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NUCLEAR EXPORT OF A PRE-mRNA SPLICING FACTOR GALECTIN-3

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NUCLEAR EXPORT OF A PRE-mRNA SPLICING FACTOR, GALECTIN-3

By

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ABSTRACT

NUCLEAR EXPORT OF A PRE-mRNA SPLICING FACTOR, GALECTIN-3

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Previous immunofluorescence and subcellular fractionation studies have documented that galectin-3 (Mr ~ 30,000) distributes differentially between the nucleus and the cytoplasm, depending on the proliferative state of the cells under analysis. Thus, it appears that controlled nuclear versus cytoplasmic distribution of galectin-3 may be one of the mechanisms regulating its function. The goal of this thesis was to study parameters that play a role in determining this distribution; one such parameter turned out to be the export of the protein from the nucleus to the cytoplasm.

Using digitonin-permeabilized 3T3 cells, we provide evidence that galectin-3 is rapidly and selectively exported from the nucleus. Although both phosphorylated and nonphosphorylated isoforms of galectin-3 are found in the nuclear fraction, only phosphorylated galectin-3 is identified in the exported fraction, implying that phosphorylation is important forthe nuclear export of the protein. The rate of galectin-3 export is decreased by cold temperature and by the addition of either wheat germ agglutinin or vanadyl cations (VO^{2+}). More strikingly, galectin-3 export can be inhibited, at least partially, by the simultaneous addition of a peptide bearing a nuclear export signal

plus a dinucleotide analog of the cap structure found at the 5'-end of mRNAs. These results suggest that galectin-3 may be exported in association with ribonucleoprotein complexes containing monomethylated cap structure as well as polypeptides containing nuclear export sequences.

The transported fraction of the nuclear export assay was analyzed in terms of the polypeptide and RNA components. Gel filtration of the exported nuclear material and analysis for galectin-3 showed that the lectin can be found in at least two sets of high molecular weight complexes (~650 kD and ~60 kD). In the presence of the saccharide ligand, lactose, both of these complexes are disrupted and galectin-3 chromatographs to a position corresponding to ~30 kD polypeptide. The polypeptide components of the high molecular weight complexes containing galectin-3 are specifically revealed by affinity adsorption on a lactose-agarose column, specific elution by lactose, and gel electrophoresis. These polypeptides are not bound to a control cellobiose column.

The transported fraction of the nuclear export assay also contains RNA. In the low molecular weight range, the RNA species include tRNA (~80 nucleotides) as well as RNAs of ~100, 300, and 650 nucleotides. High molecular weight RNAs, ranging from ~1 kb to 5 kb, include poly $(A)^+$ mRNA as revealed by hybridization with an oligo(dT) probe. Compounds containing vanadyl cations, which inhibit the export of galectin-3, also inhibit the export of RNAs. All of these results are consistent with the notion that galectin-3 is associated with a ribonucleoprotein complex during its export from the nucleus.

To my parents and Huifen

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LIST OF ABBREVIATIONS

ATP:	adenosine triphosphate
BR:	Balbiani ring
CBC:	cap binding complex
CBP:	cap binding protein
CRD:	carbohydrate recognition domain
DNA:	deoxyribonucleic acid
ER:	endoplasmic reticulum
FGF:	fibroblast growth factor
FITC:	fluorescein isothiocyanate
GDP:	guanosine diphosphate
GlcNAc:	*N*-acetylglucosamine
GTP:	guanosin triphosphate
HIV:	human immunodeficiency virus
hnRNP:	heterogeneous ribonucleoprotein complex
Lac:	lactose
MDCK:	Madin-Darby canine kidney
MIP:	M9 interacting protein
mRNA:	messenger ribonucleic acid
NEPHGE:	nonequilibrium pH gradient electrophoresis

NES:	nuclear export signal
NF:	nuclear fraction
NLS:	nuclear localization signal
NPC:	nuclear pore complex
NTF:	nuclear transport factor
PAGE:	polyacrylamide gel electrophoresis
PKI:	protein kinase A inhibitor
RAB:	Rev activation domain
RanGAP:	Ran GTPase activating protein
RIP:	Rev interacting protein
RNP:	ribonucleoprotein
SAP:	spliceosome-associated protein
SDS:	sodium dodecyl sulfate
snRNP:	small nuclear ribonucleoprotein
SV40:	simian virus 40
TB:	transported buffer
TF:	transported fraction
T-TBS:	thimerosol-Tris buffered saline
VRC:	vanadyl ribonucleoside complex
WGA:	wheat germ agglutinin

CHAPTER I

LITERATURE REVIEW

INTRODUCTION TO GALECTINS

Galectins are a family of proteins defined by their affinity toward galactose/lactose and sequence similarity at the characteristic carbohydrate binding domain (Barondes *et al.*, 1994; Kasai and Hirabayshi, 1996; Leffler, 1997). Galectins were previously found in species in the animal kingdom, including sponges, nematodes, fish, amphibian, birds and mammals. Hence, galectins were considered as a group of *animal* lectins for years (Kasai and Hirabayashi, 1996). However, the discovery of a galectin in inky cap mushroom, *Coprinus cinereus,* challenged this concept (Cooper *et al.*, 1997). This finding not only confirms that galectins can be outside the animal kingdom, but also implies that a common ancestor for animals and fungi may contain galectin-like polypeptides (Figure 1).

The evolution of galectins has been an interesting topic. For instance, they have been expanded into a large group of homologs in mammals (Table I), in which at least ten galectins have been identified (Table 1). On the contrary, it is surprising to see that galectins seem to be completely abandoned in some descendants, like yeasts and insects (Cooper *et al.*, 1997) .

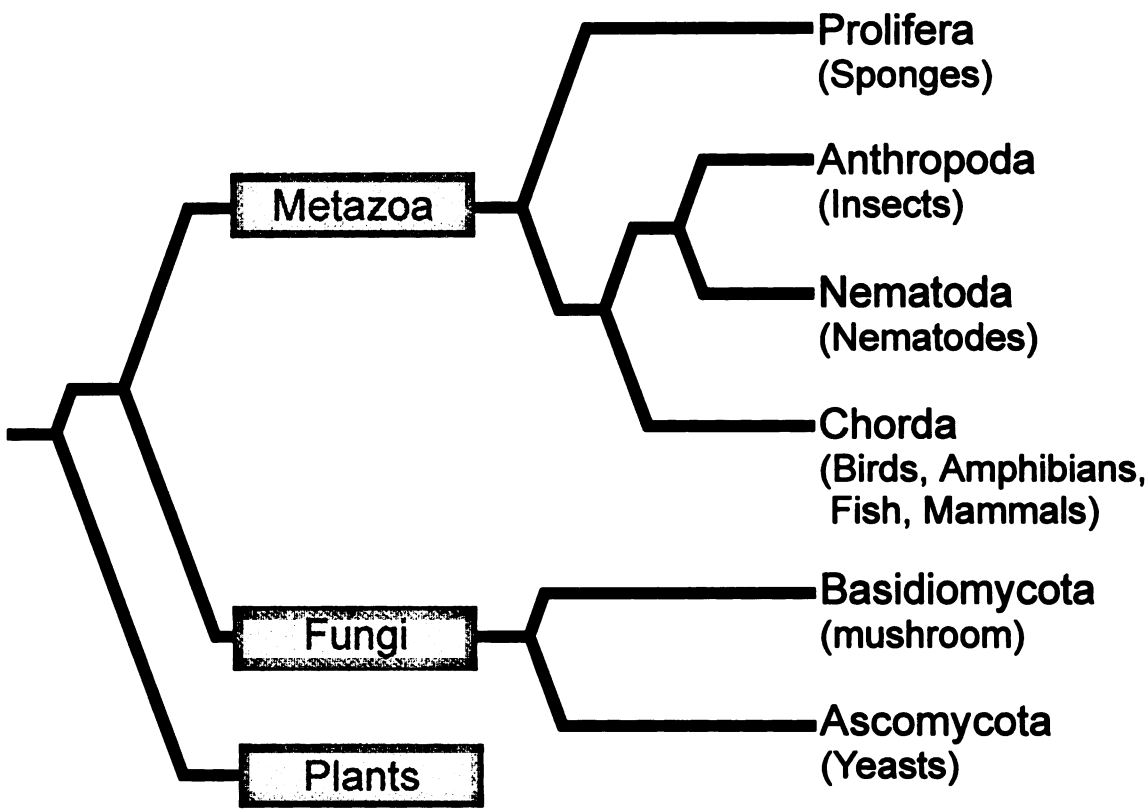


Figure 1. A phylogenetic tree for crown eukaryotes. Galectins have been found in animals, like sponges, nematodes, and vertebrates, as well as a fungus, inky cap mushroom.

Table 1. Summary of Galectins

Designations	MW (kDa)	Structural Type	Tertiary Structure	Source Organisms	Tissue/Cell Distribution
Mammals					
Galectin-1	14.5	Proto	Dimer	Human, rat, mouse, hamster, monkey, ox, pig	Muscle, heart, lung, placenta, brain, spleen, liver, lymph node, thymus, prostate, colon
Galectin-2	14.5	Proto	Dimer	Human, mouse	Small intestine
Galectin-3	29-35	Chimera	Monomer	Human, rat, mouse, dog, hamster	Macrophage, colon, leukemia cell, fibroblasts
Galectin-4	36	Tandem repeat	Monomer	Human, rat, pig, mouse	Alimentary tract epithelium
Galectin-5	17-18	Proto	Monomer	Rat	Erythrocyte
Galectin-6	34	Tandem repeat	Monomer	Mouse	Gastro-intestine
Galectin-7	14.5	Proto	?	Human, rat	Skin
Galectin-8	34	Tandem repeat	Monomer	Human, rat	Liver, lung, kidney
Galectin-9	35	Tandem repeat	?	Human, rat, mouse	Kidney, thymus, Hodgkin's lymphoma
Galectin-10	17	Proto	Dimer	Human	
Birds					
Chick 14K	14	Proto	Monomer	Chick	Skin, intestine, etc.
Chick 16K	16	Proto	Dimer	Chick	Muscle, liver, etc.
Chick 30K	30	Chimera	?	Chick	Chondrocyte
Amphibians					
Xenopus 16K	16	Proto	Dimer	*Xenopus laevis*	Skin
Bufo 15K	14.5	Proto	Dimer	*B. Arenarum*	Oocyte
Fish					
Electrolectin	16	Proto	Dimer	Electric eel	Electric organ
Congerin	16	Proto	Dimer	Conger eel	Skin mucus
Nematodes					
Nematode 32K	32	Tandem repeat	Monomer	*C. elegans*	Cuticle, pharynx
Nematode 16K	16	Proto	Dimer	*C. elegans*	?
OvaGalBP	32	Tandem repeat	?	*O. volvulus*	?
Sponges					
GcLt1/2	13-18	Proto	Dimer	*G. cydonium*	Plasma membrane
Fungi					
Cgl-I/II	15.5/ 17	Proto	Dimer	*C. cinereus*	Fruiting body

The galectin structure also went through some changes. Chimera-type galectins are only found in vertebrates but not in primitive animals, implying that they are evolved later than proto- and tandem-repeat type galectins (Table 1). In addition, it seems that activities other than carbohydrate binding has been introduced into the galectin structure. For instance, galectin-10, the Charcot-Leyden crystal protein, was reported to have a lysophospholipase activity (Weller *et* al., 1982; Ackerman *et al.*, 1993).

In the following section, I will focus on the structural characteristics, intracellular sorting, and biological functions of galectins. Since galectin-1 and galectin-3 are the most studied galectins, I will discuss properties of the whole family using them as examples.

STRUCTURAL CHARACTERISTICS

Galectins are categorized into proto-, chimera-, and tandem-repeat types according to their primary structure. According to their molecular architecture, all galectins can be grouped into the following three types: proto-, tandem-repeat and chimera types (Hirabayashi *et al.*, 1992) (Table I). Prototype, represented by mammalian galectin-1 is composed of only a single carbohydrate recognition domain (CRD) motif. Tandem-repeat type galectins, exemplified by galectin-4, consist of two homologous CRD domains. Chimera-type galectin, represented by galectin-3 is made of a CRD and a non-lectin domain.

Carbohydrate recognition domain is the signature motif for galectin family. The galectin family has a signature structural motif, carbohydrate recognition domain, which confers saccharide-binding specificity. This domain consists of about 135 amino

acids that fold into a β-sandwich with 5- and 6-stranded β-sheets as shown by X-ray diffraction studies on galectin-1 and galectin-2 (Lobsanov *et al.*, 1993; Liao *et al.*, 1994). All of the amino acid residues important for binding with the bound N-acetyllactosamine ligand are found on a continuous stretch of the polypeptide chain, which makes up four antiparallel β-strands and is designated as *carbohydrate-binding cassette* (Gitt *et al.*, 1992; Lobsanov and Rini, 1997). Galectins forming homodimers, like galectin-1 and galectin-2, may contain another structural motif, *dimer interface*. This motif is formed by two β-strands at the end away from the carbohydrate-binding cassette .

Carbohydrate-binding cassette is conserved among galectin family. Amino acid residues that are involved in carbohydrate binding are highly conserved among galectins (Lobsanov *et al.*, 1993; Liao *et al.*, 1994). Analysis of the genomic DNA structure of a number of galectins has also shown that they are all included on a single DNA exon (Barondes *et al.*, 1994; Lobsanov and Rini, 1997).

There are eight major amino acids in CRDs that directly interact with a disaccharide ligand, like N-acetyllactosamine (Lobsanov *et al.*, 1993; Liao *et al.*, 1994; Ahmed and Vasta, 1994). Among these amino acids, five residues are invariably conserved in all galectins. They primarily interact with the lactose moiety of the ligand (Figure 2). On the other hand, a lot of variations occur at the other three amino acids. They primarily interact with the N-acetylglucosamine moiety of the ligand.

Ahmed and Vasta (1994) found that complete identity with galectin-1 CRD at the latter three residues is found in a number of the galectins, whereas substitution and deletion occur at these positions in other galectins. Based on identity of these three

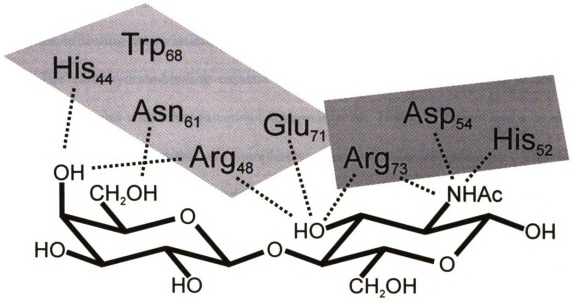


Figure 2. Interaction of bovine galectin-1 with N-acetyllactosamine. Amino acids boxed by lighter shade are completely conserved in all galectins except mammalian galectin-10. Amino acids highlighted by darker shade have greater variations among galectin family. (Modified from Ahmed and Vasta, 1994)

amino acids with the galectin-1 CRD, carbohydrate recognition domains of galectins can be classified as two types: type I (conserved) and type II (variable) CRDs (Ahmed and Vasta, 1994). So far, only chicken 14-kDa, chicken 16 kDa, and Bufo 15-kDa galectins belong to the type I category. Type I CRDs have very similar fine carbohydrate-binding specificity, presumably due to the extensive identities at the carbohydrate-binding cassette. On the other hand, type II CRDs have very different carbohydrate recognition properties, reflecting the variations in the cassette motif.

The carbohydrate-binding cassette in galectin-10 is unusually varied at these positions, compared with other galectins (Ackerman *et al.*, 1993). Different amino acids are found at the positions that are completely conserved in other galectins. This is perhaps the structural basis for its weaker sugar binding activity. Probably, in order to accommodate a lysophospholipase activity as well as lectin activity, some compromise had to be made on the primary structure.

Galectin-1/galectin-2 dimer interface mediates the homodimerization of galectin subunits. The dimer interface for galectin-1 and galectin-2 is mainly made up of hydrophobic amino acid residues, which form nonpolar surfaces (Lobsanov *et al.*, 1993; Liao *et al.*, 1994). This interface mediates the formation of a 2-fold symmetric anti-parallel dimers seen in galectin-1 and galectin-2. Recent x-ray crystallography of galectin-3 CRD (Kanigsberg et al. 1997) and N-terminal CRD of galectin-4 (Kayden *et* al., 1997) have shown that the nonpolar surfaces are essentially eliminated by the presence of hydrophilic residues. Lobsanov and Rini (1997) argued that the disruption of nonpolar surfaces prevents galectin-3 or galectin-4 from forming homodimer complex. However, whether CRD of galectin-3 or galectin-4 forms dimers is still controversial.

For instance, when isolated C-domain of galectin-3 was analyzed by gel filtration chromatography, dimer formation was found in a significant fraction of galectin-3 CRD (Wang *et al.*, 1993). Therefore, whether this dimer interface is exclusively found for prototype galectins like galectin-1 and galectin-2-needs further examination.

Galectin-3, a chimera-type galectin, contains a proline-glycine-rich domain. Galectin-3 contains a proline-glycine-rich domain at the NH_2-terminal end (Jia and Wang, 1988), in addition to a CRD. This N-domain is composed mainly of sequence repeats, each consisting of nine amino acids (PGAYPGXXX) in mammalian homologues (Jia and Wang, 1988) and eight amino acids (PGPYPGGP) in the chicken counterpart (Nurminskaya and Linsenmayer, 1996). So far, the role(s) of this domain is not clear, although similar features have also been seen in some other proteins, like a spliceosome-associated protein (SAP) SAP62 (Bennet and Reed, 1993) and an autoantigen annexin XI (Misaki *et al.*, 1994).

SAP62 is a defined component of spliceosome complexes E, A and B (Bennett *et al.*, 1992; Hong *et al.*, 1997). This splicing factor contains a C-domain of 22 proline-glycine-rich heptapeptide repeats with the consensus sequence PGVHPPA (Bennett and Reed, 1993) besides the Zn finger-containing N-domain. It has been proposed that the P-, G-rich domain may be involved in protein-protein interaction (Bennett and Reed, 1993). Nevertheless, it will be interesting to see how it is related to galectin-3 N-domain as both SAP62 and galectin-3 are involved in pre-mRNA splicing.

Annexin XI, on the other hand, was identified as a calcyclin-associated protein (Tokumitsu *et al.*, 1992), which is recognized by sera of patients with various autoimmune diseases (Misaki *et al.*, 1994). Structurally, it contains a proline-glycine-rich

N-terminal tail domain as well as a core domain that was conserved among annexin family. The N-terminal tail has been shown to be responsible for its nuclear localization (Mizutani *et al.*, 1995). Interestingly, annexin XI, a 56-KDa protein also shows a dual (nuclear and cytoplasmic) localization (Misaki *et al.*, 1994), which raises the possibility that galectin-3 N-domain may also have the same intracellular targeting capacity.

INTRACELLULAR SORTING

Subcellular localization of galectins has been extensively studied for galectin-1 and galectin-3. These two galectins, like a large number of other proteins (Smalheiser, 1996), have been found to reside in multiple cellular compartments. Protein targeting between different compartments is usually via signal-mediated pathways. It is always interesting to see how different signals are incorporated into the protein structure, and how a particular signal is selected over others. Localization studies on galectins definitely may help answer these questions, considering their relatively simple primary structures. In this section, I will concentrate on the pathways mediating the extracelluar secretion as well as nucleocytoplasmic distribution.

Galectin-1 and galectin-3 are secreted out of cells via the pathway(s) distinct from the classical pathway. Most extracellular proteins leave the cells using the classical secretory pathway, which requires a signal peptide on the substrate proteins (Rothman and Orci, 1992). The secreted proteins enter the ER, and move to the cell surface via the Golgi apparatus. Both galectin-1 and galectin-3 lack a classical secretory signal (Muesch *et al.*, 1990) and are secreted through so-called non-classical secretory

pathway(s). Examples of proteins utilizing nonclassical pathways include interleukin-1β (Siders and Mizel, 1995), FGF-1 (Tarantini *et al.*, 1995), and FGF-2 (Piotrowicz *et al.*, 1997). Both galectin-1 and galectin-3 have been used as a model system to explore these pathways.

Galectin-1 appears to be externalized from cells through extracellular vesicles derived from evaginations of plasma membrane. Galectin-1 has been found to be secreted at a higher rate in differentiated muscles cells than in myoblasts (Cooper and Barondes, 1990). Immunofluorescent localization studies showed galectin-1 is primarily cytoplasmic in myoblasts, but cytoplasmic staining is largely lost in myotubes, the well-differentiated muscle cells. Examination of differentiating myoblasts, the intermediate between two stages, showed that galectin-1 had a patchy distribution at this transition. These patches were in the outermost layer of cytoplasm and appeared to correspond to the evaginations in plasma membranes. Cooper and Barondes (1990) hypothesized that these evaginations may later mature into extracellular vesicles and serve as the secretory vehicles for galectin-1.

