow cell types may be due to the presence in abundance of growth factors (Eisenman, 1989). Transient increases in c-fos expression occur in different cell types following stimulation with serum, specific growth factors or trauma. There are cases in which addition of a mitogen leads within minutes to a more than 100-fold increase in the levels of c-fos mRNA, followed by a return to pre-stimulation levels within 30-60 minutes (Eisenman, 1989).

In earlier experiments, I tried to detect the c-fos proto-oncogene and the β-actin gene (the last was intended to be used as a standard) using the technique of Northern blotting with biotinylated probes. I interrupted this work in order to start the cloning and expression of the recombinant human galectin. Later on I decided to return to the work on the c-fos proto-oncogene, but using the method of semiquantitative PCR.

The amplification of DNA by this technique (Saiki et al., 1988) provides a non-radioactive semiquantitative method of RNA analysis. PCR has the advantage of being versatile and sensitive. Data can be obtained using total RNA and this eliminates the poly(A)+mRNA purification step of other assays.

The aim of quantitative PCR is to estimate the number of target molecules in a RNA sample that has been reverse-transcribed and amplified by PCR, and this can be absolute (the number of molecules per µg DNA), or may be relative to an external or internal standard. In our semiquantitative PCR experiments, we used both the competitive PCR technique and the technique of the coamplification of target and control genes.

The technique of competitive PCR has a reference standard being co-amplified in the same reaction as the sequence of interest. In this case, the standard is a synthetic template. This technique effectively controls for the tube-to-tube variation in amplification efficiency. The principal condition that must be fulfilled to derive absolute quantitative information using this technique is that the target and the competitor sequences are amplified with equal efficiencies using identical primer pairs (Cross, 1995).

The technique of the co-amplification of target and control genes consists of the amplification of the sequence of interest and a second control sequence from the same DNA in the same reaction as an internal standard. The standard sequence controls for
4.1 Introduction

variables such as amount and amplifiability of DNA, and for tube-to-tube variation in amplification efficiency. The quantification of the product is made by comparison of the intensity of the two products (Cross, 1995). It is known that the smaller the difference in size between the control and the target gene, the smaller is the difference in the efficiency of the amplification. The shorter fragment is amplified with higher efficiency (Saiki et al., 1988), but this should not affect the outcome of a competitive PCR quantitation, as differences in the efficiency should be constant in all the tubes (Sarkar and Bolander, 1994). I considered initially using β-actin as the control gene for the coamplification PCR, but I later discovered that this gene was containing SRE-like regulatory sequences and in consequence was not suitable for this purpose (Cahill et al., 1995). I chose gaelactin-1 as a control gene because it was not listed as having a SRE-like element.

α1-antitrypsin is a proteinase inhibitor and it has been shown to have a growth-inhibitory effect on cells (Scott and Tse, 1988; Scott and Seow, 1985). In these experiments we treated two types of tumour cells with α1-antitrypsin and gaelactin-1 in order to investigate their action on the c-fos and gaelactin-1 expression.

4.2 Materials and Methods

Materials

The pGEM-T vectors were kindly donated by Mr Geoff Gill (vector map in the Promega Technical Bulletin). The primers, enzymes, DNA-purification systems, bacterial cells, 1 kb DNA ladder, RibolClone cDNA Synthesis System with AMV reverse transcriptase, AmpliTaq DNA polymerase, dNTPs, GeneClean II kit, LB medium and agarose used for these experiments are outlined in Section 2.2.

Methods

For the method of competitive PCR, I needed to construct a standard, a synthetic template that was supposed to be amplified using the same primers as for the target gene but would give a PCR product of a different size. Initially I investigated the
possibility of cloning the fos proto-oncogene in the pGEM-T plasmid and then use a restriction site in this gene for the insertion of galectin-1 gene (a PCR product). The most convenient enzyme for this purpose was Stu I as this site was not present in the plasmid. The only problem would have been the big difference in size (640 bp) between galectin-1 (the reference gene) and c-fos (target gene) that could have resulted in a big difference in the amplification efficiency of the two fragments. Galectin-1 gene was used because we already had synthesised primers.

4.2.1 Synthesis of the c-fos PCR product

In cultured fibroblasts c-fos mRNA levels are very low and further decrease when the cells are made quiescent (Müller et al. 1984). A rapid induction of the expression of this gene takes place when quiescent fibroblasts are exposed to whole serum or polypeptide mitogens such as platelet-derived growth factor (PDGF), fibroblast growth factor (FGF), or epidermal growth factor (EGF) (Greenberg and Ziff, 1984).

Cultures of U2OS cells were incubated for 30 minutes with DMEM and 15% foetal calf serum. An RNA extraction was performed followed by a reverse transcription reaction (see Sections 2.2.4 and 2.2.5). The resulting cDNA was used as template for a PCR reaction (see Section 2.2.6) using the following primers: forward primer (fos1) 5'-CTACGAGGCGTCATC-3' and the reverse primer (fos2) 5'-TCACAGGCGAAGCGT-3'. The resulting 1.1 Kb PCR product was used for cloning.

4.2.2 Cloning of the fos and galectin-1 PCR products in a pGEM-T vector.

This process was necessary for the production of a synthetic control needed in a competitive PCR reaction. The vector pGEM-T was modified by the addition of 3' terminal thymidine at both ends. These single 3'-T overhangs at the insertion site greatly improve the efficiency of ligation of PCR products because of the non-template dependent addition of a single deoxyadenosine to the 3'-end of PCR product by thermostable polymerases (Promega Technical Bulletin).

The ligation took place in a standard mixture (see Appendix 4) containing 5 μl fos PCR product (200 ng). A transformation of Rb competent cells with the pGEM-T vector
4.2 Materials and Methods

including the fos insert was performed followed by colour and "colony PCR" screening of transformants (see Appendix 4). Digestion of pGEM-T vector containing the fos insert with the restriction enzyme Stu I was followed by an attempt to ligate the galectin-1 gene in this site (see Appendix 4).

4.2.3 Semiquantitative PCR

For both the following methods, cells that were to be used as a source for RNA were grown to confluence and then deprived of serum overnight. In order to induce the expression of the c-fos proto-oncogene another change of medium was necessary, this time containing 15 % FCS. Cultures were incubated for 30 minutes with this medium then harvested for the production of RNA. Once the optimal conditions for the induction of the c-fos proto-oncogene were found, we proceeded to treat the cells as stated above but also adding $\alpha_1$-antitrypsin (25 $\mu$g/ml) or human recombinant galectin-1 (25 $\mu$g/ml) to the medium for the 30 min incubation. It was decided that this galectin concentration was sufficient for the start, as the cost of galectin-1 production was relatively high.

4.2.4 Co-amplification of target and control genes

This method was slightly different from the one performed by Mutter and Pompino (1991) due to the fact that we had to use two different sets of primers to be able to co-amplify two different genes: the 450 bp galectin-1 gene and the 1.1 kb c-fos proto-oncogene. The primers GRP3 and 4, used for the galectin gene amplification were already described in Section 2.2.6 and the primers for c-fos were fos1 and fos2 (Section 4.2.1).

