Characterization of the Nuclear Import Pathways of Galectin-3

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Abstract
Galectin-3 (Gal-3), a pleiotropic β-galactoside–binding protein, was shown to be involved in several nuclear-dependent functions, including up-regulation of transcriptional factors, RNA processing, and cell cycle regulation. Gal-3 compartmentalization in the nucleus versus the cytoplasm affects, in part, the malignant phenotype of various cancers. However, to date, the mechanism by which Gal-3 translocates into the nucleus remains debated. Thus, we have constructed and expressed a variety of fusion proteins containing deletion mutants of Gal-3 fused with monomers, dimers, and trimers of enhanced green fluorescent protein and searched for the Gal-3 sequence motifs essential for its nuclear localization in vitro. In addition, a digitonin-permeabilized, cell-free transport in vitro assay was used to directly examine the mechanism of Gal-3 nuclear import. Partial deletions of the COOH-terminal region (114-250) of the human Gal-3 significantly decreases its nuclear translocation, whereas a peptide (1-115) was transported to the nuclei. The in vitro nuclear import assay revealed that there are at least two independent nuclear pathways for shuttling Gal-3 into the nucleus: a passive diffusion and an active transport. This is the first article providing direct evidence for the nuclear import mechanisms of Gal-3 and suggests that Gal-3 nuclear translocation is governed by dual pathways, whereas the cytoplasmic/nuclear distribution may be regulated by multiple processes, including cytoplasmic anchorage, nuclear retention, and or nuclear export. These results may lead to the development of a therapeutic modality aiming at abrogating Gal-3 translocation into the nucleus and thus hampering its activity during cancer progression and metastasis. (Cancer Res 2006; 66(20): 9995-10006)

Introduction
Galectins are an evolutionarily conserved family of β-galactoside–binding proteins that exhibit diverse biological activities (1, 2). These proteins contain at least one carbohydrate recognition domain (CRD) of ~130 amino acid residues with significant sequence homology and are categorized into three major groups: the prototype, the chimera type, and the tandem repeat type (3). Galectin-3 (Gal-3), the sole chimera type of galectin, is expressed ubiquitously and is involved in cell proliferation, cell-cell interaction, and cell-matrix adhesion mainly through binding to glycoproteins (2, 4, 5). In particular, nuclear Gal-3 is presumably associated with normal cell proliferation because it is a required splicing factor interacting with Gemin4 (5, 6). In cancer, the loss of nuclear Gal-3 expression is associated with tumor progression of colon, prostate, and squamous carcinoma cells of the tongue carcinoma (7–9). In lung carcinoma, the specific expression of nuclear Gal-3 is a predictive factor of recurrence and/or a worse clinical outcome (10, 11). In patients with esophageal squamous cell carcinoma, elevated expression of Gal-3 in the nuclei is an important biological variable related to histologic differentiation and vascular invasion (12). In an experimental prostate cancer model system, nuclear Gal-3 suppressed malignancy whereas cytoplasmic Gal-3 promoted tumorigenicity (13). In the nuclei of papillary thyroid cancer cells, Gal-3 interacts with the thyroid-specific TTF-1 transcription factor and up-regulates its transcriptional activity, contributing to the proliferation of the thyroid cells (14). In human breast epithelial cells, Gal-3 translocation into the nucleus exerts a growth-promoting effect by the induction of cyclin D1 expression through enhancement or stabilization of the nuclear protein-DNA complex formation of the cyclin D1 promoter (15). Gal-3 is a novel binding partner regulating β-catenin shuttling to the nucleus, and the Gal-3-β-catenin complex activates Tcf-reporter activity and stimulate cyclin D1 and c-myc (16, 17).

Despite the above, the mechanism of how Gal-3 is imported to the nucleus remains unclear, as it lacks classic nuclear localization signal (NLS; refs. 18, 19), whereas the nuclear export mechanism of Gal-3 is better characterized (20). Previously, it was reported that deletion of the first 11 amino acid residues of Gal-3, which contains a serine phosphorylation site, leads to a predominant cytoplasmic distribution (21), whereas others showed that an NH2-terminal deletion mutant of hamster Gal-3 could be localized in the nucleus even when up to 103 amino acid residues were deleted (22). The COOH-terminal deletion mutant of mouse Gal-3 lost its nuclear localization, suggesting that Gal-3 nuclear import was dependent on the IXT type NLS residing in the end of COOH-terminal region (23). Here, we show that the deletion mutant of the last 10 amino acids from the COOH terminus of the human Gal-3 (1-240) resulted in the abrogation of nuclear accumulation, whereas deletion mutant of the entire CRD (1-115) did not. The in vitro analyses provide direct evidence that Gal-3 can migrate into the nucleus by at least two distinct pathways: either by passive diffusion or by an active transport system.

Materials and Methods

Cell culture.
COS-7 and HeLa cells were purchased from American Type Culture Collection (Manassas, VA). The human breast cancer BT549 Gal-3 null cell line and culture conditions are as described previously (16).