Yeast cells can also secret galectin-1, when they are transformed with a galectin-1-expressing vector (Cleves *et al.*, 1996). This observation indicates that a pathway for secreting galectin-1 is conserved between budding yeast and mammalian cells. This secretion does not require any components in the classical secretory pathway nor the yeast multidrug resistance-like protein Ste6p, an **a** factor transporter. A genetic screen was designed to identify gene products which are involved in secretion. Two gene products, NCE1 and NCE2, are able to enhance galectin-1 secretion when they are

overexpressed. (Cleves *et al.*, 1996). Furthermore, *nce2* null mutants showed a great reduction in galectin-1 secretion, establishing the pivotal role of *NCE2* in nonclassical pathway for galectin-1. The primary structure of *NCE2* may allow the polypeptide to fold into a membrane protein. Whether this protein is related to the secretion mechanism seen in muscle cells awaits more documentation.

Galectin-3 is selectively secreted from apical surface, but not from the basolateral membrane, of Madin-Darby canine kidney (MDCK) cells via a nonclassical pathway. Plasma membranes of polarized epithelia cells have two domains, the apical and basolateral, which are separated from each other by tight junctions (Simons and Wandinger-Ness, 1990; Mostov *et al.*, 1992). Distinct sets of proteins are targeted to each domain. Galectin-3 in MDCK cells is specifically secreted from the apical surface through a nonclassical pathway (Lindstedt *et al.*, 1993). This conclusion is supported by the observation that this pathway was not inhibited by brefeldin A or monensin, drugs that inhibit the ER-Golgi pathway. On the other hand, the secretion is enhanced by calcium ionophore A23187 and by heat shock at 42°C. Lowered temperature as well as addition of nocodazole blocks its secretion. All of these distinguish this pathway from the classical pathway.

Galectin-3 distributes between cytoplasm and the nucleus as a function of proliferating capacity. In mouse 3T3 fibroblasts, galectin-3 was found both in cytoplasm and in the nucleus. (Moutsatsos *et al.*,1986). The distribution between these two compartments correlates with proliferative states of cells (Moutsatsos *et al.*, 1987). While in serum-starved cells, galectin-3 is primarily cytoplasmic, it becomes

predominantly nuclear as cells are reactivated by serum addition. Similar results can be seen in dense cultures versus sparse cultures. Subcellular fractionation studies showed that galectin-3 in nuclear fraction has two isoforms, a pI 8.2 phosphorylated form and a pI 8.7 dephosphorylated form. However, only phosphorylated isoform is seen in cytosolic fraction. These results suggest that phosphorylation/dephosphorylation is involved in distribution between two compartments.

Similar results have also been shown on human primary fibroblasts. These cells are susceptible to senescent change (Hamann *et al.,* 1991; Cowles *et al.,* 1989). In young cells, galectin-3 is mainly in the nucleus. As cells become senescent, galectin-3 is exclusively found in cytoplasm.

Altered intracellular distribution has also been seen in neoplastic cells (Lotz *et al.,* 1993). Paradoxically, galectin-3 is exclusively cytoplasmic in colon carcinoma cells, in contrast to its predominant nuclear localization in well-differentiated epithelial cells.

Nuclear galectin-3 is associated with the nuclear matrix defined by detergent and high-salt extraction. In immunofluorescence studies, galectin-3 was found diffusely distributed throughout the nucleoplasm excluding nucleoli. Even after detergent and high-salt extraction, it still associates with nuclear structures that contain splicing factors SC35 and Sm proteins (Wang *et al.,* 1995a; Hubert *et al.*, 1995; Vyakarnam *et al.,* 1997). The association is dependent on RNA-containing structures. Consistent with these results, galectin-3 has been found in purified nuclear matrix of adenocarcinoma cells (Wang *et al.*, 1995b).

Ultrastructurally, galectin-3 is found in nucleoplasm, interchromatin spaces excluding interchromatin granules, and on the border of condensed chromatins (Hubert *et*

al.,1995). The latter is considered as the site of mRNA synthesis and early events of pre-mRNA splicing (Spector, 1996). Therefore, this result agrees with the notion that galectin-3 is a pre-mRNA splicing factor. Most surprisingly, galectin-3 is also associated with dense fibrillar components and periphery of nucleoli, in contrast to the exclusion of galectin-3 from the nucleoli under fluorescence microscope (Hubert *et al.*, 1995). What causes this discrepancy is still not clear.

The import of galectin-3 into the nucleus has been studied and preliminary data showed that galectin-3 probably enters the nucleus through an active mechanism. Transfection experiments revealed that FLAG-tagged constructs expressing the duplicate repeat and triplicate repeat of the galectin-3 coding sequence can enter the nucleus as well as FLAG-tagged galectin-3 (Tsay and Wang, unpublished results). Inasmuch as both duplicate and triplicate repeats have molecular sizes exceeding the diffusion limit of nuclear pores, about 40~60 kDa for globular proteins (Peters, 1986), we concluded that some active mechanisms are required for the entry for these constructs. Whether there exists an import signal for galectin-3 itself still awaits further investigation.

Galectin-1 is also present in nuclear structures that contain splicing factors. Confocal microscopic experiments showed that galectin-1 is also localized in nuclear structures with Sm proteins as well as SC35, which supports the notion that it participates in pre-mRNA splicing (Vyakarnam *et al.*, 1997). It will be interesting to know whether its association with these structure is also dependent on RNAs. If the association is also sensitive to RNase treatment, this implicates that the CRD is probably responsible for the binding activity for both galectin-1 and galectin-3. However, if no RNA dependence is

seen for galectin-1, it will support the notion that the N-domain of galectin-3 perhaps plays an important role in association with nuclear matrix.

BIOLOGICAL FUNCTIONS

Galectins were initially found in the quest for proteins that recognize cell surface carbohydrates, based on the presumption that they perhaps participated in cell-cell interactions (Barondes, 1997). However, there is accumulating evidence indicating that they have many additional functions, both intracellularly and extracellularly. Whereas most activities are related to their saccharide binding activity, some are not necessarily dependent on this characteristic property.

Galectins are involved in programmed cell death. Galectin-1 has been found to induce apoptosis in activated T cell, (Perillo *et al.*, 1995) and thymocytes (Perillo *et al.*, 1997). Apoptosis is an important mechanism for the production of immunocompetent cells and for the termination of an immune response. In activated T cells, galectin-1 induces apoptosis via a Fas-independent pathway, and CD45 appears to be the cell surface receptor that interacts with galectin-1. *N*-glycans on CD45 may be essential for galectin-1 binding, since the induction by galectin-1 can be inhibited by swainsonin, an inhibitor for *N*-linked oligosaccharide processing (Perillo *et al.*, 1995). Because most of galectin-1 is in dimeric form at the concentration (10 μM) that induces apoptosis, dimeric formation was proposed as a requirement to elicit the reaction (Perillo *et al.*, 1995). Moreover, susceptibility to galectin-1-induced apoptosis is cell cycle-

dependent. Thymocytes at G_1 phase are less sensitive to apoptotic change induced by galectin-1 than at other phases (Perillo *et al.*, 1997).

A tandem-repeat type galectin, galectin-9, also was found to induce cell death in thymocytes (Wada *et al.*, 1997). The induction is cell type-specific, since hepatocytes are resistant to the stimulation. Lactose can suppress the response, suggesting that sugar-lectin interaction is involved in this process.

Galectin-3 did not elicite simliar response on thymocytes (Wada *et al.*, 1997). In contrast, it protects leukemic T cells from the apoptotic response induced by anti-Fas antibody and staurosporine (Yang *et al.*, 1996). Interestingly, galectin-3 contains NWGR motif seen in the Bcl-2 family, which is involved in heterodimer formation between Bcl-2 (a suppresser of cell death) and Bax (a cytotoxic protein) (Yin *et al*, 1994; Hanada *et al.*, 1995). Immunoprecipitation analyses showed that galectin-3 indeed complexes with Bcl-2 (Yang *et al.*, 1996), but its significance in the apoptotic pathway remains to be determined

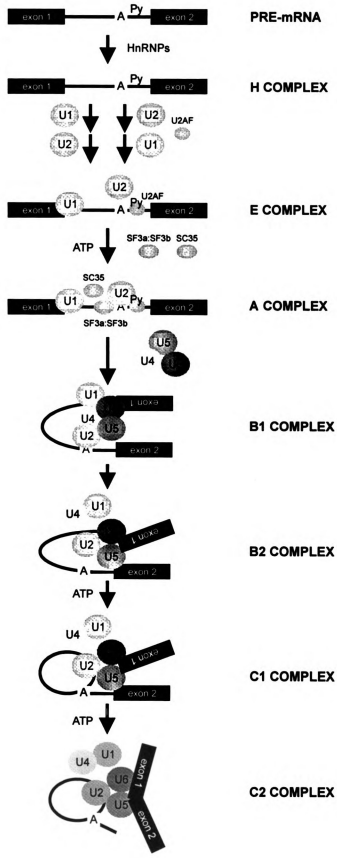
Galectin-1 participate in axon growth and guidance for olfactory neurons. Galectin-1 is expressed by primary olfactory neurons, olfactory nerve glial cell, and second-order neurons in the olfactory bulb (Puche and Key, 1995). In vitro, recombinant galectin-1 and its ligand can specifically stimulate neurite growth by cultured olfactory neurons, which were determined by increased neurite numbers as well as increased neurite lengths. (Puche *et al.*, 1996). When interneuron connection was examined in transgenic null mice lacking galectin-1 (Poirier and Robertson, 1993), connections between neurons in the nasal cavity and the olfactory bulb were defective. A subpopulation of axons failed to navigate to their target site in the olfactory bulb (Puche

et al., 1996). This is the first phenotype ascribed to the lack of galectin-1 and indicates that galectin-1 has a role in the guidance of axon growth of olfactory neurons.

Galectin-1 and galectin-3 are pre-mRNA splicing factors. The observation that saccharide ligands for galectins inhibited splicing in a cell-free assay first implicated that galectins may participate in pre-mRNA splicing (Wang *et al.*, 1991; Dagher *et al.*, 1995). When galectins were selectively depleted from nuclear extracts of HeLa cells using lactose-agarose affinity adsorption, the splicing activity was lost, showing that galectins are essential for pre-mRNA splicing. For these galectin-free extracts, either recombinant galectin-3 or galectin-1 was able to restore its splicing capacity, and this restored activity is still subjected to saccharide inhibition (Dagher *et al.*, 1995; Vyakarnam *et al.*, 1997). This result indicated that either galectin-3 or galectin-1 is sufficient to complement the splicing activity and also showing the importance of CRD. The latter notion is further supported by the observation that C-domain of galectin-3 alone also reconstitutes the splicing activity (Vyakarnam *et al.*, 1997). When these two galectins were specifically removed from nuclear extracts by dual immunodepletion, the results were the same as those using lactose affinity adsorption. This argues that galectin-1 and galectin-3 are the two major galectins present in the nuclear extracts.

How do galectins participate in the splicing pathway? Assembly of splicing substrates into splicing complexes in cell-free assay (figure 3) can be monitored by a ribonucleoprotein (RNP) gel electrophoresis system (Zillmann *et al.*, 1988). When splicing substrates were treated with galectin-free nuclear extract, only H complexes were observed on the gel. When recombinant galectin-3 was added to restore the splicing activity, it also enhanced the formation of advanced spliceosomes including A complex

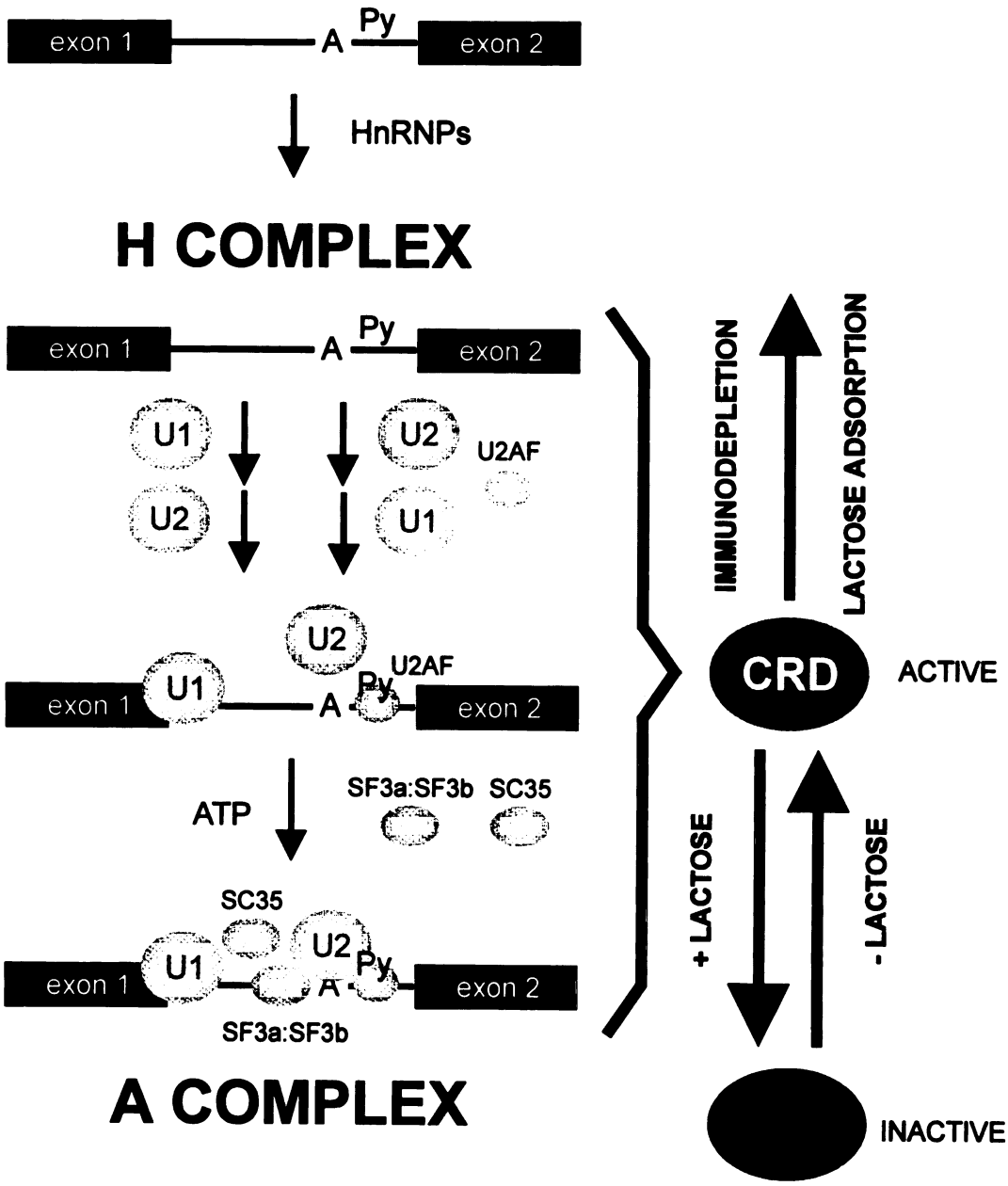
Figure 3. Schematic representation of the involvement of small nuclear ribonucleoprotein particles (snRNP) and non-snRNP proteins in pre-mRNA splicing (modified from Moore *et al.*, 1993). Pre-mRNA (top line), containing two exons separated by an intron, enters splicing complexes with snRNPs and exits as ligated mRNA (bottom line). Nascent pre-mRNA is bound by a multitude of hnRNP proteins immediately after transcription in vivo (Dreyfuss *et al.*, 1993; Weighardt *et al.*, 1996), forming H complex. E complex, the commitment complex in mammalian extracts, contains both U1 and U2 snRNPs, although U2 snRNP has a weaker affinity to the substrate at this stage (Michaud and Reed, 1991). Transition from E complex to A complex is rapid and requires ATP hydrolysis (Liao *et al.*, 1992). The SR protein family of splicing factors are involved in this conversion (Valcárcel and Green, 1996). Then, U4/U6.U5 tri-snRNP complex enter spliceosome to form complex B1. After structural arrangement, complex B1 is transformed into complex B2. Complex C1 carries out the cleavage at the 5' splice site and formation of the lariat intermediate, whereas complex C2 in the spliceosome immediately following exon ligation.


exon 1
A
Py
exon 2
PRE-mRNA
HnRNPs
H COMPLEX
U1
U2
U2AF
E COMPLEX
ATP
SF3a:SF3b
SC35
A COMPLEX
U5
U4
B1 COMPLEX
B2 COMPLEX
C1 COMPLEX
C2 COMPLEX

as well as B1/B2 and C1/C2 complexes (Dagher *et al.*, 1995; Zillmann *et al.*, 1988). This result implied that galectin-3 is at least required for the progression from H complex to A complex. It is not known whether depletion of galectins blocks the conversion from H to E complex, or from H to A complex (Figure 4).

Considering that CRD alone is sufficient to restore the splicing activity of galectin-depleted nuclear extracts, it appears that carbohydrate-recognition domain already contains sufficient elements mediating the splicing reaction. A legitimate question is whether carbohydrate-binding activity is required for splicing activity of galectins. Recent data show that the galectin-1 mutant without sugar-binding activity is still able to restore the splicing activity, although the saccharide inhibition is no longer observed (Dagher and Patterson, unpublished results). This indicates that sugar binding activity is, in fact, dispensable for splicing activity, arguing against the model that a sugar-lectin interaction is required for progression of spliceosome. A plausible explanation is that inhibition of splicing by saccharide perhaps reflects that the impact on the CRD conformation by saccharide binding. It has been shown that galectin-3 CRD have conformations of different thermal stabilities in the presence or absence of ligands (Agrwal *et al.*, 1993). It is possible that these two conformations represent active and inactive forms for splicing activities of CRDs and lactose ligands act as a switch for their interchange. Without ligand, the active form participates in splicing reaction; with bound ligand, the active form becomes converted into the inactive form and thus CRD loses its splicing activity (Figure 4). Whether there exits a switch in intact cells will be another interesting issue.

Figure 4. Roles of galectin CRD in pre-mRNA splicing. Galectin CRD participates in the conversion from H complex to A complex (Dagher *et al.*, 1995). Depletion of galectins by lactose-affinity adsorption or immunopresipitation inhibits the progression of spliceosome formation. On the other hand, saccharide ligand induces a conformational change on CRD structures, which results in inactivation of galectins and thus inhibition of splicing reaction.


exon 1
A
Py
exon 2
HnRNPs
H COMPLEX
U1
U2
U2AF
SF3a:SF3b
SC35
ATP
A COMPLEX
IMMUNODEPLETION
LACTOSE ADSORPTION
CRD
ACTIVE
+ LACTOSE
- LACTOSE
INACTIVE

INTRODUCTION TO NUCLEAR TRANSPORT

Nucleocytoplasmic transport plays a fundamental role in coordinating the functions of the nucleus and cytoplasm (Nigg, 1997; Görlich and Mattaj, 1996). It is mediated by a large molecular structure that spans the nuclear envelope called the nuclear pore complex (Davis, 1995). Proteins that function in the nucleus are synthesized on free ribosomes in the cytoplasm and imported into the nucleus. RNAs that function in cytoplasm are transcribed and processed in the nucleus and exported into the cytoplasm (Görlich and Mattaj, 1996). Both processes occur through nuclear pore complexes. In the following section, I will discuss the structure of *nuclear pore complex*, the pathways for *nuclear import*, as well as the general properties of *nuclear export*.

NUCLEAR PORE COMPLEX

Nuclear pore complexes (NPC) have a mass of 125 megadaltons and contain about 100 different polypeptides. Characteristic features of many vertebrate nucleoporins (nuclear pore proteins) are modifications with O-linked *N*-acetylglucosamine (GlcNAc) and the presence of short degenerate repeats, such as FXFG repeats and GLFG repeats. The NPC contains a passive diffusion channel about 9 nm in diameter (Peters, 1986). Small proteins with sizes less than 40~60 KDa can diffuse freely through the pore. Proteins above the diffusion limit can enter the nucleus only via an active mechanism. Nevertheless, small nuclear proteins generally enter the nucleus via active mechanism rather than by diffusion.

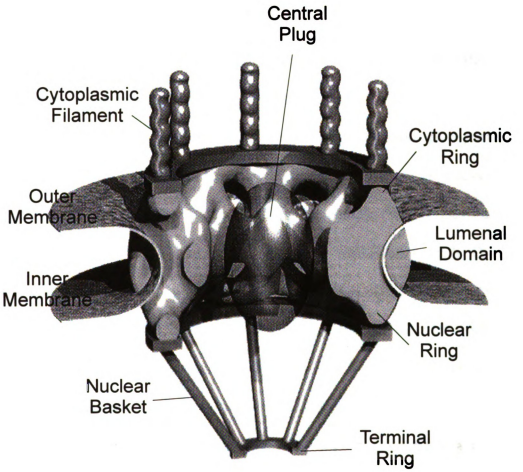


Figure 5. Schematic respresentation of a consensus model of the membrane-bound nuclear pore complex. Nuclear pore complex contains four major elements: spoke-ring assembly, central plug, cytoplasmic filaments, and nuclear basket (Bastos *et al.,* 1995).