The conditions for PCR were identical to those described in Section 4.2.2 with the exception that we used 2 sets of primers in the same tube and we had to use a correspondingly lower volume of water to bring the final reaction mixture to 50 $\mu$l.

4.2.5 The competitive PCR method

The method of Celi et al. (1993) was used to produce a synthetic standard of a different size than the target gene. In order to achieve this we needed the forward primer
(fos1; Section 4.2.1): 5'-CTACGAGGCCGTAC-3' (15 nucleotides) and a second primer, 38 nucleotide long including a segment of 18 nucleotides at the 5' end (the reverse primer for c-fos, fos2), and at the 3' end, 20 nucleotides that corresponded to the target sequence 100 nucleotides upstream from the antisense primer. The reverse primer was termed fosQ: 5'-
TCACAGGCGCAGCGTGACAGAAGAGCGTGAAGCA-3'.

Figure 4.1 Schematic representation of the technique employed for the synthesis of an artificial DNA molecule to be used as control in competitive PCR amplification (reproduced from Celi et al., 1993)

A PCR reaction using the same conditions as in Section 2.2.6, was set up using fos1 and fosQ primers. The template for this reaction was 1 μl c-fos (127 ng/μl) PCR product in 4 μl 0.15 mM PCR buffer. The resulting control DNA was termed fos-100, was 100 bp shorter than c-fos and was also amplifiable by the fos1 and fos2 primers. A larger synthetic control (only 50 bp shorter than c-fos) proved to be impossible to distinguish from c-fos. This product was not used further.

The competitive PCR consisted in the amplification of 1 μl fos100 (diluted in water to a concentration of 0.002 ng/μl) and of 4 μl cDNA (1:10 dilution in 0.15 mM MgCl2 in PCR buffer) from the treated cells, in the same tube, using fos1 and fos2 primers and the same reaction conditions from Section 2.2.6.
4.3 Results

Preliminary trials with the fos and galectin-1 primers showed that the optimum template concentration for both sets of primers was a 1:10 dilution of the cDNA preparation from U2OS cells.

Figure 4.2 Trial PCR amplifications with galectin-1 and c-fos primers.

The resulted products were loaded on a 1.5% agarose gel and visualised under UV light after staining with ethidium bromide for 30 min.

A: 20 μl of PCR products. Template: 5μl undiluted cDNA; PCR buffer 15mM
B: 20 μl of PCR products. Template: 5μl undiluted cDNA; PCR buffer 1.5mM
C: 1Kb DNA ladder
D: 20 μl of PCR products. Template: 5μl 1:10 dilution of cDNA in 0.15 mM PCR buffer; PCR buffer 1.5mM MgCl₂.

The optimal concentration of MgCl₂ in the reaction mixture was 1.5 mM and 5μl of the 1:10 diluted cDNA was the optimum amount necessary for this PCR amplification. The U2OS cells used as a source of RNA were all stimulated with 15% serum. The RNA was used as a template in a reverse transcription reaction and the cDNA which resulted was the template for the PCR reaction.
4.3 Results

Figure 4.3 Expression of the c-fos oncogene after 30 minutes treatment of U2OS cells with 15% FCS. Cells were grown to confluence before serum treatment. RNA was extracted and used as template for a reverse-transcription reaction. The resulting cDNA served as template for a PCR amplification. The resulted products were loaded on a 1.5% agarose gel and visualised under UV light after staining with ethidium bromide for 30 min.

A: 10 μl of 1 Kb DNA ladder
B,C: 20 μl of c-fos PCR product from an amplification reaction that used a 1:10 dilution of cDNA as a template.

Trial experiments in which we incubated our cultures with DMEM and 15% serum showed that there was very little expression of the c-fos oncogene at 15 min. The maximum expression was at 30 min and the c-fos expression was under the limit of detection after a 60 min incubation. (Only results for the 30 min incubation are shown).
4.3 Results

4.3.1. Coamplification of target and control genes

Figure 4.4 Coamplification of c-fos and galectin-1 genes from cDNA originating from serum-treated and serum-untreated U2OS cells.

The resulted products were loaded on a 1.5% agarose gel and visualised under UV light after staining with ethidium bromide for 30 min.

A, B: 20 μl of PCR products from serum-treated U2OS cells.
C: 1Kb DNA ladder.
D, E: 20 μl of PCR products from serum-untreated U2OS cells.

Comparing these results of the co-amplification of galectin-1 gene and c-fos proto-oncogene from U2OS cells, we noticed, as expected, that the serum-untreated cells did not have a detectable c-fos expression. A slight increase in the expression of the galectin-1 gene was seen in the serum-treated cells compared to the expression of the same gene in serum-untreated cells.
4.3 Results

Figure 4.5 Coamplification of c-fos and galectin-1 genes from cDNA originating from α1-antitrypsin-treated and from untreated U2OS cells.

The resulting products were loaded on a 1.5% agarose gel and visualised under UV light after staining with ethidium bromide for 30 min.

A, B, C: 20 μl of PCR products from cells treated with α1-antitrypsin and serum.
D: 1 Kb DNA ladder.
E, F: 20 μl of PCR products from untreated cells.

Comparing the results of the co-amplification of galectin-1 gene and c-fos from untreated and α1-antitrypsin-serum treated cells we noticed that the treated cells had a lower galectin gene expression than the untreated ones. The expression of c-fos and galectin-1 seemed to be lower in the α1-antitrypsin-serum treated cells than in the serum-only treated ones (Fig 4.4), but there was not enough of the "untreated" sample to run it on the same gel. These results were later confirmed by the data obtained using the competitive amplification method (Fig 4.10).
4.3 Results

Figure 4.6 Coamplification of c-fos and galectin-1 genes from serum only treated cells and from galectin-1 treated cells.

The resulted products were loaded on a 1.5% agarose gel and visualised under UV light after staining with ethidium bromide for 30 min.

A: 20 µl of PCR products from serum only treated cells.
B, C: 20 µl of PCR products from galectin-1 treated cells.
D: 1 Kb DNA ladder.

Comparing the results of the co-amplification of galectin-1 gene and c-fos from serum-treated and from galectin-1-treated cells, we noticed that the latter had a lower galectin-1 gene expression than the former. There was not a similar proportion between the level of reduction suffered by galectin and c-fos genes. The galectin-1 gene was obviously more affected than c-fos.

For a better comparison of the two samples, a NIH Image Analysis programme was used.
4.3 Results

Fig 4.7 Further analysis of figure 4.5 using an NIH Image Analysis programme. Lane A contains the serum-only treated samples. Lanes B and C contained the galectin-1 treated samples.

A

Peak area = 51 sq pixels

B

Peak area = 39 sq pixels

C

Peak area = 28 sq pixels

Peak area = 62 sq pixels

galectin-1 gene

c-fos gene

galectin-1 gene

c-fos gene

This analysis confirmed the fact that there was an inhibitory effect on the c-fos and galectin-1 gene expression when cells were treated with galectin-1.
4.3 Results

Figure 4.8 Coamplification of galectin-1 and c-fos using untreated, serum treated, α1-antitrypsin-treated and galectin-1-treated HE LA cells.

The resulting products were loaded on a 1.5% agarose gel and visualised under UV light after staining with ethidium bromide for 30 min.

A, B: 20 μl of PCR products from α1-antitrypsin treated cells.
C: 1 Kb DNA ladder.
D, E: 20 μl of PCR products from untreated cells.
G, H: 20 μl of PCR products from serum treated cells.
F: DNA mass ladder.
I, J: 20 μl of PCR products from galectin-1 treated cells.

At the concentration of 15% FCS in the growth medium, the expression of c-fos was under the level of detection. When treated with 20% serum, HE LA cells had shown expression of c-fos to a lesser extent than in U2OS cells treated with 15% FCS. In cells treated with α1-antitrypsin, galectin-1 or in untreated cells, c-fos expression was under the limit of detection. Galectin-1 gene expression was stimulated by serum treatment. Untreated cells and α1-antitrypsin-treated ones had a similar level of galectin-1 gene expression, while in galectin-1 treated cells this expression was significantly inhibited.
4.3 Results

4.3.2. Competitive PCR amplifications

Figure 4.9 Comamplification of variable amounts of control DNA with a fixed quantity of cDNA obtained from RNA originating from serum-treated U2OS cells.

The initial concentration of synthetic DNA control was 2 ng/μl. The resulting products were loaded on a 0.8% agarose gel and visualised under UV light after staining with ethidium bromide for 30 min.

A: 10 μl 1 Kb DNA ladder.
B: 10 μl PCR products from templates: 1 μl control DNA (1:2,000 dil.) and 4 μl cDNA from serum treated cells.
C: 10 μl PCR products from templates: 1 μl control DNA (1:5,000 dil.) and 4 μl cDNA from serum treated cells.
D: 10 μl PCR products from templates: 1 μl control DNA (1:10,000 dil.) and 4 μl cDNA from serum treated cells (equivalence point, where the concentration of control at the starting point equals the concentration of the target cDNA).

E: 10 μl PCR products from templates: 1 μl control DNA (1:100,000 dil.) and 4 μl cDNA from serum treated cells.

From the results of the amplification of increasing quantities of control DNA with a fixed quantity of cDNA, it was obvious that there is a competition between the two DNA species for the PCR substrates. The amount of control DNA that permitted the target cDNA to be equally amplified was 0.0002 ng/μl.
4.3 Results

Figure 4.10 Competitive PCR: amplification of control and target DNA from serum-treated and α1-antitrypsin-treated U2OS cells.

A 1:10,000 dilution of the control DNA was used in these reactions. The resulting products were loaded on a 0.8% agarose gel and visualised under UV light after staining with ethidium bromide for 30 min.

A: 10 μl of 1 Kb DNA ladder.
B: 10 μl PCR products from templates: 1 μl control DNA (1:10,000 dil.) and 4 μl cDNA from serum-treated cells.
C: 10 μl PCR products from templates: 1 μl control DNA (1:10,000 dil.) and 4 μl cDNA from α1-antitrypsin-treated cells.

These competitive amplifications confirmed the results obtained with the coamplification methods, that treatment of U2OS cells with α1-antitrypsin has an inhibitory effect on the expression of the c-fos proto-oncogene. Unfortunately the use of a different batch of controls had given a too high expression for the 1000bp control band and made impossible the detection of the c-fos expression in galectin-1-treated cells.
4.4 Discussion

The quantitative capability of PCR has been compared to classical means of nucleic acids quantitation such as slot blot, Northern blot and in situ hybridisation and the conclusion was that there is a good correlation between the results obtained with PCR and the ones obtained using the above mentioned methods.

The reason for choosing to use a DNA sequence to be amplified as a control is that an RNA control would have been much more difficult to prepare and would have been more prone to degradation than DNA. After visualising the RNA sample used for the synthesis of cDNA on a 1.5 % agarose gel, we noticed that our sample contained also traces of genomic DNA. I considered additional purification steps impractical because of the lability of the RNA, so I relied on the fact that if I have PCR products from both RNA and genomic DNA, I should see a clear difference in size because the primers are designed such that they span the introns within this gene.

I could have induced the expression of the c-fos proto-oncogene by heat shock and the effect would not have been transient, but the increase in c-fos expression would have been only 3-5 fold, while the serum induction can result in 30-100 fold increase (Eisenman, 1989) making easier the task of detecting changes in the levels of c-fos expression.

For the cloning of the galectin-1 gene in the Stu I site in c-fos, I tried to increase the concentration of the PCR fragment from 48 ng to 180 ng but this did not make any difference for the success of the ligation.

Comparing the results of the coamplification of galectin-1 gene and c-fos gene from both serum-treated and untreated U2OS cells I noticed, as expected, that the untreated cells did not have a detectable c-fos expression, but I also noticed a slight increase in the expression of galectin gene in serum-treated cells.

Opinions were divided over which is the optimal number of cycles that can be performed before the amplification efficiency reaches the plateau phase. Köhler (1995) calculated that the amplification efficiency of 3 different genes is reaching the plateau phase between cycle 28 and 30. Other groups consider that it is preferable to restrict competitive PCR analysis to 20 cycles (Wiesner et al., 1993; Raeymaekers, 1993). I subjected my sample to a 20 cycles amplification but c-fos expression was under the limit of detection.
4.4 Discussion

I started by using 25 μg/ml of galectin-1 for cell treatment because of the relatively high cost of production of galectin. The effect of treatment of MPS fibroblasts with the recombinant galectin in this range of concentration was well into the inhibitory range as seen in the [3H] thymidine uptake. For the U2OS cells there was a 70 % inhibitory effect on the cell growth (Fig. 3.6) which was consistent with the down-regulation observed in c-fos expression.

As explained in Section 4.1, I had no indication that galectin-1 gene might not be suitable as a standard. The coamplification experiments showed for the first time that galectin-1 gene was stimulated by serum treatment and down-regulated by galectin and α1-antitrypsin treatment in both HEla and U2OS cells. Generally, after serum treatment, the c-fos expression in HEla cells was very low. Galectin-1 treatment of U2OS cells seemed to have down-regulatory effects on the expression of c-fos as the densitometric analysis of the photo in Fig. 4.6 showed (from 51 sq pixels for the serum-treated cells to 28-39 sq pixels for the galectin-1 -treated cells). In HEla cells, c-fos expression was under the limit of detection.

There seemed to be a connection between the growth inhibitory effect of galectin-1 on U2OS cells, and the down-regulation of this proto-oncogene. I could not conclude if the growth inhibitory process was due only to inhibition of c-fos, or if it was a cumulative effect resulting from the inhibition of other genes too.

The tumour progression-like process (Section 3.4) observed in U2OS cells had as a consequence unresponsiveness to serum treatment and made the detection of c-fos impossible. This process prevented me from further investigating the effect of galectin-1 at higher concentrations. If I consider the fact that recombinant galectin seemed to be a weaker growth inhibitor than the natural lectin, this might have been the explanation for the weak down-regulation of c-fos.