Four basic elements of structure have been observed for nuclear pore complex (NPC) (Akey and Radermacher, 1993) (Figure 5). The main body is a *spoke-ring assembly* anchored on a specialized region of nuclear envelope. This structure consists of nuclear and cytoplasmic rings connected to each other by two sets of eight spokes . A *central plug* sits within the aqueous channel formed by the spoke-ring assembly. Peripheral components includes eight short *cytoplasmic filaments* that extend into the cytoplasm and *nuclear basket* that project into the nucleus.

Many vertebrate nuclear pore proteins are O-linked glycoproteins that contains characteristic peptide repeat motifs. A family of 10-20 nucleoporins has been identified as proteins that bind to wheat germ agglutinin (WGA) (Davis and Blobel, 1987). WGA binding is due to the extensive modification with O-linked *N*-acetylglucosamine moieties on the NPC structure (Hart *et al.*, 1988). cDNAs encoding six members (p62, POM121, NUP153, NUP214/CAN, NUP98 and NUP358/RanBP2) of O-linked glycoprotein family of nucleoporins have been isolated in vertebrates (Table 2). The deduced amino acid sequences showed that each member contains multiple copies of XFXFG repeats, where X is any amino acid with a small or polar side chain. This motif is also seen in a few yeast nucleoporins, like Nup1p (Bogerd *et al.*, 1994; Belanger *et al.*, 1994) and Nup2p (Belanger *et al.*, 1994). A GLFG motif, which is frequently seen on yeast nucleoporins, has only been found on NUP98 in vertebrates.

Immunoelectron microscopy has been used to determine locations of these glycoproteins within NPC. Namely, p62 is found both at cytoplasmic and nuclear sides (Panté *et al.*, 1994); POM21 is on membrane domain of NPC (Hallberg *et al.*, 1993); NUP358 as well as NUP214 are mapped to cytoplasmic filaments (Boer *et al.*, 1997; Wu

Table 2. Vertebrate nuclear pore proteins

Name	Location	Properties and possible functions
O-linked glycoprotein family		
p62	Central region; both cytoplasmic and nuclear (Panté *et al.*, 1994)	XFXFG repeats at N-terminus and heptad repeats/coiled-coil structure at C-domain.; complexed with p58 and p54; essential for NPC function; multiple *O*-GlcNAc and sialic acids (Snow *et* al., 1987; Starr *et* al., 1990; Emig *et al.*, 1995; Buss *et al.*, 1995))
POM121	Pore membrane domain	XFXFG repeats at C-terminus; integral membrane protein; membrane anchor; multiple *O*-GlcNAc (Hallberg *et al*, 1993)
NUP153	Terminal ring of the nuclear basket (Panté *et al.*, 1994)	XFXFG repeats at C-terminus; four Zn fingers; homodimerization; multiple *O*-GlcNAc ; involved in mRNA export (Sukegawa and Blobel, 1993; Bastos *et al.*, 1996))
NUP214/ CAN	Cytoplasmic filaments (Panté *et al.*, 1994) and nucleoplasmic face (Boer *et al.*, 1997)	XFXFG repeats at C-terminus; leucine zipper motif; complexed with p75; putative oncogene; docking site for karyopherins (Kraemer *et al.*, 1994)
NUP98	Nucleoplasmic side	GLFG, FG, FXFG repeats at N-terminus; docking sites for karyopherins (N-terminus); related to DNA replication; multiple *O*-GlcNAc; involved in RNA export but not protein import (Powers *et al.*, 1995; Radu *et al.*,1995; Powers *et al.*, 1997)
NUP358/ RanBP2	Cytoplasmic fibers	XFXFG, FG motifs; four Ran binding sites; leucine-rich domain; cyclophilin A homologous domain; docking sites for karyopherins and Ran (Yokoyama *et al.*, 1995; Wu *et al.*, 1995)
Others		
gp210	Pore membrane domain	Type I integral membrane protein; membrane anchor; high mannose *N*-linked oligosaccharides on lumenal domain (Gerace *et al.*, 1982; Wozniak *et al.*, 1989; Greber *et al.*, 1990; Wozniak and Blobel, 1992)
NUP107	?	Leucine zipper at C-terminus (Radu *et al.*, 1994)
NUP155	Both nuclear and cytoplasmic	Multiple potential phosphorylation sites (Radu *et al.*, 1993)
TPR/ p270	Cytoplasmic (?) and nuclear filaments (Cordes *et al.*, 1997)	Coiled-coil motif at central domain; activation of oncogenic kinases (Byrd *et al.*, 1994)
NUP180	Cytoplasmic ring and filaments	Recognized by serum form patients with autoimmune diseases (Wilken *et al.*, 1993)
NUP84 (NUP88)	Cytoplasmic face	Coiled-coil domain at C-terminus; bound with Nup214; non-glycosylated; no repeat motif; CAN-dependent NPC localization (Bastos *et al.*,1997; Fornerod *et al.*, 1177))
hCRM1 (112kDa)	?	Localized at pores and nucleoplasm; karyopherin β-like domain (Fornerod *et al.*, 1997)

et al., 1995); and NUP98 and NUP153 are on the nuclear side of NPC (Panté *et al.*, 1994; Radu *et al.*, 1995). In other words, nucleoporins with XFXFG repeats can be along the cytoplasmic-nuclear axis of NPC.

The O-linked glycoproteins are essential docking sites for nuclear transport. The majority of these proteins bind to the karyopherins, a family of transport factors, directly, and have be proposed to form an array of sites for mediated docking of transport substrates (figure 6) (Radu *et al.*, 1995; Wu *et al.*, 1995). For example, NUP358 has been considered as the site where protein import complex is initially assembled (Wu *et al.*, 1995), containing docking sites for Ran GTPase as well as karyopherin α1/α2-β complexes. In fact, more and more data suggest that anchoring sites on these nucleoporins are shared by all of the nuclear import pathways and export pathways. This is exemplified by the fact that the β-type karyopherins are all capable of binding to these glycoproteins, and their binding is competitively inhibited by each other (Bonifaci *et al.*, 1997; Yaseen and Blobel, 1997). Moreover, it was recently shown that all of the signal-mediated pathways were inhibited, when these glycoproteins in NPC were masked by mutants of karyopherin β1 (Kutay *et al.*, 1997).

Other nucleoporins. Sequence information is also available for several other vertebrate nucleoporins (Table 2). None of them contains XFXFG or GLFG repeats nor have O-linked GlcNAc. Some contain the sequence motifs that are likely to mediate protein-protein or protein-nucleic acid interactions, such as zinc fingers, leucine zippers and coiled-coil motifs.

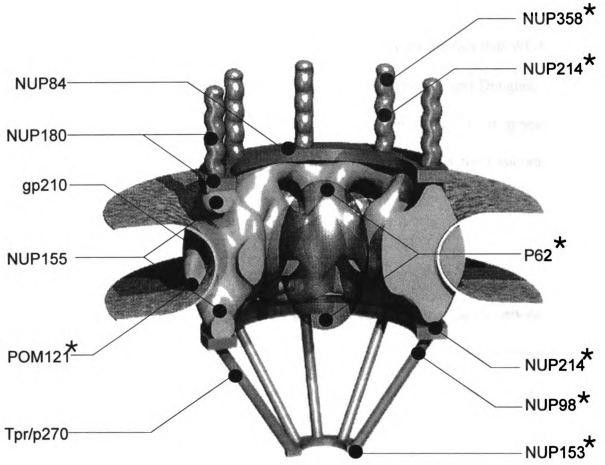


Figure 6. Diagram summarizing localizations of characterized nucleoporins within the nuclear pore complex. Nucleoporins that have been shown to bind to karyopherin complexes are placed at the right side. These proteins may serve as docking sites as import complexes move along the nuclear pore. Asterisk indicates nucleoporins that have XFXFG repeats as well as O-linked GlcNAc modifications.

Most yeast nucleoporins also have characteristic repeat motifs seen in vertebrate homologues but are not modified by sugar residues. Some yeast nucleoporins contains XFXFG repeats that are the signature motif of NPC O-linked glycoproteins of vertebrates. These yeast proteins, however, appear not to be modified by GlcNAc (Kalinich and Douglas, 1989), which is consistent with the fact that WGA does not block nuclear protein import in *in vitro* import assay (Kalinich and Douglas, 1989). A second family of nucleoporins is characterized by the repeat GLFG. It appears that yeast and vertebrate NPCs have some differences, considering that the basic structure units and posttranslational modifications are not entirely identical..

NUCLEAR IMPORT

As mentioned, molecules with sizes above the exclusion limits (40~60 kDa) primarily enter the nucleus in an active way. Usually, this kind of transport is selective and signal-dependent. Proteins or RNAs bearing import signals are recognized by specialized receptors and then targeted to nuclear pores, where these molecules are translocated across the nuclear envelope. Accumulating evidence implies that specificity of a multitude of pathway arises at the earlier steps, i.e., signal-receptor recognition. Different import pathways appear to use the same set of nucleoporins to dock import substrates and translocate them across the nuclear pores (Bonifaci *et al.*, 1997; Yaseen and Blobel, 1997). Therefore, I would like to discuss the import pathways based on the pairing between import signals and their receptors (Table 3).

Table 3. Signals and Receptors Involved in Nuclear Import

Receptor	Import Signal	Example Sequence	Substrates
Karyopherin α1 (Importin-α/NPI-1 SRP1/NTPC58/ Kap60p)	Nuclear Localization Signal Classical signal Bipattite signal	 PKKKRKV KRPAAIKKAGQAKKKK	Nuclear proteins SV40 T antigen Nucleoplasmin
Karyopherin α2 (Rch1)	Nuclear Localization Signal Classical signal Bipattite signal	 PKKKRKV KRPAAIKKAGQAKKKK	Nuclear proteins SV40 T antigen Nucleoplasmin
Karyopherin α3	?	?	?
Karyopherin β (Kap97p)	IBB domain of karyopherin α	RMRKFKNKGKDTAELRR RRVEVSVELRKAKKDE QILKRRNV	Karyopherin α
Transportin (Karyopherin β2 /Kap104p)	M9 sequence	NQSSNFGPMKGGNFGGR SSGPYGGGGQYFAKPR NQGGY	hnRNP A1
Karyopherin β3	?	?	Ribosomal proteins?
?	KNS sequence	YDRRGRPGDRYDGMVGF SADETWDSAIDTWSPSE WQMAY	hnRNP K
?	Pro, Gly-rich domain	N-terminus (aa 1-206)	ANNEXIN XI
Snurportin	Trimethylguanosine cap		U snRNAs

Karyopherin α/β1 (α1β1 and α2β1) mediated pathway for common nuclear proteins is the best characterized nuclear import pathway. Most knowledge of this pathway originates from studies in *Xenopus* and mammalian systems. Experiments based on microinjection into *Xenopus* oocytes discovered the two steps of this import process: energy-independent docking to the NPC and energy-dependent translocation through the pore (Richardson *et al.*, 1988; Newmeyer and Forbes, 1988). The major breakthrough was the development of an in vitro system based on cultured cells treated with digitonin to selectively permeabilize the plasma membrane (Adam *et al.*, 1990). Digitonin-permeabilized cells retain the capacity to import nuclear proteins with the supplement of fractionated cytosol. From these cytosolic fractions, required factors for nuclear import were purified.

Nuclear import of NLS-containing proteins has been distinguished as two steps: docking and translocation (Figure 7). First, the import substrates binds via its NLS to the karyopherin α, either α1 or α2, in the cytoplasm (Görlich *et al.*, 1994; Imamoto *et al.*, 1995), which also augments the interaction between karyopherin α and β1 subunits (Moroianu *et al.*, 1996). Karyopherin β1 contains binding sites with repeat-containing nucleoporins, and mediates the docking of the complex onto the nuclear pores in an energy-independent manner (Morioanu *et al.*, 1995). The import complex is subsequently translocated through the pore by an energy-dependent mechanism (Richardson *et al.*, 1988; Newmeyer and Forbes, 1988), which is probably achieved by sequential docking-undocking process mediated by Ran, a Ras-like GTPase (Melchior *et al.*, 1993; Moore and Blobel, 1993; Kutay *et al.*, 1997), and NTF2 (Moore and Blobel,

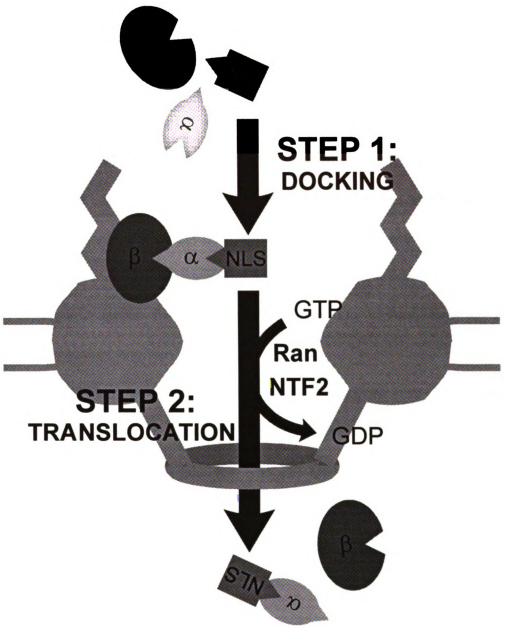


Figure 7. Nuclear import of NLS-containing proteins has two distinct steps: docking and translocation. Docking requires only karyopherin α and β, and does not need GTP. Translocation is mediated by Ran GTPase and its cofactor NTF2, which requires GTP hydrolysis.

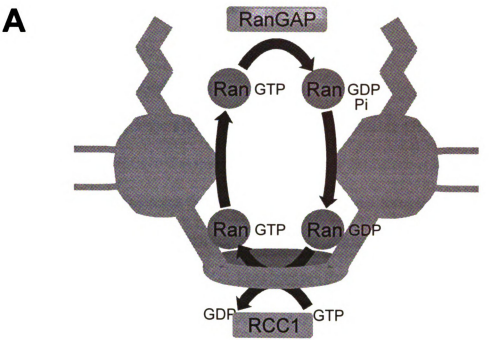
1994; Paschal and Gerace, 1995). After being transported to the nuclear side of NPC, the import complex is dissociated. As the karyopherin β1 was promptly exported by an NES-mediated pathway (Iovine and Wente, 1997), the karyopherin α-substrate complex reaches the nucleoplasm (Moroianu *et al.*, 1995).

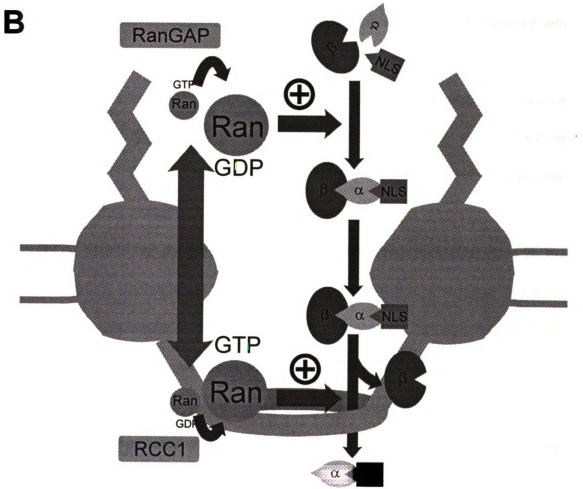
Ran GTPase cycle is coupled with the translocation of karyopherin complex. Like other GTPases, Ran has a set of effectors that modulate its GTPase activity. The only known guanine nucleotide exchange factor for Ran is a nuclear protein RCC1 (Bischoff and Ponstingl, 1991); the only known Ran GTPase activating protein is cytoplasmic (Hopper *et al.*, 1990; Bischoff *et al.*, 1995). Based on distinct locations of these two effectors, it appears that GTP exchange occurs only in the nucleus and GTP hydrolysis is only in the cytoplasm.

The Ran GTPase cycle. Koepp and Silver (1996) proposed that Ran moves out of the nucleus in a GTP-bound form and it enters the nucleus at the GDP-bound state (Koepp and Silver, 1996) (Figure 8A), assuming that Ran-GTP is enriched by nuclear RCC1 in the nucleus and Ran-GDP is formed in cytoplasm due to augmented GTPase activity by RanGAP. This model also predicted that Ran-GDP moves toward cytoplasm and Ran-GTP heads for the nuclear side so that the cycle can be completed (Figure 8). Some of the experimental data are consistent this model:

1. Ran-GDP has been found to be required for nuclear import (Weis *et al.*, 1996).
2. When RanGAP was microinjected into the nuclei, nuclear export of NES-containing protein was blocked (Richards *et al.*, 1997).

Figure 8: (A) A model illustrating Ran GTPase cycle. Two effectors for Ran are at different compartments. RCC1 is localized in the nucleus and RanGAP is in the cytoplasm. In order the complete the cycle, Ran-GDP flows toward the nucleus and Ran-GTP move toward cytoplasm. **(B) Coordination of protein import by Ran GTPase cycle.** Due to the compartmentation of RCC1 and RanGAP, Ran-GDP and Ran-GTP are enriched at different sides. Ran-GDP is more abundant at cytoplasmic side, where it promotes the docking complex for protein import. Ran-GTP is more abundant at the nuclear side, where it stimulates the dissociation of karyopherin-substrate complex. The net effect is that nuclear protein is transported from cytoplasmic side to the nuclear side.


A
RanGAP
Ran GTP
Ran GDP
Pi
Ran GTP
Ran GDP
GDP
RCC1
GTP


B
RanGAP
GTP
Ran
Ran
GDP
NLS
β
α
NLS
β
α
NLS
GTP
Ran
Ran
GDP
RCC1
α

3. GTP-bound Ran mutant lacking GTPase activity is still able to mediate nuclear export, indicating that Ran-GTP but not GTP hydrolysis directly participate in nuclear export (Richards *et al.*, 1997).

4. When nuclear import was blocked by nonhydrolyzable GTP analogues, Ran was specifically localized at the cytoplasmic surface (Melchior *et al.*, 1995).

Current data indicates that Ran GTPase cycle coordinates the import pathway. It has been shown that karyopherin α/β1 and imported substrate form a stable complex in the presence of GDP-bound Ran, but karyopherin α-ligand is released from the complex by Ran-GTP (Rexach and Blobel, 1995; Kutay *et al.*, 1997). Since the Ran-GTP is mainly nuclear and Ran-GDP is enriched in cytoplasm according to the model, the Ran-GDP mediates the import complex formation at the cytoplasmic side and Ran-GTP will dissociate the substrate from karyopherins in the nucleus (Figure 8B).

In summary, the distinct distribution of RCC1 and RanGAP across the nuclear envelope enriches GDP- and GTP-bound forms of Ran at cytoplasmic side and nuclear side respectively. This, in turn, potentially helps to establish the unidirectional movement of nuclear proteins (Figure 8B).

NTF2 assists the docking of Ran-GDP as well as import complexes to the repeat-containing nucleoporins. NTF2 was first identified as a required factor for nuclear import, complementing the functions of Ran (Moore and Blobel, 1994). In yeast, NTF2 has specific affinity to nucleoporins containing FXFG repeats, like Nup1p, Nsp1p, Nup145p , Nup57p and Nup36p. Stronger binding with some of these nucleoporins, like Nup36p requires the presence of Ran-GDP, Kap95β and Kap60α. Therefore, NTF2 has

been proposed as the regulator that coordinates Ran-dependent association and dissociation reactions underlying nuclear import (Nehrbass and Blobel, 1996). A similar interaction between karyopherins, Ran, nucleoporin, and NTF2 has also been observed in vertebrate cells (Paschal and Gerace, 1995).

Transportin-M9 sequence mediated pathway is responsible for the nuclear import of hnRNP A1 and its related proteins. HnRNPs are a set of proteins that bind to hnRNAs. They are involved in many aspects of RNA metabolism, including RNA processing and transport (Dreyfuss *et al.*, 1993). In hnRNP A1, there exists a stretch of amino acids, designated as M9 (Siomi and Dreyfuss, 1995), that is responsible for nuclear shuttling property of hnRNP A1 (Piñol-Roma and Dreyfuss, 1993). Recently, its nuclear import receptor has been identified in a variety of organisms. In vertebrates, it was designated as transportin (Pollard and Dreyfuss, 1996), MIP (*M*9 *I*nteracting *P*rotein) (Fridell *et al.*, 1997), and karyopherin β2; in yeast, it is named as Kap104p (Aitchison *et al.*, 1996). This receptor is homologous to karyopherin β1 and specifically mediates nuclear import of hnRNP A1, but not NLS-containing proteins (Pollard *et al.*, 1996; Fridell *et al.*, 1997). Transportin, like karyopherin β1, binds to a set of nucleoporins containing the characteristic peptide repeat motifs. It mediates the docking of hnRNP A1 onto the pore, but translocation also requires Ran GTPase (Bonifaci and Blobel, 1997).