α1-antitrypsin is a proteinase inhibitor and a growth-inhibitor (Scott and Tse, 1994). Competitive amplification of control DNA with cDNA obtained from U2OS cells treated with α1 antitrypsin showed that c-fos expression was down-regulated. The sample for which a galectin-1 treatment was performed was not in the range set up by the control titrations. The explanation could be the fact that I used a new batch of controls at an identical dilution not realising that in this case the "interassay limits are reliable only as long as one is using the same batch of standards within a given study" (Ferre, 1992)
The coamplification and the competitive amplification experiments have revealed for the first time the fact that a known growth inhibitor like α1-antitrypsin has an inhibitory effect on the expression of c-fos (Fig. 4.5, 4.8 and 4.10).

For future co-amplification experiments, the use of a house-keeping gene such as GAPDH as a control, might be more adequate as its level of expression is not sensitive to physiological conditions. Other genes that could be used for the same purpose are the genes for histone H3.3, or ribosomal protein L19, that have been shown to be cell-cycle independent and constitutively expressed in all tissues (Köhler, 1995).

Wong et al. (1994) recommended a new technique that minimises competition between coamplified products. In this method, the final yield of more abundant templates (or more efficiently amplified templates) was reduced below saturating levels by using fewer PCR amplification cycles than for the less abundant templates. This could be achieved by dropping different primer sets into the amplification reaction at different cycle numbers. In our case it meant that the primers for galectin-1 were supposed to be added at a later stage than the fos primers (possibly after 10 cycles). It is obvious that the galectin gene gives a very strong signal, its mRNA species competing with the mRNA species of the c-fos for the PCR substrates. This method alleviates the consequences of such competition on the outcome of the c-fos amplification.
Chapter 5

Final discussion and conclusions
Chapter 5: Final Discussion and Conclusions

5.1 Final Discussion

I expected to obtain a recombinant protein of identical molecular weight and DNA sequence and having identical properties with the original human galectin. Indeed the DNA sequencing showed that the sequence of the gene cloned in pGEX was as I expected, identical with the published sequence for human galectin (Abbot and Feizi, 1989). The polyacrylamide gel electrophoresis also confirmed that the size of the recombinant protein was approximately 14 kDa. The N-terminal sequencing of the first 6 residues gave an identical amino acid sequence with the published one. Only the MALDI-TOF determination gave a different molecular weight than the one expected from the DNA sequence (Section 2.4), and this difference in size could not be explained by extensive glycosylation because the galectin was not expressed in yeast where recombinant proteins are usually subjected to such post-translational modifications.

Initially I thought that an insertion of some kind occurred in the galectin gene but the DNA sequencing and the mass spectrometry determinations using trypsinised galectin disproved this possibility. A more probable hypothesis was that transcription failed to terminate at the first stop codon, or a frame shift occurred and activated a stop codon downstream from the initial stop codon (see Section 2.4). Both these events would have had the same consequences: a bigger recombinant protein than expected. The fact that no evidence sustaining these hypotheses was obtained and that all the tryptic peptides of the expected size were accounted for, led to the conclusion that the apparently abnormal size of the recombinant galectin was due to a calibration error that occurred in the MALDI-TOF determinations.

The protein was clearly a lectin as demonstrated by its hemagglutinating activity and by the fact that it was retained from the cleavage mixture on an asialofetuin affinity chromatography column. The hemagglutination reaction was inhibited by lactose, but the
activity of the recombinant galectin was not dependent on treatment with reducing agents (see Section 1.2.3) as is the case for most of the soluble galectins.

The minor component which resulted from the thrombin cleavage at Arg 48 was inseparable from the recombinant galectin on a polyacrylamide gel and it seemed that this peptide remained attached to the rest of the protein, probably by means of intramolecular disulphide bonding. Mass spectrometry analysis of the DTT-treated tryptic peptides provided no evidence for this hypothesis. The protein sequencing also revealed the presence of galectin-1 dimers.

It has been suggested that galectin-1 resembles interferons in its cytostatic effect (Wells and Mallucci, 1991). They both seem to be acting by arresting the cells in G0. Galectin also prevents the cells from traversing from G2 into M phase (Wells and Mallucci, 1991) while interferon seems to arrest growth also by preventing cells from traversing from G1 to S (Shaun and Thomas, 1996). As opposed to galectin, transforming growth factor β (TGFβ) has been shown to arrest cells in G1 (Ewen et al., 1993), but the mode of action of galectin-1 may be comparable with TGFβ because galectin-1 also appeared to have both inhibitory and stimulatory effects on the proliferation of different types of cells. TGFβ was shown to inhibit the growth of normal epithelial, endothelial, neuronal, lymphoid, haematopoietic cells and also neoplastic cells from these kinds of tissues (Moses et al., 1990) while the natural and the recombinant galectin-1 was shown to act as a growth inhibitor for human fibroblasts, and for human tumour cells like Hep2 and U2OS, but not for HELA cells. It has been observed that natural galectin had a growth stimulatory effect on bovine corneal endothelial cells at concentrations that caused growth inhibition in other human cells (Manilal et al., 1993). This observation suggested that different types of cells have different concentration response ranges for galectin. Possible changes in levels of galectin-1 expression are comparable with some effects seen in TGFβ. In experiments on MPS fibroblasts I noticed the fact that galectin-1 is expressed constitutively, while Chiarotti et al. (1994) have discovered that in normal quiescent rat thyroid cells galectin-1 expression was transiently induced by thyrothropin which has a cell proliferative effect.

Overexpression of TGFβ has been reported in many types of carcinomas including the colon, lung, breast, uterus and stomach (Tahara et al., 1996). This raised the possibility that TGFβ might exert some tumour-promoting effects via its activities in stromal cells (Tahara et al., 1996).
5. Final Discussion

Overexpression of galectin-1 gene was seen in several thyroid carcinoma-derived cells (Chiariotti et al., 1995). I have not observed an overexpression of galectin-1 gene in U2OS and HEla compared with the MPS fibroblasts in the preliminary experiments in which I amplified cDNA in order to clone this gene in pGEX (Appendix 5). However the unavailability of viable fibroblasts, made it impossible to further investigate this matter. It is easy to conclude that galectin -1 is differently expressed in different types of normal cells and not only in tumour cells. The overexpression of galectin in some tumour cells seems to be more related to the role that galectin-1 plays in mediating invasion and metastasis, than to the growth inhibitory properties of this molecule.

It is worth mentioning that, while some tumour cells overexpress galectin-1, others like KM12c human colon carcinoma cells require treatment with a differentiation agent in order to express galectin-1 (Ohannesian et al., 1995). At the same time, treatment with a differentiation agent like retinoic acid causes a 30-fold decrease of galectin expression in transformed neural cells (Chiariotti et al., 1994). In conclusion, in order to fully understand the contribution of galectin-1 to tumorigenesis, we have to consider the fact that this lectin might play different roles in different type of tumour cells.

I considered Lotan's conclusion (1992) that there are no modifications in the galectin-1 gene in tumour cells compared with the normal cells, when I decided to use U2OS cells as the source of RNA for the amplification of this gene.

Both galectin-1 and TGFβ seem to share the capacity to induce cellular transformation. Overexpression of a rat galectin-1 in mouse fibroblasts by stable induction of a galectin-1 cDNA expression plasmid resulted in the acquisition of the transformed phenotype (Yamaoka et al., 1991) while TGFβ itself was firstly discovered as a growth factor which causes transformation of normal rat kidney cells (Tahara et al., 1996). A novel transforming growth factor (TGFγ2) proved to be in fact rat galectin-1 (Yamaoka et al., 1991). In the case of this protein there seems to be a connection between the oxidation state of the Cys residues, the carbohydrate-binding properties and the mitogenic effect of TGFβ on cells: the presence of free sulfhydryl residues seems to make rat galectin-1 a poorer mitogenic agent while its carbohydrate-binding site becomes available (Yamaoka et al., 1996). These observations are similar to those of Tracey et al., (1992) and Abbot and Feizi (1991), that an alteration of the native structure of the molecule due to formation of random disulfide bonds is responsible for the lack of carbohydrate-binding properties. In this context the hypothesis of Yamaoka et al. (1996), that different
properties of these molecules are regulated by the structural changes caused by intramolecular disulfide bond breakage and reformation, seems reasonable. It also seems that the binding of a carbohydrate to galectin protects it from oxidation (Cho and Cummings, 1995).

Our recombinant galectin-1 had at least one disulfide bond (Cys 42 - Cys 60) and retained carbohydrate-binding properties in solution. This was consistent with the observation that oxidation of Cys 2 was the one most affecting the stability of the galectin-1 molecule (Hirabayashi and Kasai, 1991). The formation of the disulfide bonds at these Cys residues probably affects one of the invariant HFNPRF (47-52) residues which are involved in galactoside-binding, while the other invariant residues (WGTEQRE, 71-77) should be still functional. This might mean that as long as the last of these two conserved sequences is available, galectin-1 would still have a functional carbohydrate-binding site. We were aware that Tracey et al. (1992) detected two major disulfide-bonded peptides, one containing a Cys 42 - Cys 60 disulfide bond, the other containing two disulfide bonds (Cys 2 - Cys 130 and Cys 16 - Cys 88) and a small amount of a peptide containing the Cys 60 - Cys 130 bond. Although we calculated the sizes of all the possible combinations of disulfide bonds that can form in the galectin-1 molecule (Appendix 3), we could detect only a Cys 42 - Cys 60 bond and a small amount of the T13-T1-T8 peptides linked through 2 disulfide bonds (Cys2 - Cys130 and Cys16 - Cys88).

The thymidine incorporation experiments showed that human recombinant galectin-1 has a strong mitogenic effect which is downregulated in the presence of lactose (Section 2.3). In this case there seems to be a connection between the availability of the carbohydrate-binding site and the mitogenic properties of the lectin.

The fact that the recombinant lectin has a growth inhibitory effect, while the fusion protein does not, even though it has full lectin properties, suggests that the growth inhibitory properties do not depend on the carbohydrate-binding site but the availability of the C-terminal region might be crucial for this purpose. There is data showing that exclusion of the saccharide binding region of the molecule by competing sugars does not affect the growth inhibitory effect of mGBP (Wang and Hsu, 1987). Moreover, Wells and Mallucci (1991) have shown that the association of the murine galectin-1 with a glycan complex confers greater efficiency in cell growth inhibition. The physiological significance of the endogenous glycan component might be that the glycan itself is a modulator of galectin growth-modulatory activity, having the role of minimising non-productive or
mitogenic interactions and maximising growth inhibition. Our growth inhibition experiments on U2OS cells have shown that when cells are treated with galectin in the presence of lactose, there is a trend suggesting a stronger inhibitory effect than in the absence of lactose. As has already been discussed, this could be because the lactose blocks non-productive binding to pericellular glycans.

The murine recombinant galectin seemed to be a more effective growth inhibitor than the human recombinant galectin, but it was hard to make a direct comparison between the two because the first one was expressed in eukaryotic cells and tested on murine fibroblasts (Wells and Mallucci, 1991), while the second one was expressed in prokaryotic cells and tested on human fibroblasts and tumour cells.

The recombinant human galectin-1 that I obtained was about 5-10-fold less effective as a growth inhibitor than the natural protein. However, this difference was not connected to the presence of a glycan similar to that reported by Wells and Mallucci (1991), because both the natural and the recombinant proteins have been purified on the basis of their affinity for β-galactosides.

A direct comparison between the natural and the recombinant protein was hard to make because of the restricted availability of the natural protein.

I think that the biphasic effect of galectin-1 treatment of cells might be due to the fact that at low concentration dimeric galectin cross-links cell surface oligosaccharides and initiates a mitogenic effect (this has been seen on lymphocytes; Sharon, 1994), while at high concentration there might be enough galectin available to participate in a protein-protein interaction with a putative cell surface receptor possibly responsible for the growth inhibition.

Nicholson and Lacorbiere (1973) have suggested that when the cells are getting closer to confluence and the number of galactosyl residues increases, the cell-cell cross-linking mediated by galectin-1 is responsible for the phenomenon of contact inhibition. This, however, is an effect which depends upon the lectin activity, unlike the effect that we have observed.

The gene expression experiments (Chapter 4) have shown that probably inhibition of c-fos by galectin treatment is not the only process necessary for the onset of growth inhibition. Even though expression of c-fos seems to be downregulated in both U2OS and HELA cells, only the growth of the first tumour cell type is inhibited. We have no proof that galectin-1 is overexpressed in these two types of tumour cells, but the down-regulatory effect of galectin-1 on the expression of its own gene when induced by serum
5. Final Discussion

treatment is a proof that a feedback regulatory mechanism is still functional in both HELA and U2OS cells.

It is possible that galectin-1 gene overexpression, when cells are treated with serum in order to induce c-fos, is due to transcription activation by the AP1-transcription factor (the result of the c-fos and c-jun proto-oncogenes products). A possible scenario for the processes that could take place in vivo could be that tumour cells overexpress galectin-1 gene for metastatic purposes but once galectin is released from the cells it could activate macrophages and blood monocytes to produce TNF.

The fact that 3 among 6 galectins are homodimers with an identical subunit of 14 500 Da makes possible the existence of immuno-cross-reactivities and might explain the fact observed by Poirier and Robertson, (1993), that galectin-1 knock-out mice present a normal phenotype: one of the 3 types of galectin mentioned previously could take over the function of the missing protein.

As there was no evidence for rapid disappearance of galectin-1 from the growth medium, the hypothesis that galectin-1 might have been a substrate for the GRP was disproved. The fact that treatment of cells with galectin-1 actually downregulated its own gene expression meant that there was not likely to be a subsequent accumulation of galectin followed by growth inhibition. This was another argument against the GRP/galectin hypothesis.