There are a multitude of nuclear import pathways yet to be defined. For example, KNS sequence in hnRNP K is responsible for its nuclear shuttling property, and, interestingly, this sequence can dock proteins to the nuclear pore without the supplement of cytosolic factors (Michael *et al.*, 1997). What nucleoporin mediates its

docking still remains to be studied. Some other potential receptor proteins are also identified, like karyopherin α3 (Takeda *et al.*, 1997) and karyopherin β3 (Yaseen and Blobel, 1997), but no substrates have been characterized yet. There is also a report on the identification of the receptor that mediates nuclear import of U snRNAs, which has a trimethylguanosine cap that interacts with a receptor, snurportin (Lührmann, 1997). This receptor mediates nuclear import of U snRNPs in oocyte cells but only plays a minor role in somatic cells (Marshallsay and Lührmann, 1994).

NUCLEAR EXPORT

The list of signals and receptors involved in nuclear export is relatively small, compared to that for nuclear import (Table 4). Discovery of the cap-binding complex (Izaurralde *et al.*, 1995) provided the first chance to examine the molecular mechanisms underlying RNA export and to explore the interplays between nuclear import and export. Nuclear export signals have also been recently identified in several nuclear shuttling proteins (Gerace, 1995).

Cap-binding complex (CBC) is involved in RNA export. Monomethylated cap structures have been found to participate in several steps of RNA metabolism, including nuclear export (Hamm and Mattaj, 1990), initiation of translation (Sachs *et al.*, 1997) and RNA processing (Izaurralde *et al.*, 1994). Microinjection of cap analog, the dinucleotide m^7GpppG, was shown to specifically inhibit nuclear export of U1 snRNA. For mRNAs, a monomethylated cap conferred a higher export rate than a dimethylated or trimethylated cap structure (Hamm and Mattaj, 1990; Jarmolowski *et al.*, 1994), although the

Table 4. Signals and Receptor Involved in Nuclear Export

Receptor	Export signal	Example Sequence	Substrate
Cap-binding complex	7-methylguanosine cap	mGpppG, mGpppA	U snRNAs; mRNAs
hRIP	Nuclear Export Signal (NES)	LPPLERLTL (Rev) LALKLAGLDI (PKI)	Nuclear proteins
?	M9 sequence	NQSSNFGPMKGGNFGGR SSGPYGGGGQYFAKPR NQGGY	hnRNP A1
?	KNS sequence	YDRRGRPGDRYDGMVG FSADETWDSAIDTWSP SEWQMAY	hnRNP K

monomethylated cap was not essential for their export (Izaurralde *et al.*, 1997). A cap-binding complex (CBC) was identified from nuclear extracts, which consists of two subunits: CBP20 and CBP80 (Izaurralde *et al.*, 1992; Izaurralde *et al.*, 1995). Antibodies raised against recombinant CBP20 indeed inhibited the nuclear export of U snRNAs, demonstrating that this complex is an nuclear export factor for RNA (Izaurralde *et al.*, 1995).

Although CBC seems not to be required for export of mRNAs, it does accompany mRNPs through nuclear pores on a particular pre-mRNA particle, the Blabiani ring (BR) RNP. BR particle is the product of the Balbiani ring (BR) gene in the salivary glands of the dipterin *Chironomus tentans* (Visa *et al.*, 1996), whose giant size (35-40 kb) makes it possible to be visualized under electron microscope. CBC binds to the BR transcript as it was transcribed, and accompanies this RNP particle to travel through the nucleoplasm until it meets nuclear pores. During translocation through the channel, CBC remains at the 5' end of the particle and is not detached until the RNA molecule extends into the cytoplasm (Figure 9) (Visa *et al.*, 1996). Thereafter, it appears to be released from the BR RNP immediately, probably reentering the nucleus. Recent data showed that karyopherin α-β1 pathway is responsible for the nuclear import of this NLS-containing CBC (Görlich *et al.*, 1996).

Nuclear export signals (NES) in certain nuclear shuttling proteins are responsible for their rapid exit from the cell nucleus. Nuclear export signals have been identified in several nuclear shuttling proteins. They were initially found in the HIV-1 Rev protein (Wen *et al.*, 1995; Fischer *et al.*, 1995) and in the Protein kinase A

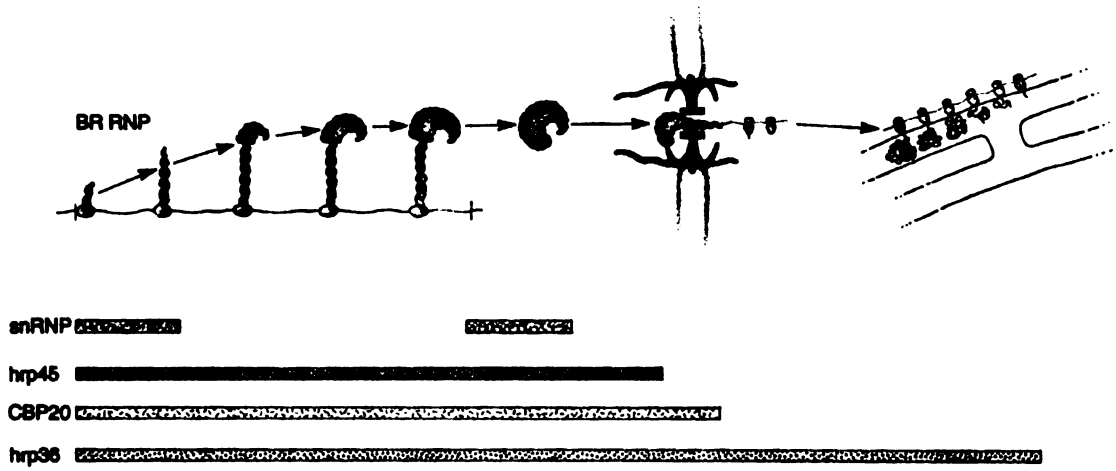


Figure 9: The nuclear export of Balbiani Ring RNP particle. Three pre-mRNA splicing factors, hrp45, CBC20 and hrp36, travel with the transcript to different locations (modified form Danholt (1997)).

inhibitor (PKI) (Wen *et al.*, 1995). Both are short sequences and enriched in hydrophobic residues, especially leucines, with particular spacing. NES-mediated export is energy and temperature dependent and confers a rapid export property to heterologous proteins (Wen *et al.*, 1995). A receptor that interacts with this group of signals has been identified. The NES receptor, hRIP (*h*uman *R*ev *I*nteracting *P*rotein) or RAB (*R*ev *A*ctivation domain *B*inding protein), is a 59-kDa nucleoporin-like protein with FG peptide repeat motifs (Fritz *et al.*, 1995; Bogerd *et al.*, 1995). It is localized in the nucleus as well as the nuclear envelope (Fritz *et al.*, 1995), and probably also cytoplasm (Bogerd *et al.*, 1995). Known nuclear proteins containing NES include (1) components for nuclear transport of macromolecules, like Rev (Wen *et al.*, 1995), Rex (Bogerd *et al.*, 1996; Kim *et al.*, 1996), karyopherin β1 (Iovine and Wente, 1997), and RanBP1 (Zolotukhin *et al.*, 1997), and (2) proteins involved in signal transduction pathways, like PKI (Wen *et al.*, 1995), and MAPK kinase (Fukuda *et al.*, 1996).

M9-mediated pathway is related to nuclear export of mRNAs. Using a heterokaryon cell system, it has been shown that hnRNP A1 has a shuttling capacity across nuclear envelope (Piñol-Roma and Dreyfuss, 1993). The same conclusion is derived from the observation on its yeast homologue Npl3p (Flach *et al.*, 1994). A domain was found to mediate the nuclear import and export activities, called M9 sequence (Siomi and Dreyfuss 1995; Michael *et al.*, 1995). Although transportin is considered as M9 receptor, it seems not to be responsible for its nuclear export (Pollard *et al.*, 1996; Bonifaci *et al.*, 1997). When hnRNP A1 was microinjected into either cytoplasm or the nucleus, it indeed saturates the export pathway for mRNAs, but not for

U snRNAs (Izaurralde *et al.*, 1997) This inhibition is independent of M9 sequence and may be exerted in cytoplasm, and the underlying mechanism is not clear at present. *C tentans* hrp36, a homologue of hnRNP A1, is a component of BR RNP particle (Visa *et al.*, 1996). Its localizaiton has been studied using immunoelectron microscopy. This study showed that it was added to BR RNA concomitant with transcription, and remained on this RNP particle, while traveling through nucleoplasm. After leaving the nucleus, hrp36 was still present in this RNP even when polysomes were assembled on BR particles (Visa *et al.*, 1996). Although hnRNP A1 has extensive association with RNAs, direct evidence indicating that it is essential for mRNA export is still lacking.

A novel signal conferring nuclear shuttling activities is found in hnRNP K. This KNS sequence, like M9 sequence, is responsible for the nuclear export as well as nuclear import property of hnRNP K1, as demonstrated in a heterokaryon system (Michael *et al.*, 1997). The molecular mechanism for its export awaits further investigation.

Some of the pre-mRNA splicing factors are exported with processed transcripts. Both hnRNP A1 (Mayeda *et al.*, 1992; Cáceres *et al.*, 1994) and CBC (Izaurralde *et al.*, 1994; Lewis *et al.*, 1996) are well documented pre-mRNA splicing factors. Although both of these proteins travel out of the nucleus with mRNAs, the timing of their release form the RNP is quite different. CBC appears to be released from the RNP complexes immediately following their arrival at cytoplasmic side of nuclear pores (Visa *et al.*, 1996a), while hnRNP A1 probably accompanies RNAs from transcription to translation (Visa *et al.*, 1996b). However, not every pre-mRNA splicing factor is exported with mRNP complex. For example, the hrp45 protein, a SR protein of

C. tentans, is found to associate with nascent RNAs concomitant with their synthesis via the binding with the exon. It is released from BR RNP particles in the nucleoplasm when the particle make contacts with the nuclear pores (Alzhanova-Ericsson *et al.*, 1996) (Figure 9). Briefly speaking, different splicing factors seem to have distinct fates for binding with the pre-mRNA/mRNA particles. Based on distinct timing of release, this process seems to be a regulated process. How and why their binding with mRNPs is terminated will be interesting subjects to explore.

CHAPTER II

NUCLEAR EXPORT OF GALECTIN-3 IN MOUSE 3T3 FIBROBLASTS: I. PARAMETERS OF THE TRANSPORT IN DIGITONIN-PERMEABILIZED CELLS

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SUMMARY

Galectin-3 is a galactose-/lactose-binding protein (M_r ~30,000), identified as a required factor in the splicing of pre-mRNA. Immunofluorescence staining revealed that galectin-3 distributes differentially between the nucleus and the cytoplasm, depending on the proliferative state of the cells under analysis. Using digitonin-permeabilized mouse 3T3 fibroblasts, we provide evidence that galectin-3 is rapidly and selectively exported from the nucleus. Although both phosphorylated and nonphosphorylated isoforms of galectin-3 are found in the nuclear fraction, only phosphorylated galectin-3 is identified in the exported fraction, implying that phosphorylation is important for the nuclear export of the protein. In addition to galectin-3, we found that several other proteins are also rapidly exported in the digitonin-permeabilized cell assay. These include galectin-1, Ran, and karyopherin-β. In contrast, the Sm B and U1 A polypeptides of the small nuclear ribonucleoprotein complexes (snRNPs) are retained in the nuclear residue during the course of the same assay. The behavior of heterogeneous nuclear RNP (hnRNP) protein A1 is somewhat intermediate, exhibiting very slow export. The rate of galectin-3 export is decreased by cold temperature and by the addition of wheat germ agglutinin. More strikingly, galectin-3 export can be inhibited, at least partially, by the simultaneous addition of a peptide bearing a nuclear export signal plus a dinucleotide cap analogue found at the 5'-end of mRNAs. These results suggest that galectin-3 may be exported in association with RNP(s) containing monomethylated cap structure as well as polypeptide(s) bearing nuclear export signals.

INTRODUCTION

Galectin-3 belongs to a family of widely distributed proteins that: (a) bind to β-galactosides; and (b) contain characteristic amino acid sequences in the carbohydrate recognition domain of the polypeptide (for reviews, see Barondes *et al.*, 1994; Kasai and Hirabayashi, 1996; Leffler, 1997). Of the presently known 10 members of this family, galectins-1 and -3 have been shown to be factors involved in the splicing of pre-mRNA, assayed in a cell-free system (Dagher *et al.* 1995; Vyakarnam *et al.*, 1997). This conclusion is based on several key findings: (i) nuclear extracts (NE) derived from HeLa cells, capable of carrying out splicing, contain galectins-1 and -3; (ii) saccharides that bind the galectins with high affinity inhibit the cell-free splicing reaction; (iii) depletion of both galectins from NE, either by lactose affinity adsorption or by double antibody adsorption, resulted in the concomitant loss of splicing activity; (iv) depletion of either galectin-1 or galectin-3, by specific antibody adsorption, failed to remove all of the splicing activity and the residual activity was still sensitive to saccharide inhibition; and (v) either galectin-1 or galectin-3 alone is sufficient to reconstitute, at least partially, the splicing activity of NE depleted of both galectins. All of the results suggest that the activities of galectin-1 and galectin-3 in the nucleus may be redundant (Patterson *et al.*, 1997).

A number of lines of evidence have been accumulated to indicate that galectin-3 is found in both the cytoplasm and nucleus of cells. Immunofluorescence staining, using

monoclonal, as well as polyclonal, antibodies specifically directed against galectin-3, was carried out on formaldehyde fixed, Triton X-100 permeabilized 3T3 fibroblasts. There was prominent labeling of the nucleus and variable staining of the cytoplasm (Moutsatsos *et al.* 1986; Wang *et al.*, 1995). This dual localization of galectin-3, in the nucleus and cytoplasm, has been confirmed by immunoelectron microscopy (Hubert *et al.*, 1995). Moreover, the nuclear staining of galectin-3, at the light and electron microscopic levels, was sensitive to treatment of the cells with ribonuclease A, but not to parallel treatment with deoxyribonuclease I, implying galectin-3 is associated with RNA-containing nuclear structures. Finally, the predominance of nuclear versus cytoplasmic distribution of galectin-3 depended on the proliferative state of the cells: sparse, proliferating cultures showed nuclear localization of galectin-3 whereas confluent, quiescent cultures yielded mostly cytoplasmic staining (Moutsatsos *et al.*, 1987). Galectin-3 expression and its intracellular distribution have also been shown to vary along the crypt-to-surface axis of human colonic epithelia (Lotz *et al.*, 1993). The protein is concentrated in the nuclei of differentiated colonic epithelial cells. The progression from normal mucosa to adenoma to carcinoma is characterized by a paradoxically distinct absence of galectin-3 in the nuclei of adenoma and carcinoma cells.

These observations on the dual localization of galectin-3 raise the possibility that the protein might shuttle between the cytoplasm and nuclear compartments. The rapidly expanding list of proteins that exhibit this nucleocytoplasmic shuttling property includes: (i) nucleolar proteins nucleolin and B23/No38 (Borer *et al.*, 1989); (ii) heat shock protein hsp70 (Mandell and Feldherr, 1990); (iii) transcription factors such as the steroid

receptors (Guiochon-Mantel *et al.*, 1991; Chandran and DeFranco, 1992) and TFIIIA (Fridell *et al.*, 1996); (iv) proteins of the heterogenenous nuclear ribonucleoprotein complex (hnRNP) such as hnRNP A1, A2, E (Piñol-Roma, and Dreyfuss, 1993) and hnRNP K (Michael *et al.*, 1997); (v) proteins involved in nucleocytoplasmic transport, including karyopherin-/importin-β (Iovine and Wente, 1997), Ran-binding Protein (Richards *et al.*, 1996), the HIV Rev protein (Wen *et al.*, 1995; Fischer *et al.*, 1995), and Npl3p (Flach *et* al., 1994; Lee *et al.*, 1996); and (vi) various signal transduction proteins and their regulators, such as the heat stable inhibitor of cAMP-dependent protein kinase (PKI) (Wen *et al.*, 1995) and mitogen-activated protein kinase kinase (Fukuda *et al.*, 1996).

Studies on nuclear protein import have identified some of the key components that function in recognition, docking, and translocation steps of the process. Karyopherin-/importin-α is one subunit of the receptor that recognizes the nuclear localization signal (NLS) and, along with karyopherin-β, mediates the docking of import-competent substrates to the nuclear pore complex (Görlich *et* al., 1994; Radu *et al.*, 1995). A GTP-binding protein Ran/TC4 and the associated factor, p10/NTF2, are required for the translocation of the substrate proteins through the nuclear pore complex (Moore and Blobel, 1993; Melchior *et al.*, 1993). These advances in our understanding of nuclear protein import have been possible mainly as a result of the development of an *in vitro* assay, using digitonin-permeabilized cells (Adam *et al.*, 1990). This assay takes advantage of the fact that digitonin treatment of cultured cells permeabilizes the plasma

membrane to macromolecular substrates while retaining the structural integrity of the nuclear envelope. Thus, the nuclei retain the ability to transport and accumulate proteins bearing the appropriate NLS. The system is particularly amenable to fractionation and biochemical analysis of the components involved in the transport process.

We have taken advantage of this digitonin-permeabilized cell system to test the possibility that galectin-3 might be exported from the cell nucleus, a requirement for a nuclear protein to shuttle between the nucleus and the cytoplasm. In the present communication, we report that, indeed, galectin-3 is rapidly and selectively exported from the nucleus of digitonin-permeabilized cells. In the accompanying paper, we report the results of our initial characterization of the exported components.

EXPERIMENTAL PROCEDURES

Cell Culture and Reagents

NIH mouse 3T3 fibroblasts were obtained from the American Type Culture Collection (Rockville, MD). The cells were grown as monolayers in Dulbecco's Modified Eagle Medium containing 10% calf serum, 100 U/ml penicillin, and 100 μg/ml streptomycin at 37 °C in a humidified atomsphere of 10% CO_2. Routinely, the cells were used in the export assay at a density of ~ 1.5 x 10^4 cells/cm^2, about 30% of the saturation density. The nuclear export assay was also carried out using 3T3 cells metabolically labeled with $^{32}PO_4$ (New England Nuclear, Boston, MA; 50 μCi/ml culture medium, 5 ml/ culture dish).

A rat monoclonal antibody was developed against the Mac-2 antigen (Ho and Springer, 1982), which has been shown to be galectin-3 (Cherayil *et al.*, 1989). The antibody was prepared from serum-free cell culture supernatant derived from the hybridoma line as described (Wang *et al.*, 1995); this antibody preparation is hereafter designated as rat anti-galectin-3. The polyclonal rabbit antiserum against recombinant rat galectin-1 (rabbit anti-galectin-1) (Cooper *et al.*, 1991) was a gift from Drs. Sam Barondes, Hakon Leffler, and Doug Cooper (University of California, San Francisco). The mouse monoclonal antibody 9H10/4B10 (Pinol-Roma *et al.*, 1988), directed against

hnRNP A1 (M_r ~34,000), was a generous gift of Dr. Gideon Dreyfuss (University of Pennsylvania) and is designated as mouse anti-hnRNP A1.

The following mouse monoclonal antibodies were purchased from Transduction Laboratories (Lexington, KY): (a) mouse anti-Ran was used to detect the GTP-binding-protein Ran (M_r ~ 25,000), involved in nucleocytoplasmic transport; and (b) mouse anti-karyopherin-β was used to detect the β-subunit (M_r ~97,000) of the NLS receptor. Human autoimmune serum anti-Sm, used to detect Sm B (M_r ~29,000) representing a core polypeptide of small nuclear ribonucleoprotein complexes (snRNPs), was purchased from The Binding Site (San Diego, CA). Human autoimmune serum used to detect the A polypeptide (M_r ~33,000) specific to U1 snRNP, anti-U1 RNP, was also purchased from The Binding Site. Rabbit polyclonal antibody against phosphoserine was purchased from Zymed Laboratories (South San Francisco, CA).

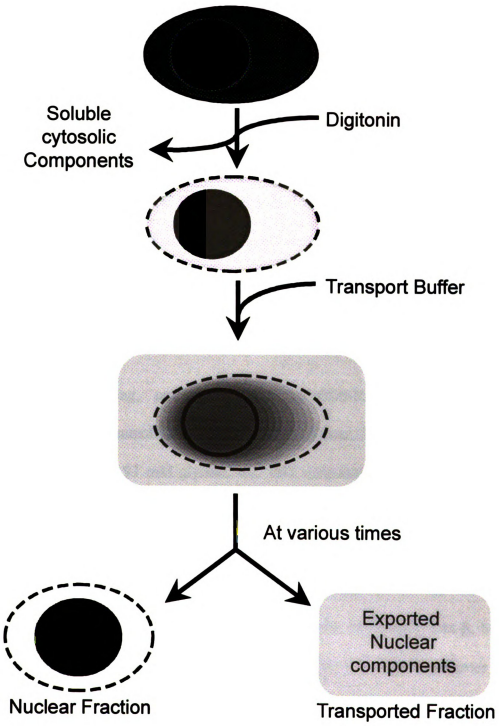
Wheat germ agglutinin (WGA) was purchased from Sigma (St. Louis, MO). Nucleotides corresponding to the monomethylated cap structures at the 5'-end of RNA polymerase II transcripts, m^7GpppA and m^7GpppG, were purchased from Boehringer Mannheim (Indianapolis, IN). The peptide NELALKLAGLDINKTG corresponds to residues 34-50 of PKI and has been shown to serve as the latter's Nuclear Export Signal (NES), with the underlined leucine and isoleucine residues being critical for activity (Wen *et al.*, 1995). This peptide was synthesized by the Macromolecular Structural Facility at Michigan State University and is hereafter designated the NES peptide.