In some systems, apoptosis and proliferation are coordinately regulated, such that cessation of proliferation coincides with the onset of apoptosis (Rotello et al., 1991). The similarity in functions between galectin and TGFβ, and the fact that TGFβ is known to induce programmed cell death in certain cancer cells (Yanagihara and Tsumuraya, 1992), determined us to investigate the possibility that treatment of tumour cells with galectin-1 might have not only a growth inhibition but also an apoptotic one. We were familiar with the fact that galectin-1 was overexpressed during the induction of apoptosis in the glucocorticoid-sensitive human leukemia cells (Goldstone and Lavin, 1991), that Viscum album lectins had an apoptotic effect on human lymphocytes (Bussing et al., 1996) and that Perillo et al. (1995) observed the fact that the dimeric form of galectin was necessary for induction of apoptosis in active T cells. In our case the recombinant galectin was present in a very small percentage in the dimeric form. I thought that if growth inhibition and apoptosis are initiated through the same mechanism then this might explain why the growth inhibition in the presence of the recombinant galectin was so low compared with the natural galectin. The absence of an apoptotic effect of galectin-1 on
5. Final Discussion

U2OS cells was not surprising as it seems that so far the apoptotic effect of this lectin had been seen only in lymphocytes as part of an immunomodulatory process.

It seems though, that galectin-1 exerts different types of action on the same type of cells as a function of the stage of development and activation of those cells as seen in the experiments of Perillo et al. (1995) on T lymphocytes. At the same time, the effect of galectin treatment seems to differ in various types of cells, for example, in the same concentration, galectins had a different effect on T and B lymphocytes (Sharon, 1994), and on HELA and U2OS cells (see Section 3.2). It could be that, in some cells, galectin has only a growth inhibitory effect while in others it might trigger the onset of apoptosis.

We think that our difficulties in obtaining results when using late passage-number cells was due to the fact that some tumour cells seem to undergo a change in sensitivity towards treatment with different effectors like serum, proteinase inhibitors and galectin-1.

This was somehow similar to the process of tumour progression. It is worthwhile mentioning that the same phenomenon was encountered by Scott and Tse (1994) when working with human colon carcinoma cell lines, and with the melanoma cell line MM96 (Scott, personal communication).

5.2 Conclusions

We could conclude that this study does not confirm the initial hypothesis that galectin-1 is a substrate for the growth-related proteinase as there was no evidence for an active degradation of galectin-1 when incubated on tumour cells. The results of the cell growth experiments indicate that galectin-1 acts as a biphasic growth modulator exerting either mitogenic or inhibitory activity as a function of its concentration. Even though we experienced difficulties in the synthesis of a galectin identical with the native protein, our galectin had fully retained its carbohydrate-binding and mitogenic properties and partially its growth inhibitory properties.

The carbohydrate-binding site seems to be responsible for the mitogenicity of galectin-1 and for its capacity to mediate cell-cell recognition and adhesion processes. The growth inhibitory properties seem to be due to a different site from the earlier mentioned one, a site that remains to be investigated.
5.3 Future Directions

Galectin-1 has not only been shown to release the tumour necrosis factor from macrophages (Kajikawa et al., 1986), but it also suppresses experimental autoimmune myasthenia gravis (Levi et al., 1983) and autoimmune encephalomyelitis (Offner et al., 1990).

As an inhibitor of tumour cell growth, the therapeutic potential for application is obvious, as long as the concentrations at which galectin-1 acts as a growth inhibitor in vivo are elucidated. It could be that through site-directed mutagenesis, some modifications could be made to the original molecule in order to enhance the inhibitory action whilst eliminating the mitogenic one.

An attempt was made to make truncated galectin-1 and use it in growth inhibition experiments, but the fusion proteins had poor solubility (Cameron, 1995). New directions for this project would be to create a deletion or substitution at the Arg-48 site which seems to be the most important site for carbohydrate-binding, and to investigate the properties of this mutated galectin in cell growth inhibition experiments. If cell growth inhibition is independent of lectin activity, as shown in this study, then this mutant should retain growth inhibitory activity.

Another direction would be to investigate the role of the overexpression of the galectin gene in tumour cells and to investigate the levels of galectin-1 present in vivo in order to establish if the levels are mitogenic or growth inhibitory. A study investigating at the same time gene and protein expression, using semiquantitative PCR and immuno-detection techniques, might make this investigation complete.
Appendix 1

DNA sequencing data
Plurality: 3.00  Threshold: 1.00  AveWeight 1.00  AveMatch 1.00  AvMisMatch 0.00

PRETTY of: list-seql.msf(*)  October 2, 1996 15:28

list-seql.msf(galectin1)
list-seql.msf(seql-r)
list-seql.msf(seql-f)  Consensus

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ATGG-TTGTG GTCGTGTCGC CAGCAACCTG AACCTCAAC CTTGAGAGTG

51
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101
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ACCTGGGCAA AGACACCAAC AACCTTGCC TGCACTTCAA CCGTTGCTTC

151
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AACGCCCAGG GCCACCGCGCA CACAATCCTG GCACACTGCGA AGGACGCGGG

201
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251
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301
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351
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CATCACTAC ATGGCAGCTT AGCAGCTT GACATCAGCA TGGTGGGCC

401
TTTGACTGA TTTGACTGA TTTGACTGA TTTGACTGA

Consensus
Figure 3: Plasmid map for pCNNinv vector.
The sequencing profile for standard amino acid residues is depicted in the initial trace. The sequence profile for the 14 kDa protein from the retarded fraction PK1, fig. 2.11, was:

\[ \text{G-S-M-A(-)}-G-L-V \text{ (major fraction)} \]

\[ \text{F-N-A-H-G-D-A-N} \text{ (minor fraction)} \]

This sequence was identical with the protein sequence of human galectin-1 as the protein database search revealed.
The sequencing profile for standard aminoacid residues is depicted in the initial trace. The sequence profile for the 29 kDa protein from the unretarded fraction (fig 2.14 A) was:

M-X-P-I-L-G-Y
P-I-L-G-Y

This sequence was identical with the protein sequence of glutathione-S transferase (Smith et al., 1986)
The sequencing profile for standard aminoacid residues is depicted in the initial trace. The sequence profile for the 29 kDa protein from the retarded fraction (fig 2.14 A) was:

\[ \text{G} - \text{S} - \text{M} - \text{A} - (\text{G}) - \text{L} \]

This sequence was identical with the protein sequence of human galectin-1 as the protein database search revealed. This fraction was the galectin-1 dimer.
Appendix 3

Mass spectrometry data
Spectra of tryptic digests of galectin-1 resulted from a mass spectrometry determination in which sinapinic acid was used as a matrix.
Spectra of tryptic digests of a DTT-treated galectin-1 resulted from a mass spectrometry determination in which sinapinic acid was used as a matrix.
Spectra of tryptic digests of galectin-1 resulted from a mass spectrometry determination in which α-CHC was used as a matrix.
Spectra of tryptic digests of DTT-treated galectin-1 resulted from a mass spectrometry determination in which α-CHC was used as a matrix.
Spectra of tryptic digests of galectin-1 resulted from a mass spectrometry determination in which DHB was used as a matrix.
Spectra of tryptic digests of DTT-treated galectin-1 resulted from a mass spectrometry determination in which DHB was used as a matrix.
Molecular weight of all possible combinations of peptides that could result from disulfide-bond formation in galectin-1

<table>
<thead>
<tr>
<th>Tryptic peptides</th>
<th>Cys bonds</th>
<th>Theoretically calculated molecular weight</th>
</tr>
</thead>
<tbody>
<tr>
<td>T1</td>
<td>2 - 16</td>
<td>2129.8</td>
</tr>
<tr>
<td>T1 - T6</td>
<td>2 - 60</td>
<td>3721.2</td>
</tr>
<tr>
<td>T1 - T5</td>
<td>2 - 130</td>
<td>2684.0</td>
</tr>
<tr>
<td>T1 - T8</td>
<td>2 - 42</td>
<td>3560.4</td>
</tr>
<tr>
<td>T1 - T13</td>
<td>2 - 88</td>
<td>4941.6</td>
</tr>
<tr>
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<td>16 - 42</td>
<td>3560.0</td>
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<tr>
<td>T1 - T5</td>
<td>16 - 60</td>
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<td>60 - 130</td>
<td>2142.3</td>
</tr>
</tbody>
</table>
Appendix 4

Various Protocols
1. Preparation of competent cells

Preparation of *E. coli* competent cells

The method used to prepare competent cells was developed by Nishimura *et al.* (1990). For this a 50 ml culture inoculated with 0.5 ml over night culture was grown with aeration in LBW medium supplemented with 10 mM MgSO$_4$·7H$_2$O and 0.2 % glucose (solution A) to mid-logarithmic phase. The cells were kept on ice for 10 minutes, then pelleted at 3,000 r.p.m. for 10 minutes at 4 °C in a Cool-Spin centrifuge. Cells were resuspended gently in 0.5 ml of solution A, precooled on ice, then 2.5 ml of storage solution B [36 % glycerine, 12 % PEG (MW 7500), 12 mM MgSO$_4$ was added to LB broth (pH 7) and sterilised by filtration], was added and mixed without vortexing. The competent cells were divided and stored at -80 °C until use. The transformation efficiency determined for the *E. coli* strain used (DH5α) was 2x10$^7$/µg (aliquot kindly donated by Dr Christie). The competent cells prepared by us had a transformation efficiency of 2x10$^5$/µg and they were successfully used for transformations.

Preparation of *S. cerevisiae* competent cells by the lithium acetate (LiOAc) method

The method used to prepare yeast competent cells was developed by Hill *et al.* (1991). 10 µl of 1/10 dilution from a 5 ml overnight yeast culture grown in YPD (2 % glucose, 2 % Bacto-peptone, 1 % yeast extract) was used to inoculate 100 ml cultures which were grown over night. The light scattering value of 0.9 represented a concentration of 10$^7$ cells/ml.

A culture containing 4x10$^7$ cells/ml was centrifuged at 3 000 r.p.m. for 5 min at 4°C in a Cool Spin Centrifuge. The pellet was resuspended in 10 ml TE, pH 8, and centrifuged again at 3000 r.p.m. for 5 min. The pellet was resuspended gently in 10 ml sterile lithium acetate 0.1 M in TE buffer, pH 8.0. and the cells were stored in a -20°C freezer.
2. **Ligation**

**Blunt end ligation**

For a blunt end ligation a 1:10 molar ratio between the vector and the insert was needed. The formula used to calculate amount of insert necessary was:

\[
[(\text{ng of vector} \times \text{kb size of insert}) / \text{kb size of vector}] \times (\text{molar ratio of insert/ vector}) = \text{ng of insert.}
\]

The standard reaction for the ligation is:

- 2 μl T4 DNA ligase 10x Buffer,
- 1 μl (50 ng) vector,
- X μl PCR product,
- 1 μl T4 DNA ligase (1 Weiss unit/μl)

and water to a final volume of 20 μl.

**Ligation of cohesive termini**

For a ligation of cohesive termini, the molar ratio between the vector and the insert should be 1:1.

3. **Transformation of bacterial cells**

Transformation of bacterial cells using the method of Nishimura (1990). LB/Amp plates were prepared and brought to room temperature. Half of the ligation mixture (10 μl) was added to a vial 100 μl of competent cells thawed for 5 min and kept on ice at all times. Content of the tube was mixed gently and incubated on ice for 20 min. The cells were heat shocked for 50 seconds at 42 °C. The tubes were returned to ice for 2 min, then 1 ml LB without ampicillin was added and cells were incubated for 1 hour at 37 °C to develop ampicillin resistance. 100 μl of this culture was spread on a plate, the rest was centrifuged and resuspended in 100 μl LB/Amp and also plated out. Plates were incubated over night and the results were scored next day.
4. Synthesis of Rb DH5α competent cells (personal communication from Dr Jo Putteril)

A single colony from a plate of DH5α E.coli cells, less than a week old, was used to inoculate 5 ml growth medium. This was grown at 37°C overnight. 1:200 dilution of overnight culture was used to inoculate 100 ml growth medium. This was grown at 37°C for 2-3 hours until the light scattering values were between 0.5 and 0.6 at 600 nm. The culture was chilled on ice for 15 minutes and centrifuged at 3-4,000 r.p.m. for 10 minutes. While chilled on ice the cells were gently resuspended in 20-30 ml Tfb I very gently and then incubated on ice for further 5-10 minutes. Culture was centrifuged at 3-4,000 r.p.m. in a Cool Spin Centrifuge, for 10 minutes at 4°C. The pellet was resuspended in 4 ml Tfb II very gently and aliquoted (200 µl/ aliquot). Aliquots were frozen in liquid nitrogen and stored at -80°C.

Growth medium: bacto-yeast extract, 0.5% (w/v),
bacto-tryptone, 2% (w/v),
MgSO₄ x 7 H₂O, 0.5% (w/v), adjusted to pH 7.6 with 2M KOH.

**Tfb I**: 30 mM K acetate,
100 mM RbCl,
10 mM CaCl₂ x 2 H₂O,
50 mM MnCl₂ x 4 H₂O to which we added glycerol to 15% v/v.
The pH was adjusted to 5.8 with 0.2 M acetic acid and then the buffer was filter sterilised.

**Tfb II**: 10 mM MOPS,
75mM CaCl₂ x 2 H₂O
10 mM RbCl
15% glycerol, (v/v).
The pH was adjusted to 6.5 with 2M KOH and then the buffer was filter sterilised.
5. Transformation of Rb competent cells with the pGEM-T vector including the fos insert

10 µl ligation mixture was added to an aliquot of Rb competent cells and incubated on ice for 30 minutes, then incubated at 42°C for 90 seconds and returned on ice for 2-5 minutes. 1 ml LB growth medium was added and then the cultures were incubated for 1 hour at 37°C and 100 µl culture were plated out on a X-gal-IPTG-Amp plate and grown over night.