The Nuclear Export Assay in Digitonin-Permeabilized Cells

The following protocol is described for a typical assay (Fig. 1) derived from one tissue culture dish (28 cm^2 surface area). Cells, at ~30% of confluent density, were washed with transport buffer (TB; 20 mM Hepes, pH 7.3, 110 mM KOAc, 2 mM EGTA, and 2 mM $Mg(OAc)_2$) The cells were permeabilized in TB containing digitonin (1.75 μM) for 5 min. at 4 °C. After removal of the digitonin solution containing the soluble cytosolic components, 3 ml TB, supplemented with 1 mM dithiothreitol, 5 μg/ml aprotinin, 5 μg/ml pepstatin, and 10 μg/ml leupeptin, were added onto the permeabilized cells and incubated for various times of the export assay (Fig. 1). The supernatant was then collected as the Transported Fraction (TF). To 1.5 ml of TF, 15 μl deoxycholate (10%, w/w) and 150 μl trichloroacetic acid (100%) were added and the mixture was incubated at 4 °C for 30 min. The suspension was then centrifuged (12,000 x g; 15 min.) and the precipitate was washed with acetone. The precipitate was resuspended in 40 μl 1% deoxycholate, 0.1 N NaOH; 10 μl of 5X SDS-PAGE sample buffer was then added and the entire sample was subjected to electrophoresis.

In each experiment, the corresponding nuclear fraction (NF) was harvested from the same culture dish by solubilization in 50 μl of SDS-PAGE sample buffer. The components of the nuclear residue were analyzed by SDS-PAGE.

Figure 1: Schematic illustration of the digitonin-permeabilized cell system for the assay of nuclear export. Monolayer cultures of mouse 3T3 cells were treated with digitonin and then incubated with transport buffer. The presence of galectin-3 in the nuclear fraction was monitored by immunofluorescence and by Western blotting. The exported nuclear proteins were analyzed by Western blotting.


Soluble
cytosolic
Components
Digitonin
Transport Buffer
At various times
Exported
Nuclear
components
Nuclear Fraction
Transported Fraction

One- and Two-dimensional Gel Electrophoresis

Proteins were resolved on one-dimensional gels, using 12.5% SDS-PAGE as described by Laemmli (1970). For two-dimensional gels, nonequilibrium pH gradient electrophoresis (NEPHGE) in the first dimension and SDS-PAGE in the second dimension were used as described by O'Farrell *et al.* (1977) with the following minor modifications. The NEPHGE gels were made using only pH 3-10 ampholines and the electrophoresis was carried out at 400 V for 5 h. Standard proteins for two-dimensional gels (BioRad, Richmond, CA) were used to track the pH gradient.

Western Blotting and Quantitation

For immunoblotting, proteins resolved by SDS-PAGE were transferred electrophoretically onto Immobilon-P membranes (Millipore, Bedford, MA) in buffer containing 25 mM Tris, 193 mM glycine, and 10% methanol. The membranes were blocked for several hours at room temperature with 10% non-fat dry milk in T-TBS (10 mM Tris, pH 7.5, 0.5 M NaCl, 0.05% Tween-20). After two brief washes with T-TBS, the membranes were incubated with primary antibody (rat anti-galectin-3, rabbit anti-galectin-1, mouse anti-hnRNP A1, mouse anti-Ran, mouse anti-karyopherin-β, human anti-U1 RNP and human anti-Sm) at room temperature for 1 h. The membranes were rinsed twice and then washed three times with T-TBS (one 15-min. wash and two 5-min washes). They were then incubated with the appropriate alkaline phosphatase-conjugated

secondary antibody (goat anti-rat immunoglobulin, goat anti-rabbit immunoglobulin, goat anti-mouse immunoglobulin, or goat anti-human immunoglobulin, all at 1:2000 dilution) for 30 min. at room temperature. After rinsing twice and washing in T-TBS (one 15-min wash and two 5-min washes), the blots were developed using colorimetric substrates for alkaline phosphatase, nitroblue tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate.

For quantitation, the blots were scanned using a Hewlett-Packard ScanJet 3C and the DeskScan II program (Hewlett Packard, Palo Alto, CA). The image was then analyzed using Image Quant (Molecular Dynamics, Sunnyvale, CA) and the pixel values of the individual bands were integrated to yield a value for intensity.

Immunofluorescence

For immunofluorescence microscopy, 3T3 cells were seeded onto glass coverslips placed in 6-well (8 cm^2/well) cluster dishes. In the basic protocol, cells were washed twice with phosphate-buffered saline (PBS, 140 mM NaCl, 2.68 mM KCl, 10 mM Na_2HPO_4, 1.47 mM KH_2PO_4, pH 7.4). and fixed for 20 min. at 4 °C with 4% paraformaldehyde in PBS. Cells were washed twice in PBS and then permeabilized with 0.2% Triton X-100 in PBS for 5 min. at 4 °C. After permeabilization, the cells were again washed twice with PBS and incubated with 0.2% gelatin in PBS for 1 h at room temperature.

Several variations of this basic protocol were also performed: (i) the cells were treated with digitonin as in the export assay (1.75 μM; 5 min.; 4 °C), followed immediately by fixation with paraformaldehyde and permeabilization with Triton X-100 as described above; (ii) the cells were treated with digitonin as in the export assay, fixed with paraformaldehyde but without the additional permeabilization with Triton X-100; and (iii) the cells were permeabilized with digitonin, incubated with TB for 30 min., followed by fixation with paraformaldehyde and permeabilization with Triton X-100 as described for the basic protocol.

In all cases, the cells, after the "blocking" incubation in 0.2% gelatin in PBS, were washed in T-TBS. They were then incubated with rat anti-galectin-3 (250 μg/ml in T-TBS containing 0.2% gelatin) for 1 h at 4 °C. The cells were washed three times (15 min. each) with T-TBS and then incubated with fluorescein-labeled goat anti-rat immunoglobulin (10 μg/ml in T-TBS containing 0.2% gelatin) for 30 min. at room temperature. The coverslips were washed three times in T-TBS (15 min. each) and then mounted in Perma Fluor (Lipshaw Immunon, Pittsburgh, PA) on glass slides. Fluorescence staining was analyzed using a Meridian Instruments (Okemos, MI) Insight confocal laser scanning microscope.

RESULTS

Export of Galectin-3 from Nuclei of Digitonin-permeabilized cells

The digitonin-permeabilized cell system, used to assay the import of protein bearing NLS (Adam *et al.*, 1990; Moore and Blobel, 1993) was adapted to analyze the export of galectin-3 from the nucleus. The presence of galectin-3 in the nuclear residue was monitored by immunofluorescence and by Western blotting of NF. In parallel, TF, containing the exported nuclear components, was also analyzed for galectin-3 (Fig. 1).

In accord with our previous observations (Laing and Wang, 1988; Wang *et al.*, 1995), mouse 3T3 fibroblasts, fixed with paraformaldehyde and permeabilized with Triton X-100, yielded prominent nuclear and discernible cytoplasmic staining with anti-galectin-3 (Fig. 2A). When 3T3 cells were first treated with digitonin to permeabilize the plasma membrane, then fixed and treated with Triton X-100 to permeabilize the nuclear membrane, a similar staining pattern of the cell nucleus was observed (Fig. 2B). Cytoplasmic galectin-3 was lost upon permeabilization of the plasma membrane. The galectin-3 detected by this procedure (Fig. 2B) is inside the nucleus. This conclusion is based on the observation that digitonin-permeabilized cells, fixed with paraformaldehyde but without Triton X-100 permeabilization of the nuclear membrane to allow antibody accessibility, yielded no staining (Fig. 2C). When the digitonin-permeabilized cells were incubated in TB for 30 minutes prior to fixation and Triton X-100 permeabilization, galectin-3 staining was diminished drastically (compare Fig. 2D with Fig. 2B). These

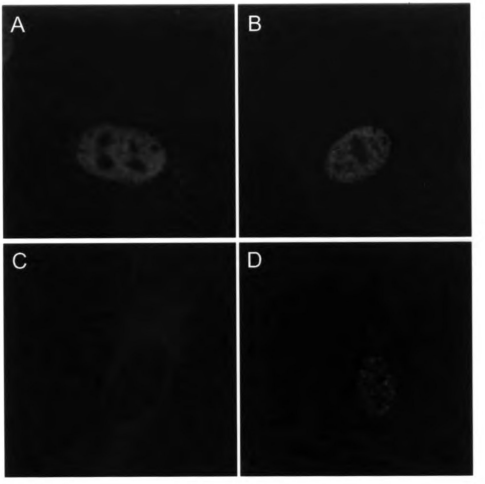


Figure 2: Comparison of the immunofluorescence staining for galectin-3 in 3T3 cells before and after differential permeabilization. (A) Cells fixed with paraformaldehyde (4%; 20 min; 4 °C) and permeabilized with Triton X-100 (0.2%; 5 min; 4 °C); (B) Cells permeabilized with digitonin (1.75 μM; 5 min; 4 °C), followed immediately by fixation with paraformaldehyde and permeabilization with Triton X-100; (C) Cells permeabilized with digitonin and fixed with paraformaldehyde; and (D) Cells permeabilized with digitonin, incubated with transported buffer for 30 min, prior to fixation with paraformaldehyde and treatment with Triton X-100. Galectin-3 was detected with rat anti-galectin-3(250 μg/ml) and fluorescein-conjugated goat anti-rat immunoglobulin.

results suggest that nuclear galectin-3 can be exported during the 30 minute incubation in TB.

This conclusion is corroborated by Western blot analysis of the galectin-3 polypeptide, both in the nuclear residue as well as in the soluble transported fraction. The level of galectin-3 in the nuclear residue is decreased considerably after the 30-minute incubation (Fig. 3, NF). Over the same time period, the level of galectin-3 in the supernatant rose correspondingly (Fig. 3, TF). Thus, both the immunofluorescence and immunoblotting analyses yielded consistent data, indicating that there was a rapid export of the galectin-3 polypeptide from the nuclei of digitonin-permeabilized 3T3 cells. This export assay has also been carried out using simian Cos-1 cells, with essentially the same results.

Kinetics of Export and Temperature Dependence

The rate of export of galectin-3 from digitonin-permeabilized 3T3 cells was temperature dependent. It was rapid at 37 °C; the half-life of galectin-3 in the nuclear residue was less than 5 minutes and less than 15% of the original nuclear galectin-3 remained after a 30-minute incubation in TB (Fig. 4). Parallel assays carried out at 25 °C and 0 °C showed that the rate of exit for galectin-3 decreased with lowering of the temperature. At 0 °C, for example, at least 40% of the galectin-3 remained with the nuclear residue over the 30-minute time course monitored. Because of the rapidity of the

Figure 3: Western blotting analysis for galectin-3 in the nuclear fraction (NF) and the transported fraction (TF) before and after a 30-min. export assay. Galectin-3 was detected using rat anti-galectin-3 (250 ng/ml) and alkaline phosphatase-conjugated goat anti-rat immunoglobulin. The solid and dotted lines on the right highlight the positions of migration of the doublet corresponding to galectin-3 in NF at the beginning of the assay. The galectin-3 in TF consists of only the upper band of the doublet. The asterisk highlights the presence of a band in TF reactive with anti-galectin-3, most probably representing a degradation product. The numbers on the left indicate the positions of migration of molecular weight markers.


NF
TF
t (min)
0
30
0
30
93
67
56
42
28
23
17
11
*

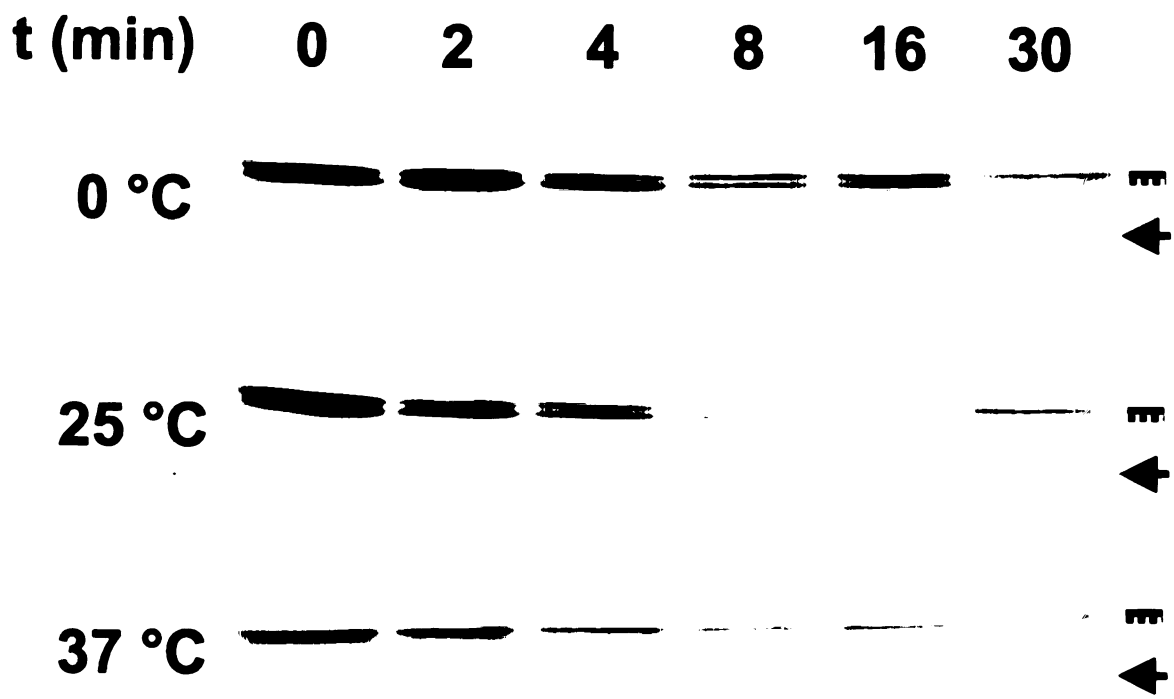


Figure 4: Temperature dependence of the kinetics of galectin-3 export in the digitonin-permeabilized cell assay. The amount of galectin-3 remaining in the nucleus was monitored by Western blot analysis of NF at various times. The solid and dotted lines on the right highlight the positions of migration of the doublet corresponding to galectin-3. The arrowhead indicates the position of migration for Sm B, a nuclear protein that is not exported in the assay, and thus provides an indication that material from approximately the same number of nuclei were electrophoresed in each lane.

export system at 37 °C, all subsequent experiments used samples after an 8-minute incubation in TB.

In these experiments, the export assay was carried out on cells incubated with fresh TB, after the removal of the digitonin solution used to permeabilize the plasma membrane of the cells. We have found that we can obtain essentially the same results by simply supplementing the digitonin solution with dithiothreitol and protease inhibitors, without the removal of the detergent. Thus, it appears that the continued presence of digitonin during the export assay does not extract or deplete factors affecting the transport itself.

The Exit of Nuclear Galectin-3 is Selective

In the course of these studies, careful analysis of Western blots indicated that, consistently, galectin-3 is revealed as a doublet in the nuclear residue whereas only the upper band of the doublet can be found in the transported fraction (Fig. 3). We had previously observed that, in 3T3 fibroblasts, galectin-3 can be found in two isoforms: (a) a phosphorylated form (pI ~8.2); and (b) a non-phosphorylated species (pI ~8.7). The former is present both in the nucleus and in the cytoplasm, while the latter is found exclusively in the nucleus (Cowles *et al.*, 1990). On this basis, the nuclear residue and the transported fraction were subjected to two-dimensional gel electrophoresis, followed by Western blotting. The nuclear residue, which contained the galectin-3 doublet on

single-dimension SDS-PAGE, yielded two spots on the two-dimensional gels, with the upper band corresponding to the pI ~8.2 species and the lower band corresponding to the pI ~8.7 isoform (Fig. 5A). In contrast, the transported fraction, which contained only the upper band of galectin-3, yielded exclusively the pI ~8.2 spot (Fig. 5B).

On this basis, we conclude that the upper band of the galectin-3 doublet represented the phosphorylated form while the lower band corresponded to the nonphosphorylated species. This conclusion is supported by two additional lines of evidence. First, when the nuclear residue is immunoblotted with anti-phosphoserine, only the upper band of the galectin-3 doublet showed reactivity (data not shown). This is consistent with the identification of Ser-6 as the major site of phosphorylation of the canine homolog of galectin-3 (Huflejt *et al.*, 1993). Second, an export assay was carried out using 3T3 cells that had been metabolically labeled in the presence of $^{32}PO_4$. The TF was subjected to lactose affinity chromatography and the bound material was resolved on SDS gels. The single galectin-3 band, identified by immunoblotting, was also radioactive upon autoradiography (data not shown).

Although the exported galectin-3 consists of only the phosphorylated form, the majority of the galectin-3 molecules was found to be ultimately lost from the nuclear residue. Moreover, on the basis of the intensity of the immunoblotted bands, the amount of galectin-3 lost from the nucleus (Fig. 3, NF) can be approximately accounted for by the amount appearing in the transported fraction (Fig. 3, TF). Thus, it appears that the non-phosphorylated galectin-3 polypeptide (lower band) is first phosphorylated (to

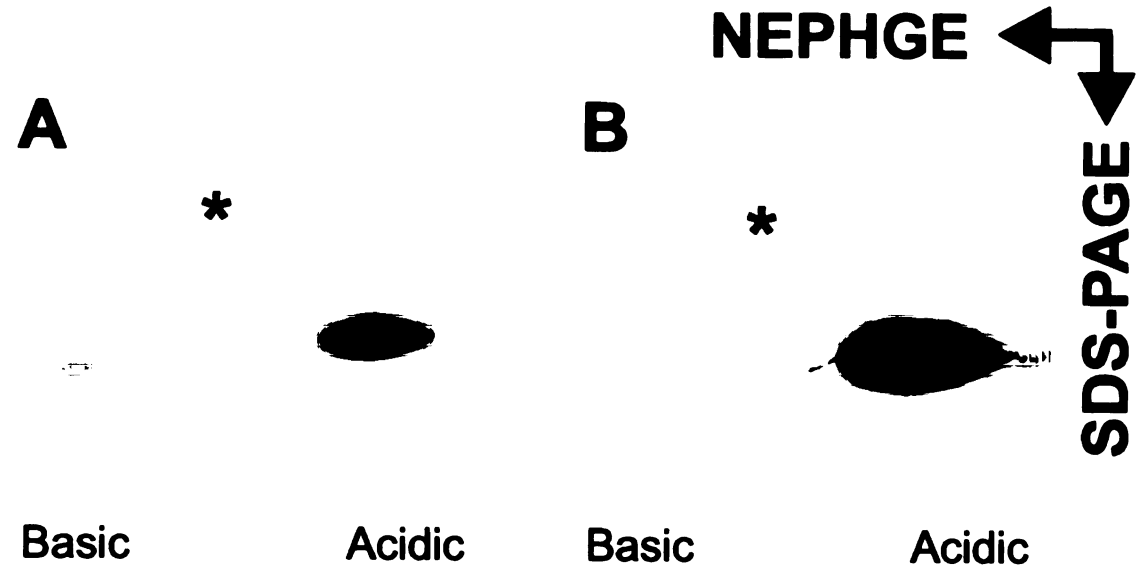


Figure 5: Two-dimensional gel electrophoretic analysis for galectin-3 in NF before the export assay (A) and in the TF after an 8-minute export assay (B). The samples were subjected to two-dimensional NEPHGE/SDS-PAGE and Western blotting. The asterisk marks the reference protein rabbit muscle glyceraldehyde 3-phosphate dehydrogenase, which yielded a pI value of 8.5.

become the upper band) prior to export. This notion is supported by a comparison of the ratios of the upper to lower bands at various time points during transport at 37 °C (Fig. 4). These results suggest that phosphorylation may be important for the exit of galectin-3 from the nucleus.

The observation that only the phosphorylated form of galectin-3 appears to be exported suggests the system is highly selective in terms of substrates for export. Thus, we determined the export or retention of several nuclear proteins, on the basis of antibody reagents available. Karyopherin-β and Ran, two factors required for the import of NLS-bearing proteins, were also found to be exported. This conclusion can be derived by comparing the initial and 8-minute samples of either the nuclear residue (Fig. 6, lanes 1-2 and 5-6) or the exported material (Fig. 6, lanes 3-4 and 7-8). Galectin-1, belonging to the same lectin family as galectin-3, has also been identified as a nuclear splicing factor (Vyakarnam *et al.*, 1997). Like galectin-3, it is exported from the nucleus in our assay (Fig. 6, lanes 9-12). On the other hand, polypeptides of the snRNPs, Sm B representing the core polypeptide of several of the snRNPs and the A polypeptide specific to U1 snRNP, were not exported in our assay (Fig. 6, lanes 13-20). Finally, the hnRNP protein A1 is exported very slowly (relative to galectin-3), if at all (Fig. 6, lanes 21-24). Thus, the specificity of the galectin-3 transport system is manifested by: (a) comparing galectin-3 with other components of the nuclear splicing machinery; and (b) comparing the isoelectric variants of the galectin-3 polypeptide itself.