6. Screening for transformants

Plasmid pGEM-5Zf(+) contains a multiple cloning region within the α-peptide coding region for the enzyme β-galactosidase. The insertional inactivation of the α-peptide allows recombinant clones to be directly identified by colour screening on indicator plates (X-gal-IPTG-Amp plates) (Promega data sheet).

The method of "colony PCR" was also used to check the white colonies for the presence of the fos insert. The template in this case was a small part of the transformant culture inoculated in the PCR mixture using a sterile Gilson pipette tip.

7. Digestion of pGEM-T vector containing the fos insert

6 µl vector pGEM-T (234 ng) containing a fos insert was digested with µl Stu I (10 U/µl) under the same conditions specified in Section 2.2.9.

The reaction mixture was incubated at the end for 10 minutes at 65°C to inactivate Stul. A dephosphorylation of this vector with calf intestinal alkaline phosphatase was performed, the galectin-1 PCR product was backfilled and phosphorylated, and the two components were subjected to ligation and used in transformation reactions but due to the fact that one of these steps did not work we do not give the detailed protocols.

For the both following methods cells that were used as a source for RNA were grown to confluence, then deprived of serum over night. For the expression of the fos proto-oncogene another change of medium was necessary, this time containing 15 % FCS. Cultures were incubated for 30 minutes with this medium then harvested for the
oncogene were found we proceeded to treat the cells that were used as source of RNA with α1-antitrypsin (25 μg/ml) and respectively with human recombinant galectin (25 μg/ml) in order to assess the effect of this treatment on the expression of c-fos. We decided that 25 μg/ml of galectin was initially enough for our studies considering also the cost of production of recombinant galectin.

8. Transformation of yeast cells

Transformation of yeast cells using the method of Hill et al (1991). 100 μl of yeast competent cells suspension was added to 4 μl (8.6 μg) transforming DNA. The contents were mixed gently and left at room temperature for 5 min. To this 280 μl PEG solution (50 % PEG 4000 in lithium acetate solution, filter sterilised) was added and mixed by inverting the tubes. Subsequently the tube was incubated at 30°C for 45 min without shaking. 45 μl DMSO were added and mixed by inversion, then heat shocked at 42°C for 5 min and 1 ml YPD was added. Cells were centrifuged at 6500 r.p.m. and resuspended in fresh YPD to remove the DMSO. Cells were centrifuged again and resuspended in 100 μl YPD. This mixture was plated out on selective media (SD) supplemented with histidine, uracil and tryptophan and transformants were scored after 3 days at 30°C (YPD medium contained 2 % glucose, 2% bacto-peptone and 1% yeast extract; SD medium contained 0.67 % yeast nitrogen base without amino acids and 2 % glucose). The transformation efficiency for the S. cerevisiae strain using this method is 41070±6700 LEU2 transformants per μg DNA.

9. Coupling of asialofetuin to CNBr Sepharose

The coupling of asialofetuin to CNBr-Sepharose was based on the method of Axen and Ernback (1971) and Ostrove (1990). Fetuin (50 mg) was dissolved in 10 ml 0.01 M HCl (the pH was adjusted to 2 with NaOH) and incubated at 80°C for an hour (desialylation). The pH was adjusted again to 5.6 and the solution was dialysed overnight in coupling buffer (0.1 M NaHCO₃ / 0.5 M NaCl, pH 8.3). CNBr Sepharose (2 g) was left to swell in 50 ml 1 mM ice-cold HCl for 1h. The Sepharose was washed then with 800 ml 1 mM ice-cold HCl and transferred in the same vial with the asialofetuin. The two components were left to react for 2h at room temperature and then centrifuged. The gel was washed with
100 ml of coupling buffer. 6 ml of 1M ethanolamine were incubated for 2h at room temperature with the Sepharose to block the uncoupled linkages. The gel was finally washed with 125 ml of the following buffers:

a) Coupling buffer
b) 0.1 M Na Borate/1M NaCl
c) 0.1 M Na acetate/1M NaCl
d) 0.1 M NaHCO₃/glycine
e) repeated b)
f) repeated c)
g) 0.01 M Na acetate pH 4.1
h) 30 mm Tris-HCl pH 7.5.

and stored at 4°C in Tris-HCl Buffer containing 0.02% azide.

10. Coupling of glutathione (reduced) to epoxy-activated Sepharose

The method of Simons and Van der Tagt (1977) was used to perform this coupling. 100 ml Sepharose 4B-CL was thoroughly washed with water (1 litre) and suction dried on a glass sinter funnel. 100 ml of 1,4-butanediol-diglycidylether (Araldite RD-2) and 100 ml of 0.6 M NaOH containing 2 mg/ml of sodium borohydride was added to the Sepharose and mixed by rotation in a 1 litre round-bottomed flask for 8 h at 25°C. After incubation the Sepharose was washed extensively with water, then the gel was resuspended in 500 ml of 44 mM phosphate buffer (8 ml 0.2M KH₂PO₄ mixed with 28 ml of 0.1 M Na₂HPO₄ and brought to a final volume of 100 ml with water), pH 7.0.

10 g of glutathione was dissolved in 80 ml H₂O (adjusted to pH 7 using 7.5 M KOH) then the solution was brought to a final volume of 100 ml.
The glutathione solution was combined with Sepharose and mixed by rotation overnight at 37°C. The next day the gel was washed again extensively with water and then was incubated for 4 hours with 1 M ethanolamine, pH 9.5.

At the end the gel was washed again with water and stored in PBS with 0.02% azide.
11. Preparation of samples for SDS-PAGE

Samples and molecular weight markers for electrophoresis were boiled for 5 min in 1x loading buffer (0.0625M Tris-HCl, pH 6.8, 2.3 % (w/v) SDS, 5 % (v/v) 2-mercaptoethanol/10 % (v/v) glycerol, 0.001 % (w/v) bromophenol blue ), centrifuged at 10,000 r.p.m. for 3 min and loaded onto gels.

The high molecular weight markers were used for the 10% gels and they contained (in daltons): myosin (205,000), β-galactosidase (116,000), phosphorylase b (97,400), bovine serum albumin (66,000), ovalbumin (44,000), glyceraldehyde-3-phosphate dehydrogenase (36,000), carbonic anhydrase (29,000), trypsinogen (24,000), trypsin inhibitor-soybean (20,100), lactalbumin (14,200).

The low molecular weight markers were used for the 15% gels and contained (in daltons): albumin (66,000), ovalbumin (45,000), glyceraldehyde-3-phosphate dehydrogenase (36,000), carbonic anhydrase (29,000), trypsinogen (24,000), trypsin inhibitor (20,000), α-lactalbumin (14,200), aprotinin (6,500).
Appendix 5

Galectin-1 PCR products
Figure: Amplification of cDNA originating from RNA of tumour and normal cells.

A: 1Kb DNA ladder.
B: PCR product from MG 63 (osteosarcoma) cells.
C: PCR product from HELa (epidermal carcinoma) cells.
D: PCR product from U2OS (osteosarcoma) cells.
E: PCR product from MPS (fibroblast) cells.
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