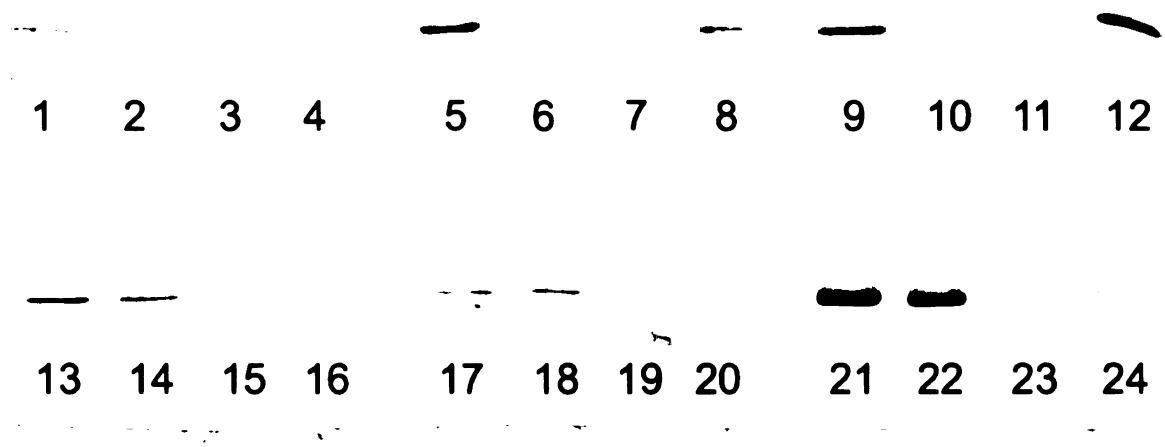


Figure 6: Comparison of several nuclear proteins in terms of their export or retention in the digitonin-permeabilized cell assay. Samples were subjected to Western blotting using the following antibodies: lanes 1-4, mouse anti-karyopherin-β (250 ng/ml); lanes 5-8, mouse anti-Ran (250 ng/ml); lanes 9-12, rabbit anti-galectin-1 (1:200 dilution); lanes 13-16, human anti-Sm (1: 10,000 dilution); lanes 17-20, human anti-U1 RNP (1: 10,000 dilution); and lanes 21-24, mouse anti-hnRNP A1 (1: 1000 dilution). The first two lanes of each set represent NF, at the beginning of the assay and after an 8-minute export period, and the last two lanes of each set represent the corresponding samples of TF.

Inhibition of Galectin-3 Export

The plant lectin WGA, which binds N-acetyl-D-glucosamine (GlcNAc)-containing glycoconjugates, has been shown to inhibit the nuclear import of proteins (Finlay *et al.*, 1987), as well as nuclear export of RNA (Yoneda *et al.*, 1988; Bataille *et al.*, 1990; Neuman de Vegvar and Dahlberg, 1990; Dargemont and Kuhn, 1992). The presence of WGA in our assay inhibited the export of galectin-3 from the nucleus (Fig. 7). This was the case when WGA was added either to the permeabilization buffer containing digitonin or to TB. The effect of WGA was most pronounced when it was present during both permeaibilization and transport phases of the assay. Moreover, the effect of WGA can be reversed by its saccharide ligand, GlcNAc, but not by a non-binding saccharide, D-galactose (Gal) (Fig. 7). Similar results were obtained when the export of Ran was analyzed. Inasmuch as it has been shown that WGA has no effect on diffusion of fluorescent dextrans in and out of the nuclear pores (Jiang and Schindler, 1987; Wolff *et al.*, 1988; Yoneda *et al.*, 1988), these results further argue against the notion that the observed export of galectin-3 was due to leakage and diffusion.

On the basis of our previous documentation that galectin-3 is required for the splicing of pre-mRNA (Dagher *et al.*, 1995; Vykarnam *et al.*, 1997) and that its detection in the nucleus is sensitive to ribonuclease (Laing and Wang, 1988; Hubert *et al.*, 1995), we tested the effects of the nucleotides m^7GpppG and m^7GpppA. These correspond to the monomethylated, inverted guanosine cap structure, m^7G(5')ppp(5')N, of RNA polymerase II transcripts and have been implicated to facilitate RNA export from the

Figure 7: The effect of wheat germ agglutinin (WGA) on the export of galectin-3 and Ran in the digitonin-permeabilized cell assay. Samples of NF, at the beginning of the assay and after an 8-minute export period, were subjected to Western blotting using antibodies against galectin-3 (rat anti-galectin-3, 250 ng/ml) and against Ran (mouse anti-Ran, 250 ng/ml). The concentration of WGA was 500 μg/ml and the concentrations of GlcNAc and Gal were 100 mM.

nucleus (Hamm and Mattaj, 1990). Although neither nucleotide, at a concentration of 2 mM, had an observable effect on galectin-3 export, we were surprised to find that m^7GpppA (2 mM) in combination with the NES peptide (50 μg/ml) yielded partial inhibition (Fig. 8A). The sequence of the NES peptide corresponds to residues 34-50 of PKI, found to be responsible for the latter's export from the nucleus, but the addition of NES peptide (50 μg/ml) by itself had little effect on transport in our assay (data not shown). Higher concentrations of m^7GpppA (4 mM) and NES peptide (700 μg/ml) yielded greater (but still only partial) inhibition (Fig. 8B). This effect was not observed with the combination m^7GpppG plus NES peptide (Fig. 8A and 8B). Moreover, the export of Ran was not similarly affected by the combination of m^7GpppA plus NES peptide (Fig. 8B). These results suggest that at least some of the galectin-3 is exported in association with an RNP and that this RNP contains both the monomethylated cap structure m^7GpppA and polypeptide(s) bearing NES-like sequences. This notion is schematically illustrated in Figure 9.

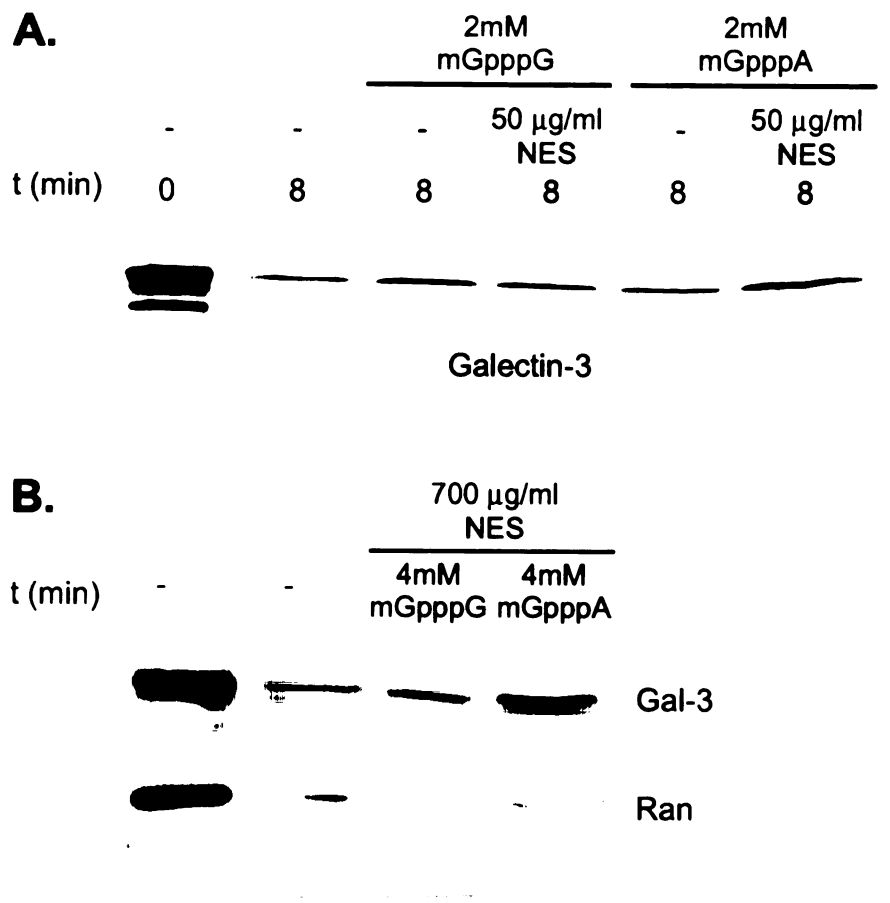


Figure 8: The effects of 5'-cap structure nucleotide analogs and of a peptide bearing a nuclear export signal (NES) on the export of galectin-3 and Ran in the digitonin permeabilized cell assay. Samples of NF, at the beginning of the assay and after an 8-minute export period, were subjected to Western blotting using antibodies as described in the legend to Figure 7. In panel A, the nucleotides, m^7GpppG and m^7GpppA, were used at a concentration of 2 mM and the NES peptide was used at a concentration of 50 µg/ml. In panel B, the corresponding concentrations of nucleotides and peptide were 4 mM and 700 µg/ml, respectively.

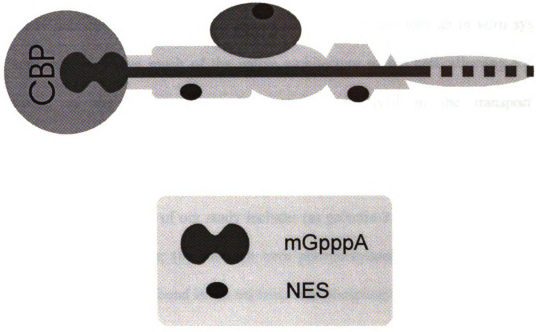


Figure 9: Schematic diagram illustrating the association of galectin-3 with a ribonucleoprotein complex in TF of the export assay. Galectin-3 is represented by an oval (Gal3) containing a nuclear export signal (NES). The horizontal line depicts a mRNA, with a cap structure (mGpppA) at the 5'-end and a poly A tail (dotted line) at the 3'-end. The sphere (CBP) represents cap-binding protein(s). Other proteins associated with the RNA may also carry NES sequences.

DISCUSSION

Since its initial development by Adam *et al.* (1990), the digitonin-permeabilized cell system has contributed much to our understanding of nuclear protein import. By selectively permeabilizing the plasma membrane, digitonin provides an *in vitro* system that appears to preserve much of the native architecture of the cell, allowing for the elucidation of the components and interactions involved in the transport of macromolecular substrates across the nuclear envelope. We have now taken advantage of this selective permeabilization system to analyze the export of galectin-3 from the cell nucleus. The key findings of our study include: (a) galectin-3 is rapidly and selectively exported from the nucleus; (b) although both phosphorylated and nonphosphorylated isoforms of galectin-3 are found in the nucleus, only phosphorylated galectin is identified in the exported fraction; and (c) while the Sm B and U1 A polypeptides of snRNPs are retained in the nuclear residue, galectin-1, Ran, and karyopherin-β are also exported in the same assay; (d) the rate of export is decreased by cold temperature and by the addition of WGA; and (e) galectin-3 export is at least partially inhibited by the addition of cap-like m^7GpppA nucleotide plus NES peptide.

At least three types of nuclear export signals (NES) have now been identified: (i) short sequences enriched in hydrophobic residues, especially leucines (leucine-rich NES), as originally identified in PKI (Fantozzi *et al.*, 1994) and HIV Rev protein (Meyer and Malim, 1994); (ii) a 38-amino acid region containing many glycine residues (designated

M9), identified on human hnRNP A1 (Michael *et al*, 1995) and on its insect homolog, hrp 36 (Visa *et al.*, 1996); and (iii) a KNS sequence, identified on hnRNP K that, like the M9 region, can function both for import and export (Michael *et al*, 1997). A sequence matching the leucine-rich NES, with distinct spacing of the leucine residues, can be found in galectin-3 homologs from various species. If this sequence indeed serves as an NES for galectin-3, then one might expect that a peptide corresponding to the NES of PKI would compete for the export machinery and inhibit the galectin transport. The addition of the NES peptide failed to affect galectin-3 export. Moreover, in transfection experiments using a FLAG epitope-tagged galectin-3 construct, we have carried out site-directed mutagenesis on the leucine residue correponding to Leu-44 of PKI. This leucine residue had been demonstrated by Wen *et al.* (1995) to be critical for the export activity of the NES. We found no difference, however, in the nuclear versus cytoplasmic distribution of the FLAG-tagged galectin-3 in cells transfected with the wild-type and the mutant constructs (Y.G. Tsay, unpublished results).

One difficulty in interpreting such experimental results may be related to the complex nature of many of the molecular species that are exported from the nucleus. It is generally believed that the RNA substrates for nuclear export are probably all transported as RNP complexes (see reviews by Izaurralde and Mattaj (1995), by Gerace (1995), and by Moore (1996)). Most of these RNPs contain multiple polypeptides so it seems possible that mutational knockout of the putative NES on galectin-3 will not alter its export properties as long as it remains associated with an RNP that carries other NES-bearing polypeptide(s) (see Fig. 9). Indeed, this model is supported by the observation

that a combination of NES peptide and nucleotide analogs recognized by cap-binding proteins are required to inhibit galectin-3 export (Fig. 9). Furthermore, this model may help account for the previous results that the monomethylated cap structure is a facilitating, but not a required, factor for mRNA export (Hamm and Mattaj, 1990; Jarmolowski *et al.*, 1994). The presence of multiple export signals on these RNPs probably circumvents the absolute requirement of a particular signal during nuclear export.

Our present results need to be discussed in the context of two other studies that have used the digitonin-permeabilized cell system to study nuclear export. Moroianu and Blobel (1995) used digitonin-permeabilized buffalo rat liver cells to preload the nuclei with fluorescently-labeled human serum albumin covalently conjugated with the NLS of SV40 large T-antigen. These preloaded nuclei were then incubated to investigate the conditions for the export of the fluorescent substrate. They found that nuclear export in their system required Ran and GTP hydrolysis. In contrast, the export of galectin-3 in our system was not dependent on any exogenously added Ran or GTP. In fact, we found that Ran was also exported from the nuclei during the course of the transport assay, and , to the best of our knowledge, this represents the first demonstration on the nuclear export of Ran. Thus, it seems possible that depletion of Ran (and GTP) during the preloading of the fluorescently labeled transport substrate and subsequent washing steps may account for the apparent requirement for these factors, as observed by Moroianu and Blobel (1995).

More recently, Yang *et al.* (1997) have used digitonin-permeabilized GrH2 rat hepatoma cells, which express elevated levels of glucocorticoid receptors, to study the effect of hormone withdrawal on receptor dissociation from chromatin and a novel nuclear export process that is stimulated by sodium molybdate (Na_2MoO_4) and ATP. The effect of sodium molybdate can be mimicked by protein tyrosine phosphatase inhibitors, including sodium tungstate, sodium vanadate, and heparin; it is counteracted by inhibitors of tyrosine kinase such as genistein and tyrphostin AG126 (Yang *et al.*, 1997). The stimulation of *in vitro* export by these compounds is not unique to glucocorticoid receptors but can also be observed with heat shock proteins hsp70 and hsp90, as well as hnRNP A1. Several similarities and differences with our present results become immediately obvious.

First, hnRNP A1 is exported very slowly, if at all, in our 3T3 cell assay. This was true even in the presence of ATP and sodium molybdate. It should be noted that hnRNP A1 is known to shuttle between the cytoplasm and the nucleus (Piñol-Roma and Dreyfuss, 1993) and a nuclear export signal, designated M9, has been identified on the polypeptide (Michael *et al.*, 1995). The fact that hnRNP A1 failed to be exported rapidly in our present assay argues strongly against non-specific leakage being responsible for the observed exit of nuclear components such as galectin-3.. This notion is supported by the observation that while the nucleus contains both phosphorylated and nonphosphorylated galectin-3, only the phosphorylated isoform appears in TF. The export of galectin-3 is also blocked by WGA, similar to the latter's inhibition of the transport of preloaded NLS-

human serum albumin (Moroianu and Blobel, 1995), and of the ATP- and molybdate-stimulated export of glucocorticoid receptors (Yang *et al.*, 1997).

Second, the export of glucocoticoid receptors from the nuclei of GrH2 rat hepatoma cells required ATP, in addition to molybdate (Yang *et al.*, 1997). On the other hand, the addition of either ATP or its nonhydrolyzable analog ATPγS had little or no effect on the export of galectin-3 in our assay. This apparent discrepancy on energy requirements may reflect the monitoring of different export substrates. ATP hydrolysis may be required for staging of the glucocorticoid receptors into a subnuclear compartment for export whereas galectin-3 transport may not need such preparation. In any case, neither the export of galectin-3 nor the export of glucocorticoid receptors appear to be sensitive to inhibition by GTPγS, suggesting that GTP hydrolysis was not required. On the other hand, Moroianu and Blobel (1995) had reported such a GTP dependence in the export of preloaded NLS-human serum albumin.

Finally, in contrast to the stimulatory effect of molybdate, tungstate, and vanadate on glucocorticoid receptor export (Yang *et al.*, 1997), we found no effect of these oxyanions on galectin-3 transport. More strikingly, we observed inhibition of the export of galectin-3 by vanadyl cations. The basis of the vanadyl versus vanadate difference may again be related to our idea that at least some of the galectin-3 is exported from nucleus in the form of an RNP (Fig. 9). Thus, the details of the vanadyl inhibition will be

presented in the accompanying manuscript, as a part of the analysis of the chemical components in the exported fraction (Tsay *et al.*, accompanying paper).

CHAPTER III

NUCLEAR EXPORT OF GALECTIN-3 IN MOUSE 3T3 FIBROBLASTS

II. CHARACTERIZATION OF THE EXPORTED COMPONENTS

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SUMMARY

The nuclear components collected in the transported fraction of a nuclear export assay (see accompanying manuscript) was analyzed in terms of the polypeptide and RNA components. Gel filtration of the exported nuclear material and analysis for galectin-3 showed that the lectin can be found in at least two sets of high molecular weight complexes (~650 kD and ~60 kD). In the presence of lactose, both of these complexes are disrupted and galectin-3 chromatographs to a position corresponding to ~ 30 kD polypeptide. In contrast, the overall elution profile of galectin-1 in the transported fraction is not drastically altered by lactose. The polypeptide components of the high molecular weight complexes containing galectin-3 are revealed by affinity adsorption on a lactose-agarose column, specific elution by lactose, and gel electrophoresis. These polypeptides are not bound to a control cellobiose column. The transported fraction of the nuclear export assay also contain RNA. In the low molecular weight range, the RNA species include tRNA (~ 80 nucleotides) as well as RNAs of ~100, 300, and 650 nucleotides. High molecular weight RNAs, ranging from ~1 kb to 5 kb, include poly A^+ mRNA as revealed by hybridization with an oligo(dT) probe. Compounds containing vanadyl cations (VO^{2+}), such as vanadyl ribonucleoside complex, inhibit the export of both galectin-3 and RNA in our assay. All of these results are consistent with the notion that galectin-3 is associated with a ribonucleoprotein complex during its export from the nucleus.

INTRODUCTION

In the previous manuscript, we documented that, in digitonin-permeabilized 3T3 fibroblasts, galectin-3 as well as several other nuclear proteins, is rapidly and selectively exported from the nucleus (Tsay *et al*, 1997). Several key findings were made: (a) although both phosphorylated and nonphosphorylated isoforms of galectin-3 are found in the nucleus, only phosphorylated galectin-3 is identified in the exported fraction; (b) while Sm B and U1 A of snRNPs are retained in the nuclear residue, galectin-1, Ran, and karyopherin-β are also exported in the same assay; and (c) galectin-3 export is inhibited by the addition of cap-like m^7GpppA nucleotide plus a peptide bearing the nuclear export signal (NES) of the inhibitor of cAMP-dependent protein kinase (PKI). On the basis of these observations, the possibility was raised that galectin-3 may be exported in association with a ribonucleoprotein complex (RNP), with many polypeptides bound directly or indirectly with an RNA species.

One major advantage of the digitonin-permeabilized export assay lies in the fact that the exported material can be collected in a soluble form, amenable for fractionation and biochemical analysis. We have thus carried out preliminary physico-chemical characterization of the components of the exported nuclear components, with two main goals: (a) to obtain additional evidence that nuclear galectin-3 is associated with a high molecular weight complex; and (b) to delineate the number and, if possible, the identity

of the polypeptide and RNA species of the galectin-3 complex. The results of these studies are documented in the present communication.

EXPERIMENTAL PROCEDURES

Cell culture and reagents

NIH mouse 3T3 fibroblasts were purchased form the American Type Culture Collection (Rockville, MD). The cells were grown in Dulbecco's Modified Eagle Medium containing 10% calf serum, 100 U/ml penicillin, and 100 μg/ml streptomycin at 37°C in a humidified atmosphere of 10% CO_2. Generally, cells were seeded at a density of ~1.5 x 10^4 cells/cm^2, about 30% of the saturation density. Metabolic labeling with ^{32}P-labeled orthophsophate was carried out at the concentration of 50 μCi/ml culture medium.

Anti-Galectin-3 antibody is a rat monoclonal antibody developed against the Mac-2 antigen (Ho and Springer, 1982); anti-galectin-1 antibody is a polyclonal rabbit antiserum raised against rat galectin-1 (a generous gift from Dr. Barondes, University of California, San Francisco); anti-Ran antibody is a mouse monoclonal antibody, purchased from Transduction Laboratories (Lexington, KY).

Vanadyl sulfate hydrate was from Aldrich (Milwaukee, WI), vanadyl ribonucleoside complex (VRC) was from GIBO-BRL (Gaithersburg, MD), and ribonucleosides were from Boehringer-Mannheim (Indianapolis, IN). Agents purchased from Sigma (St. Louis, MO) include sodium metavanadate ($NaVO_3$), tungstic acid (H_2WO_4), sodium molybdate (Na_2MoO_4), and all of the secondary antibodies used for immunofluorescence and immunoblotting analyses.

Nuclear export assay

The details of the assay are describe in the accompanying manuscript (Tsay *et al.* , 1997). For transported fraction (TF) used in gel filtration chromatography and immunoprecipitation analysis, VRC was added at a concentration of 10 mM immediately following the collection of TF. TF used for gel filtration and immunoprecipitation was concentrated approximately 200-fold in Ultrafree-15 centrifugal filter device (Millipore, Bedford, MA) centrifuged at 3,000 x g.

For immunofluorescence experiments, cells seeded on coverslips were incubated with 40 μg/ml digitonin at 0°C for 5 min. Reagents to be tested for stimulatory or inhibitory effects then added without removal of the digitonin solution. The cells were incubated at 25 °C for 8 min. To stop the reaction, cells were incubated in 3.7% of formaldehyde for 20 min at 0 °C. Fixed cells were washed in phosphate-buffered saline (PBS) twice for 10 min. Permeabilization with 0.2% Triton X-100 was carried out at room temperature for 5 min. After two 10-min washes, 0.2% of gelatin was added to the cells. Staining with antibodies was described in the accompanying manuscript (Tsay *et al.*, 1997). Concentrations for primary antibodies are 2.5 μg/ml for anti-Ran and 25 μg/ml for anti-galectin-3. FITC-conjugated anti-mouse IgG and anti-rat IgG antibodies were both used at 1:250 dilution.

For immunoblotting experiments, cell were treated as described above. After the incubation at 25°C, the cell residue, designated as the *nuclear fraction* (NF), was

collected in SDS-PAGE sample buffer. The sample was sonicated for 1 min at room temperature and heated at 100 °C before loading to a 12.5% polyacrylamide gel. The polypeptides resolved on polyacrylamide gels were electrotransferred onto Immobilon-P (Millipore, Bedford, MD) and immunoblotted with the indicated antibodies. Concentrations of primary antibodies for immunoblotting are 250 ng/ml for anti-Ran, 250 ng/ml for anti-galectin-3 and 1:1000 for anti-galectin-1. All of the secondary antibodies were used at 1:2000 dilution.

Gel filtration chromatography

About 24 ml of Sephacryl S-300 HR (Pharamacia, Piscataway, NJ) were packed in a 1 x 30 cm Econo-Column column (Bio-Rad, Hercules, CA) according to the manufacturer's guide. The column is rinsed with transport buffer (TB) at the rate of 24 ml/hour and then equilibrated with the same buffer at the rate of 8 ml/hour before application of the sample. Molecular weight standards (100 μg each) include thyroglobulin (670 kDa), alcohol dehydrogenase (150 kDa) and carbonic anhydrase (29 kDa). Eluted components were collected in 13-drop (~ 0.75 ml) fractions. Protein concentration in each fraction was determined by the Bradford method (Bradford, 1976). Typically, TF derived from 4 x 10^6 cells, in a volume of 200 μl, was loaded onto the column and then eluted with TB. Polypeptide components in each fraction were precipitated in the presence of 10% trichloroacetic acid and 1% deoxycholate at 4°C for 30 min. The pellet was collected after centrifugation at 12,000 x g for 15 min. After

rinsing with acetone, the pellet was resuspended with 0.1% deoxycholate-0.1 M NaOH and mixed with 5X SDS sample buffer. The samples were analyzed by SDS-PAGE and immunoblotting.

Affinity adsorption chromatography

For saccharide adsorption experiments, 200 µl of concentrated TF were incubated with 40 µl of 1:1 slurry of lactose- or cellobiose-agarose (Sigma) overnight at 4°C. These sample was centrifuged at 12,000 x g for 10 sec, and the supernatant (*unbound fraction*) was removed from the beads. The agarose beads were then washed four times in TB containing 1% NP-40, 0.5% deoxycholate and 0.1% SDS. After the last wash, bound materials were eluted with 0.2 M lactose in TB and designated as *eluted fraction.* The eluted fraction was resolved on a 12.5% polyacrylamide gel and analyzed by silver staining.

For antibody adsorption experiments, concentrated TF was incubated with 3 µg of anti-galectin-3 overnight at 4°C. Twenty microliters of 1:1 protein G-agarose were added to the solution and mixed with rocking at room temperature for 2 hours. The sample was centrifuged at 12,000 x g for 10 sec, and the supernatant (unbound fraction) was removed and subjected to RNA analysis.

Analysis of RNA components

To samples containing ^{32}P-labeled RNAs were added 0.1% NP40, 0.1% SDS, and 10 µg/ml tRNA and then treated with 10 µg/ml of proteinase K at 37 °C for 10 min.

RNAs were then isolated by standard phenol extraction and ethanol precipitation procedures (Sambrook *et al.*, 1989). RNA pellets were resuspended in formaldehyde loading buffer and resolved on a formaldehyde-1.2% agarose gel or an 8% polyacylamide gel. RNAs were then visualized by autoradiography.

For detection of poly(A)$^+$ RNAs, unlabeled RNAs were prepared as described and then resolved on a formaldehyde-8% agarose gel. RNAs were hybridized in dried gels with ^{32}P-labeled oligo(dT)$_{12\text{-}18}$. (GIBCO-BRL, Gaithersburg, MD) at room temperature using the standard procedure (Sambrook *et al., 1989).* RNAs hybridized with oligo(dT) were visualized by autoradiography.

RESULTS

Gel Filtration of Exported Nuclear Components and Analysis for Galectin-3

In the previous manuscript, we documented the use of digitonin-permeabilized 3T3 fibroblasts to analyze the export of galectin-3 from the nucleus (Tsay, accompanying manuscript). We defined the material remaining in the nuclear residue after a given period of incubation as the Nuclear Fraction (NF). The exported material is collected in the Transported Fraction (TF). One key observation was that the export of galectin-3 can be inhibited, at least partially, by the simultaneous addition of the cap-like nucleotide m^7GpppA plus a peptide bearing the NES from PKI. The possibility was raised that galectin-3 may be exported in association with an RNP, which contain signals that facilitate nuclear export in both the 5'-cap structure of the RNA species as well as peptide sequences of component polypeptide(s). Because one major advantage of the digitonin-permeabilized export assay lies in the fact that the exported material can be collected in a soluble form amenable for fractionation and biochemical analysis, we have carried out preliminary physical and chemical characterization of the components of TF.

First, TF was concentrated approximately 200-fold and subjected to gel filtration on a column of Sepharcryl S-300 HR. Individual fractions eluting from the column were subjected to SDS-PAGE and Western blotting for galectin-3. The elution profile for galectin-3 in TF showed three zones, with peaks centered around: (a) ~650 kD; (b) ~60

kD; and (c) ~15 kD (Fig. 1A). Previous gel filtration analyses of galectin-3, purified from 3T3 cells (Roff and Wang, 1983) and from *E.coli* expression system (Hsu *et al.*, 1992), showed that the protein chromatographed as a ~30 kD species, corresponding to the monomeric polypeptide. On this basis, we can immediately conclude that galectin-3 in TF existed in at least two sets of complexes: (a) the ~650 kD group; and (b) the ~60 kD group. The presence of galectin-3 in the zone centered around ~15 kD may complicate the molecular weight estimates because it suggests that either galectin-3 or an associated component may interact with the Sephacryl backbone and get retarded in the chromatographic column. We have carried out similar gel filtration studies using a number of other matrices (e.g. Sepharose, Sephadex); the same overall conclusion, that galectin-3 in TF was associated with high molecular weight complexes, was obtained.

The individual fractions from the Sepharcryl S-300 HR column were also subjected to Western blotting for galectin-1. There was a broad zone of elution, with a peak slightly higher in molecular weight and another one just slightly lower in molecular weight than the 29 kD marker (Fig. 1B). This result is consistent with the report of Cho and Cummings (1995), who showed that galectin-1, on gel filtration, yielded two poorly resolved peaks corresponding to the monomeric polypeptide (~15 kD) and a dimer (~30 kD). The ratio of monomer to dimer depended on the concentration of the protein in solution. Because the members of the galectin family exhibit very similar carbohydrate-binding specificities, the fact that galectin-1 chromatographed at the expected position suggests that the retardation of galectin-3 in the ~15 kD zone (Fig. 1A) is most probably not due to protein interactions with the carbohydrate backbone of the Sephacryl matrix.

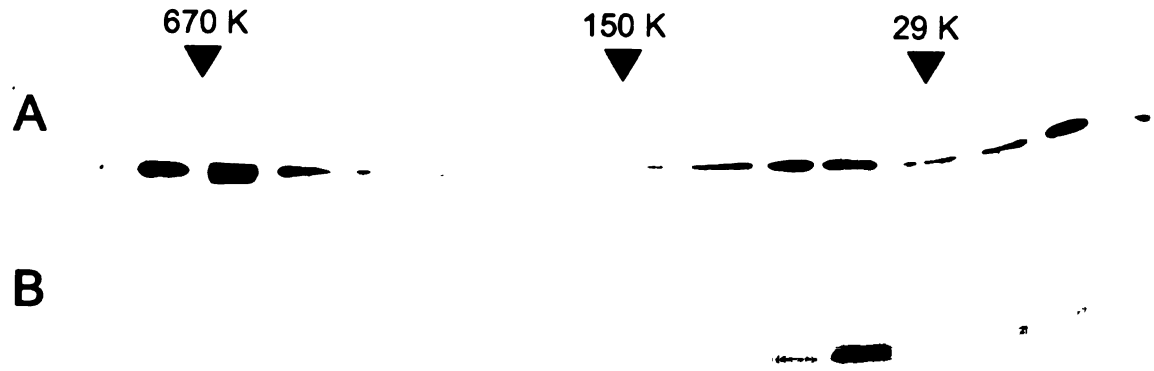


Figure 1: Gel filtration of the exported nuclear components in TF and analysis for galectins-1 and -3. The column (1.3 x 29 cm) was packed with Sephacryl S-300 HR and was equilibrated with TB (containing supplements?). HOW MUCH TF LOADED? IN WHAT VOLUME? Fractions of 0.5 ml were collected, subjected to SDS-PAGE and immunoblotting with anti-galectin-3 (panel A) or anti-galectin-1 (panel B). The numbers at the top indicate the positions of migration of molecular weight markers: thyroglobulin (670 K), alcohol dehydrogenase (150 K), and carbonic anhydrase (29 K).

Using purified galectins-1 and -3 , previous studies had shown that the disaccharide ligand lactose has little or no effect on the chromatographic behavior of these lectins (Roff and Wang, 1993; Hsu *et al.*, 1992; and Cho and Cummings, 1995). The inclusion of Lac in the chromatographic medium had little or no effect on the overall position of elution for galectin-1 in TF (Fig. 2). There appeared to be some diminution of the peak >29 kD and accentuation of the peak < 29 kD. One possibility is that Lac shifted the monomer-dimer equilibrium in favor of subunit dissociation. An alternative possibility is that the > 29 kD peak represents some association of galectin-1 with another component and that this complex is disrupted by Lac binding to galectin-1.

For galectin-3 in TF, however, the inclusion of Lac in the chromatographic medium drastically altered its elution profile (Fig. 2). The peak at ~650 kD zone disappeared. The peak at ~60 kD zone was also diminished. The majority of the galectin-3 chromatographed to a position slightly lower in molecular weight than the 29 kD marker. These results suggest that the high molecular weight complexes with which galectin-3 is associated in TF is sensitive to Lac-binding. Individual fractions in the ~650 kD zone derived from columns carried out in the absence and presence of Lac were compared by SDS-PAGE and silver staining. The polypeptide compositions of these fractions from the column with Lac were quite distinct from the corresponding fractions from columns without the disaccharide (data not shown). These results are consistent with the notion that Lac removed not only galectin-3 from the ~650 kD zone but also other polypeptides in the high molecular weight complex. Finally, it should be noted

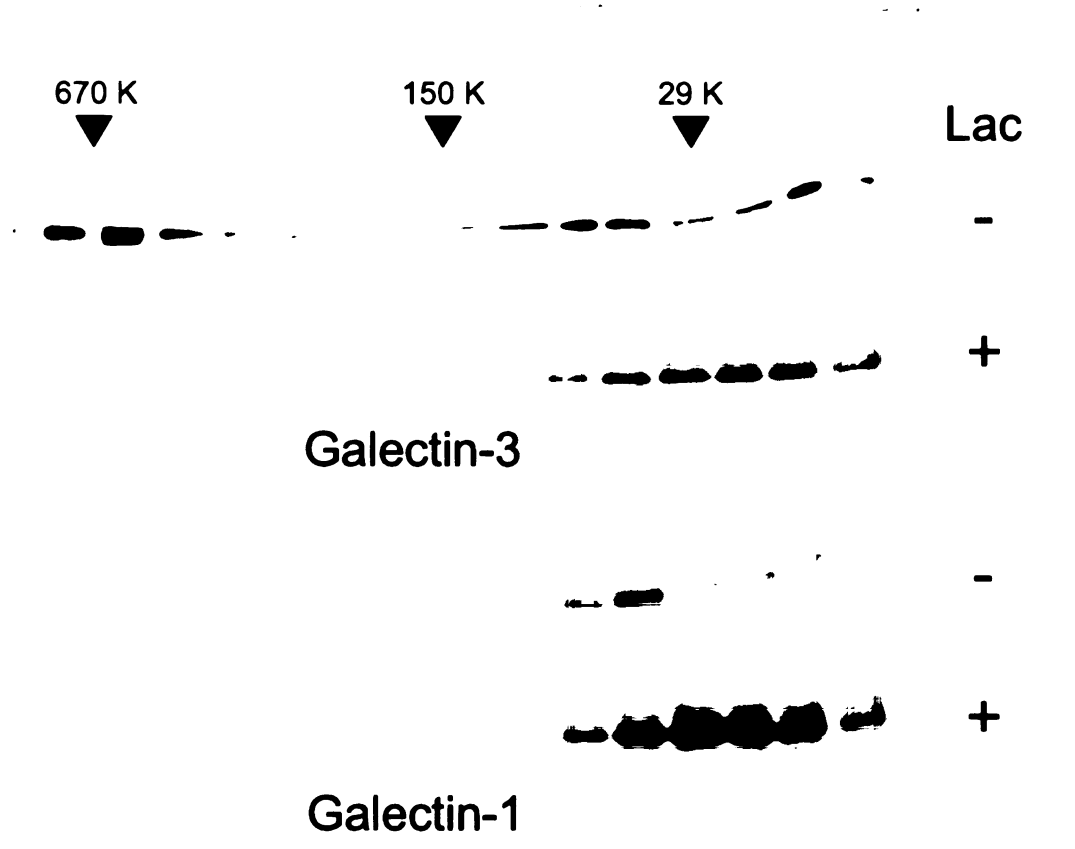


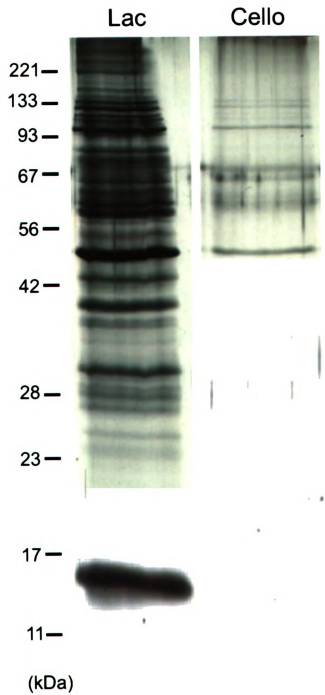
Figure 2: The effect of Lac on the gel filtration profile of galectins-1 and -3 in TF. The column and the conditions of chromatography are as described in the legend to Figure 1. In experiments testing the effect of Lac, the column was equilibrated and developed with TF containing 0.2 M Lac.

that, even in the presence of Lac, traces of galectin-3 could still be observed over a broad region of the column, ranging in molecular weight from <600 kD to ~60 kD (Fig. 2).

Affinity Chromatography of Transported Fraction on a Lactose Column

The exported material in TF was also subjected to affinity adsorption on Lac-agarose beads. After extensive washing, the bound material was eluted with Lac. This material revealed a very complex composition of polypeptides upon SDS-PAGE and silver staining (Fig. 3). The most prominent bands corresponded to polypeptides of : (i) ~15 kD; (ii) ~30 kD; (iii) ~40 kD; (iv) ~50 kD; (v) ~58 kD; and (vi) ~93 kD. With the exception of the ~50 kD band, none of these prominent bands can be found when TF was subjected to a parallel adsorption on cellobiose-agarose beads (Fig. 3). On the basis of the Lac-binding property of the galectin family members and on the basis of immunoblotting, galectins-1 and -3 can be identified. The other bands most probably represent polypeptides of a complex that have bound to the Lac beads through their association with either galectins-1 or -3. The complexity of the polypeptide profile precludes a detailed comparison. However, it should be noted that the pattern presented in Figure 3 (Lac affinity adsorption) is very similar to: (a) that obtained from fractions of the ~650 kD zone of the Sephacryl S-300 HR column; and (b) that observed when nucleoplasm of 3T3 fibroblasts is fractionated on an N-(e-aminocaproyl)-D-galactosamine-Sepharose column and eluted with galactose (Laing and Wang, 1988).

Figure 3: Affinity adsorption of the polypeptide components in TF on Lac-agarose and cellobiose-agarose. Parallel samples of 200 µl were incubated with 40 µl of Lac-agarose or cellobiose-agarose. The material bound to the beads were eluted with TB containing 0.2 M Lac, subjected to SDS-PAGE and silver staining. The numbers at the left indicate the positions of migration of molecular weight markers.


Lac
Cello
221
133
93
67
56
42
28
23
17
11
(kDa)

The gel filtration studies on TF in the presence and absence of lactose suggested that the binding of the disaccharide disrupted the high molecular weight complexes containing galectin-3 (Fig. 2). Therefore, the question may be raised as to how galectin-3-associated polypeptides can still be isolated on the basis of Lac-affinity adsorption (Fig. 3). One possible explanation may lie in our previous parenthetical note that, even in the presence of Lac, traces of galectin-3 could still be observed over a broad range of the gel filtration column, from <600 kD to ~60 kD (Fig. 2). This may reflect dynamic and continuous reequilibration of galectin-3 with the high molecular weight complex and it is on this basis that the Lac affinity beads can trap some of the associated polypeptides. It may also be worthy to note that the efficiency of galectin-3 isolation (amount of galectin-3 in the bound and Lac-eluted fraction as a percent of galectin-3 in the unfractionated material) from TF was much lower than the efficiency of adsorption of purified recombinant galectin-3 onto the same Lac-agarose matrix. One possible implication of this observation is that the affinity of galectin-3 for its saccharide ligand is decreased in complex formation. Alternatively, galectin-3 in TF may already be occupied with carbohydrate ligands with which Lac-agarose must compete for binding.

Effect of Vanadyl Cations on the Export of Galectin-3

The inhibition of galectin-3 export by the combination of 5' cap-like nucleotide and NES peptide had suggested the possibility that the lectin was co-exported with RNA(s) (Tsay *et al.*, 1997). The present finding that a significant portion of the exported galectin-3 can be found in a high molecular weight complex lends support to the

hypothesis. On the basis of previous reports documenting that VRC can stabilize RNA species from nuclease degradation (Berger and Birkenmeier, 1979), we tested the effect of VRC on the preservation of the galectin-3 high molecular weight complex in TF. To our surprise, we found that VRC (10 mM) was a potent inhibitor of nuclear export in our assay (Fig. 4A). The active ingredient appears to be vanadyl (VO^{2+}) cations, inasmuch as vanadyl sulfate (10 mM) also inhibited nuclear export but little or no inhibition was observed with a mixture of the four ribonucleosides (10 mM). The oxoanion of vanadium, vanadate (VO_3^-), did not show inhibition at the same concentration. Nor did other transition metal oxoanions, molybdate and tungstate (data not shown).

A comparison of the dose-response curves of inhibition showed that VRC was potent at concentrations as low as 5 mM (Fig. 4B) while strong inhibition was observed only with higher concentrations (10 mM or greater) of vanadyl sulfate (Fig. 4C). The conclusion that VRC was more effective than vanadyl sulfate in suppressing nuclear export can also be derived from immunofluorescence analysis for galectin-3 in the nuclear residue. The level of galectin-3 appeared to be higher in the nuclear residue of VRC (10 mM)-treated cells (Fig. 5D) than the corresponding cells treated with vanadyl sulfate (10 mM) (Fig. 5E). Surprisingly, the simultaneous application of VRC and vanadyl sulfate (10 mM each) resulted in even greater inhibition than either reagent alone (Fig. 5F). This suggests the possibility that the effects of the two reagents may be additive and thus may be acting via different pathways or different populations of molecules. Parenthetically, the inhibitory effect of vanadyl cations is more potent that

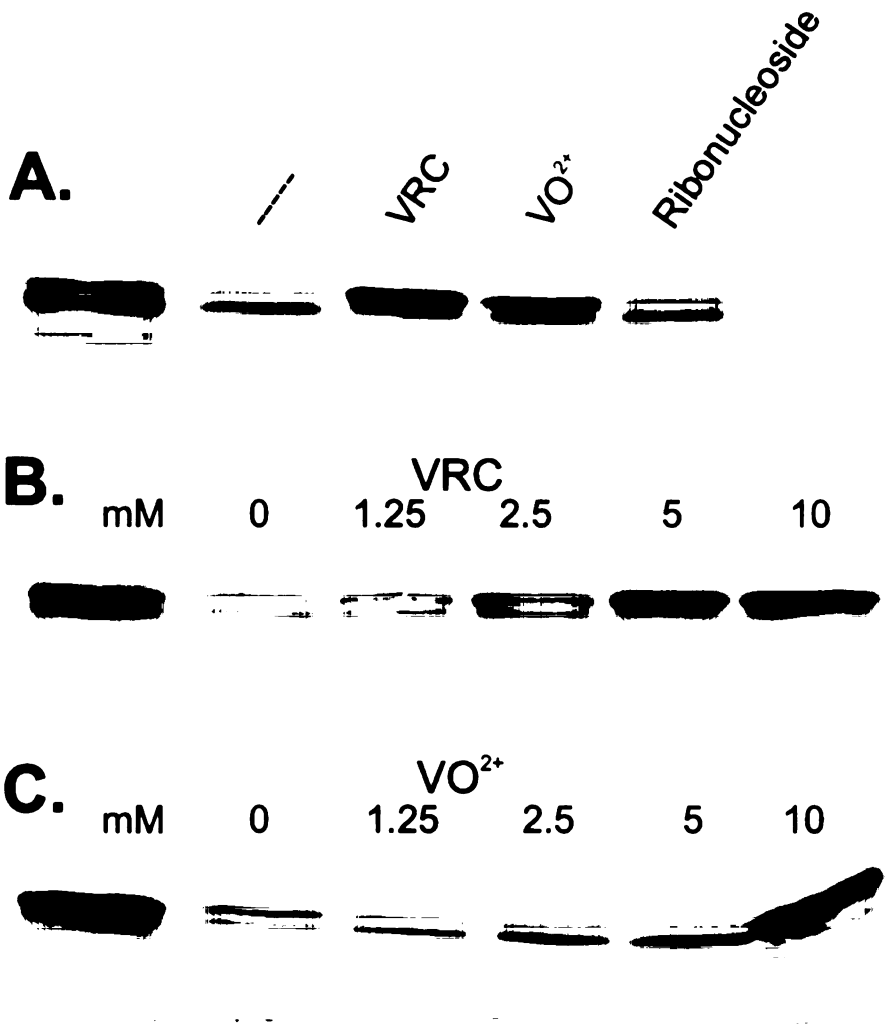


Figure 4: The effect of vanadyl cations (VO^{2+}) on the nuclear export of galectin-3, assayed by immunoblotting NF. In panels A-C, the first left hand lane represents NF at the beginning of the export assay. All the other lanes in each panel represent NF at the end of an 8-minute export period. Panel A: -, no addition; VRC, vanadyl ribonucleoside complex (10 mM); VO^{2+}, vanadyl sulfate (10 mM); Ribonucleoside, mixture of four ribonucleosides (2.5 mM each). Panels B and C: dose-response curves for VRC and Vanadyl sulfate, respectively.

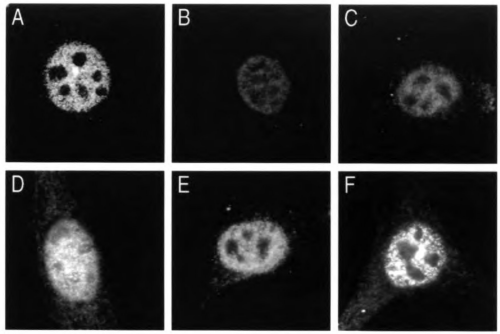


Figure 5: The effect of vanadyl cations (VO^{2+}) on the nuclear export of galectin-3, assayed by immunofluorescence analysis of NF. Galectin-3 was detected using rat anti-galectin-3 (250 ng.ml) and fluorescein-conjugated goat anti-rat immunoglobulin. A: NF at the beginning of the export assay. B-F: NF at the end of an 8-minute export period. B: no addition; C: wheat germ agglutinin (500 μg/ml); D: VRC (10 mM); E: vanadyl sulfate (10 mM); and F: VRC and vanadyl sulfate (10 mM each).

that of wheat germ agglutinin (Fig. 5C), a conclusion also corroborated by subjecting NF to Western blot analyses.

Finally, the inhibitory effect of vanadyl cations on nuclear export is not restricted to galectin-3; a similar set of results was also obtained by analyzing for Ran. Immunofluorescence analysis of NF at the beginning of the export assay (Fig. 6A) and after an 8-minute export period (Fig. 6B) showed that Ran was rapidly exported. This is in agreement with the immunoblotting data presented in the accompanying manuscript (Tsay *et al.*, 1997). To the best of our knowledge, the immunoblotting and immunofluorescence data together represent the first rigorous documentation that Ran is exported from the nucleus. Like that for galectin-3, the export of Ran was sensitive to inhibition by wheat germ agglutinin (Fig. 6C), by VRC (Fig. 6 D), and by vanadyl sulfate (Fig. 6E), with the strongest effect observed with a combination of VRC and vanadyl sulfate (Fig. 6F).

Analysis of RNA Components in the Transported Fraction

An export assay was carried out using 3T3 cells that had been metabolically labeled with [^{32}P]orthophosphate. The RNA components in TF collected from this assay were extracted, precipitated, and resolved on separate polyacrylamide and agarose gels. Autoradiography of the polyacrylamide gels revealed low molecular weight RNAs, with bands corresponding to: (i) ~80 nucleotides; (ii) ~100 nucleotides; (iii) ~300 nucleotides; (iv) ~650 nucleotides; (v) ~800 nucleotides; and (vi) >1000 nucleotides (Fig. 7A). With

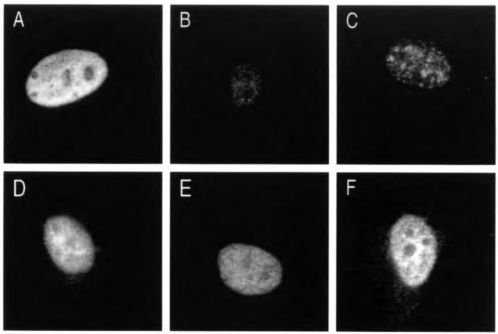
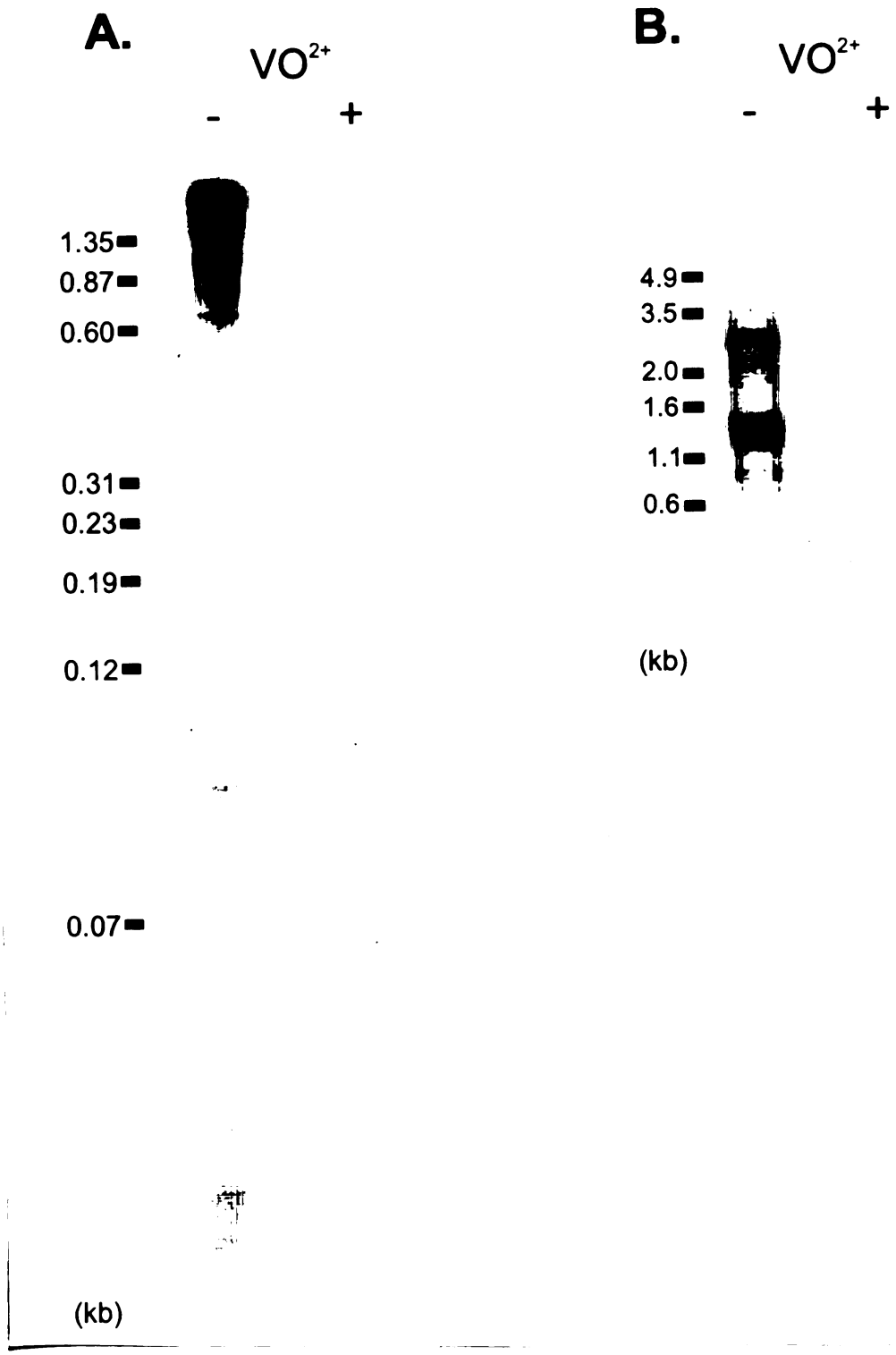


Figure 6: The effect of vandyl cations (VO^{2+}) on the nuclear export of Ran, assayed by immunofluorescence analysis of NF. Ran was detected using mouse anti-Ran (250 ng/ml) and fluorescein-conjugated goat anti-mouse immunoglobulin. A: NF at the beginning of the export assay. B-F: NF at the end of an 8-minute export period. B: no addition; C: wheat germ agglutinin (500 μg/ml); D: VRC (10 mM); E: vanadyl sulfate (10 mM); and F: VRC and vanadyl sulfate (10 mM each).

Figure 7: Gel electrophoretic analysis of the RNA components in TF when the export assay is carried out in the absence and presence of vanadyl sulfate. The export assay was carried out using 3T3 cells metabolically labeled with [^{32}P]orthophosphate and the RNA components of TF were isolated and resolved on an 8% polyacrylamide gel (panel A) or a 1.2% agarose gel (panel B). The radioactive RNA components were revealed by autoradiography. The numbers on the left indicate positions of migration of size markers in nucleotides (panel A) or in kb (panel B). In experiments testing the effect of vanadyl cations, the export assay was carried out in the presence of 10 mM vanadyl sulfate.


A.
VO^{2+}
-
+
1.35
0.87
0.60
0.31
0.23
0.19
0.12
0.07
(kb)
B.
VO^{2+}
-
+
4.9
3.5
2.0
1.6
1.1
0.6
(kb)

the exception of tRNA (75-85 nucleotides), the identities of the other small RNAs are not known. Autoradiography of the agarose gel revealed a smear ranging from 0.7 kb to ~5 kb, with prominent bands at ~ 1.2 kb and ~ 3 kb (Fig. 7B). An oligo dT probe was used to hybridize to the high molecular weight RNA; on this basis, RNA species ranging from 1 kb to ~5kb can be identified as poly(A)$^+$ mRNA (data not shown). Therefore, it appears that both low molecular weight RNA, as well as mRNA, are exported in our assay and can be collected in TF.

We have made several attempts to delineate the RNA species associated with the high molecular weight galectin-3 complex, using either Lac affinity adsorption or immunoprecipitation. Due to technical difficulties that we have yet overcome, however, we have been unsuccessful in isolating and identifying the RNA components of this complex. By comparing the level of radioactivity and the RNA species in the unbound fractions the Lac-beads or anti-galectin-3-beads with the corresponding unbound fractions of control beads (e.g. cellobiose-beads), we have ascertained that radioactive RNA is indeed bound to those beads that should trap galectin-3. Thus, what remains is the delineation of the number and identity of the RNA species.

Just as we had demonstrated for the export of galectin-3 (and Ran) in terms of polypeptides, vanadyl cations also inhibited the export of RNA in our assay. The transport of both low molecular weight RNA species (Fig. 7A), as well as high molecular weight mRNA (Fig. 7B), were suppressed.

DISCUSSION

Taking advantage of the fact that the material in TF of the digitonin-permeabilized nuclear export assay can be collected in a soluble form, we have carried out initial characterization of the components of TF. The key findings of the present study include: (a) gel filtration of TF and analysis for galectin-3 showed that the lectin is associated with at least two high molecular weight complexes (~650 kD and ~60 kD); (b) both complexes are sensitive to disruption by the presence of the galectin ligand, Lac; (c) the polypeptide composition of the high molecular weight galectin-3 complex appears to be very complicated, with prominent bands of about 15 kD, 30 kD, 40 kD, 50 kD, 58 kD, and 90 kD; (d) TF also contains RNA, including small nuclear RNAs, as well as poly A^+ mRNA; and (e) vanadyl cations (VO^{2+}) inhibit the export of galectin-3 and the RNAs.

These results provide new levels of information to two important previous observations. First, galectin-3 has been identified as a required factor in the splicing of pre-mRNA, assayed in a cell-free system (Dagher *et al.*, 1995; Vyakarnam *et al*, 1997). Saccharides such as Lac that bind to galectin-3 with high affinity inhibit the splicing reaction. The present finding that the high molecular weight galectin-3 complex is disrupted by Lac may provide a mechanism for the effect of the saccharide in the splicing reaction. In both the inhibition of splicing and in the disruption of the high molecular weight galectin-3 complex, it still remains to be determined whether Lac is competing

with a glycoconjugate ligand for the carbohydrate-binding site of galectin-3 or it is inducing a conformational change upon binding to the galectin-3 polypeptide. Using differential scanning calorimetry, it has in fact been demonstrated that Lac binding to galectin-3 induces a conformation change in the polypeptide (Agrwal *et al.*, 1993). Coupled with our failure, despite various attempts, to identify a glycoconjugate ligand for galectin-3 in the nuclear fraction, we tend to favor the notion that a conformational change in the galectin polypeptide upon Lac binding dissociates the protein from its complex. This may, in turn, lead to loss of splicing activity.

Second, we had documented, in the accompanying manuscript, that the nuclear export of galectin-3 was sensitive to inhibition by a combination of a nucleotide mimicking the 5' cap structure of mRNAs and of a peptide bearing the NES from PKI. Coupled with its required role in the splicing reaction, the results suggested that galectin-3 may be exported in the form of an RNP, with many polypeptides bound directly or indirectly with an RNA species. Thus, signals that facilitate nuclear export in both the 5' cap structure of the RNA species and the peptide sequences of the component polypeptides needed to be neutralized before inhibition of export could be observed. Indeed, we found in the present study that gel filtration of the exported material yielded galectin-3 in at least two sets of high molecular weight complexes. It should be noted that although Lac disrupts the galectin-3 complex(es), the saccharide actually has little or no effect on the rate of the protein's export from the nucleus (Y.G. Tsay, unpublished observations). Even in the presence of Lac, however, traces of galectin-3 could be observed over a broad range of the gel filtration column (from <600 kD to ~60 kD) and

these putative complexes may continue to serve the role of providing multiple signals for nuclear export.

Although we have obtained some information on the complexity of the polypeptide composition of the galectin-3-associated particle and although we can document that TF contains RNA, we have yet to delineate the number and identity of the RNA species. If galectin-3 is indeed exported in the form of an RNP, then it would join two other pre-mRNA splicing factors that accompany their RNA substrates out of the nucleus. Both hnRNP A1 (Mayeda *et al.*, 1992; Caceres *et al.*, 1994) and the cap-binding complex that recognize the monomethylated guanosine cap structures at the 5'-end of RNA polymerase II transcripts (Izaurralde *et al.*, 1994; Lewis *et al.*, 1996) travel out of the nucleus with mRNAs (Visa *et al.*, 1996a and Visa *et al.*, 1996b). Once out of the nucleus, whether the RNA stays with galectin-3, as is with hnRNP A1 (Visa *et al.*, 1996b), or the splicing factor is dissociated from the RNP, as is with the cap-binding complex (Visa *et al.*, 1996a), are issues that need to be studied.

In the course of the present study, we found that vanadyl cations strongly inhibited the nuclear export of both galectin-3 and RNAs. However, no effect could be demonstrated with vanadate, molybdate, or tungstate. This is to be contrasted to the stimulatory effect of these oxyanions on the nuclear export of the glucocorticoid receptor (Yang *et al.*, 1997). Vanadium exhibits complex chemistry, fluctuating between vanadyl (oxidation state +4) and vanadate (oxidation state +5), with possible reduction-oxidation interconversions (Macara *et al.*, 1980; Li *et al.*, 1996). Over the short time course of our

export assays, however, the inhibitory effect on galectin-3 export appears to be restricted to compounds that give rise to vanadyl cations (VRC, vanadyl sulfate) whereas no such effects were observed with vanadates. This is consistent with recent observations that while both vanadyl cations and vanadate oxyanions can target protein phosphotyrosine phosphatases, distinct vanadate-independent and vanadyl-dependent pathways can be dissected (Li *et al.*, 1996).

Beside protein phosphotyrosine phosphatases, another set of targets susceptible to inhibition by ribonucleoside-vanadyl complexes are nucleases (Berger and Birkenmeier, 1979). Thus, it seems possible that VRC indirectly affects galectin-3 export through inhibition of a ribonuclease(s). This enzyme may have specifically digested an RNA that anchors galectin-3-containing RNPs to the nuclear structure. Galectin-3 export is not allowed, when the anchor remains intact due the inhibition of the nuclease. This notion is consistent with our previous observation that association of galectin-3 with nuclear structure was sensitive to treatment with ribonuclease (Laing and Wang, 1988; Wang *et al.*, 1995). Since VRC has been shown to have similar inhibitory effects on nuclear export of Ran and galectin-1, if this peculiar mechanism does exist, it may also regulate the export of Ran and galectin-1. So far, crucial evidence is still lacking and this possibility awaits further investigation.

CONCLUSIONS

In this manuscript, we demonstrate that galectin-3 has nuclear shuttling activity; i.e., it can move between the nucleus and cytoplasm bidirectionally. The nucleocytoplasmic distribution of galectin-3 is , in fact, a net result of counteraction between nuclear import and export. When nuclear import is predominant, this will lead to nuclear localization. In contrast, when nuclear export dominates over import, cytoplasmic localization will be seen.

As a pre-mRNA splicing factor, galectin-3 shows differentiation localization as a function of proliferative states of cells, which distinguishes galectin-3 from other splicing factors. The major implication is that the splicing process may be modulated by nuclear accumulation of galectin-3. In conjunction with the fact that galectin-3 can shuttle between the nucleus and cytoplasm, it is possible that nuclear import may serve as a mechanism that upgrades RNA splicing activities, and nuclear export is a mechanism that down-regulates RNA splicing activities.

On the other hand, we also report that galectin-3 appears to be exported in complexes that contain RNAs capped with mGpppA dinucleotide and polypeptides bearing NES signals. This finding appears to suggest that galectin-3, along with other components in these complexes, may participate in RNA metabolism or other cellular processes subsequently. In other words, nuclear export of galectin-3 is not only a way to

turn off nuclear activities, but also a mechanism to *turn on* certain cytoplasmic activities. It will be interesting to study the fate of these ribonucleoprotein complexes in cytoplasm as well to explore the roles of galectin-3 in the process.

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