Plasmid construction for expression in mammalian cells. To create the constructs for monomer enhanced green fluorescent protein (EGFP; ×1GF) fused Gal-3 and its deletion derivatives, the following frames were amplified by a PCR using Pfu turbo (Stratagene, La Jolla, CA) and pBK-CMV/Gal-3 (21) as a template with a set of appropriate primers [a 3′ primer for aa 1-250 (wild type, 1-250/G), 5′-CAGGATCCATCTGATGTAATGGA-3′; a 3′ primer for aa 1-115 (1-115/G), 5′-GGTACCACTACTATCCCGC-3′; a 3′ primer for aa 1-135 (1-135/G), 5′-GGGTACCGAGATCTATCC-3′;
a 3′ primer for aa 1-150 (1-150/G), 5′-GGGGATCCCTGGAAGACTAAGGA-3′; a 3′ primer for aa 1-175 (1-175/G), 5′-GGGATCCCTGGAAGACTAAGGA-3′; a 3′ primer for aa 1-200 (1-200/G), 5′-GGGATCCCTGGAAGACTAAGGA-3′; a 3′ primer for aa 1-222 (1-222/G), 5′-GGGATCCCTGGAAGACTAAGGA-3′; a 3′ primer for aa 1-229 (1-229/G), 5′-GGGATCCCTGGAAGACTAAGGA-3′; a 3′ primer for aa 1-240 (1-240/G), 5′-GGGATCCCTGGAAGACTAAGGA-3′; a 3′ primer for aa 1-245 (1-245/G), 5′-GGGATCCCTGGAAGACTAAGGA-3′; a 3′ primer for aa 1-250 (1-250/G), 5′-GGGATCCCTGGAAGACTAAGGA-3′; and then subcloned into pEGFP-N3 vector (BD Bioscience, Palo Alto, CA) after digesting with BamHI or KpnI and EcoRI. For the constructs of wild-type Gal-3 and its deletion mutant was inserted into pEGFP-N3 vector (BD Bioscience, Palo Alto, CA) after digesting with BamHI or KpnI and EcoRI. For the constructs of wild-type Gal-3 fused with x1EGFP in a GST fusion system (pGEX/Gal-3/GFP), the fragment from 1-250/G vector cut with EcoRI/NotI was subcloned into the vector pGEX-6p-3. For the construction of x1EGFP in a GST fusion system (pGEX/x1EGFP), the larger fragment from pGEX/Gal-3/GFP cut with BamHI were self-ligated. To construct the expression vectors of the pGEX/1-115/x2EGFP and pGEX/139-193/x2EGFP, the fragments from (139-193/×3G) cut with EcoRI/NotI and from (1-115/×3G) cut with BamHI were subcloned into the vectors pGEX-6p-3 and pGEX/x1EGFP, respectively, in the sense direction. The expression plasmids of pGEX/CAN(NG-F repeat) were derived from CAN/Nup214 was constructed as described previously (25).

**Plasmid construction for expression in bacteria.** To construct the expression vectors for wild-type Gal-3 in a glutathione S-transferase (GST) gene fusion vector pGEX-6p-2 (Amersham, Piscataway, NJ) was described previously (24). This vector is called the pGEX/Gal-3. To create the constructs for wild-type Gal-3 fused with x1EGFP in a GST fusion system (pGEX/Gal-3/GFP), the fragment from 1-250/G vector cut with EcoRI/NotI and then subcloned into pEGFP-N3 vector (BD Bioscience, Palo Alto, CA) after digesting with BamHI or KpnI and EcoRI. For the constructs of wild-type Gal-3 fused with x1EGFP in a GST fusion system (pGEX/x1EGFP), the larger fragment from pGEX/Gal-3/GFP cut with BamHI were self-ligated. To construct the expression vectors of the pGEX/1-115/x2EGFP and pGEX/139-193/x2EGFP, the fragments from (139-193/×3G) cut with EcoRI/NotI and from (1-115/×3G) cut with BamHI were subcloned into the vectors pGEX-6p-3 and pGEX/x1EGFP, respectively, in the sense direction. The expression plasmids of pGEX/CAN(NG-F repeat) were derived from CAN/Nup214 was constructed as described previously (25).

**Transfection.** To express the proteins in cultured cells, a variety of plasmids for Gal-3 deletion mutants were transfected into COS-7 and BT549 cells using Lipofectamine 2000 reagent (Invitrogen). Transfection mixtures were prepared according to the instructions of the manufacturer. Leptomycin B (LMB; Sigma, St. Louis, MO) was used in part of the experiments at concentration of 30 ng/mL for 6 hours at 37°C after transfection.

**Western blotting.** To confirm the protein expression, the plasmid-transfected COS-7 cells were collected and processed as described (26).

**Fluorescence microscopy.** The fluorescence of the plasmid-transfected cells, cells on the chamber slide were fixed with 4% paraformaldehyde in PBS for 20 minutes after 24 hours after transfection. The cells were washed with PBS and counterstained with 5 μg/mL Hoechst 33258 (Molecular Probes, Eugene, OR). After washing extensively with PBS, the cells were mounted in 80% glycerol and analyzed using an Olympus BX60 microscope and MCID software.

**In vitro nuclear import assay.** Digitonin-permeabilized HeLa cells were prepared as described previously (27-29). The reaction for nuclear import assay was done in 10 μL testing solution containing the substrates dissolved in transport buffer [20 mmol/L HEPES (pH 7.3), 110 mmol/L potassium acetate, 2 mmol/L magnesium acetate, 5 mmol/L sodium acetate, 0.5 mmol/L EGTA, 2 mmol/L EDTA, 1 μg/mL aprotinin, leupeptin, and pepstatin A]. The composition and concentration of the recombinant proteins are described in each figure legend. Where indicated, an ATP-regenerating system (1 mmol/L ATP, 5 mmol/L phosphocreatine, 20 units creatine kinase; Sigma) and/or cytosol prepared from HeLa cells were included in the above 10 μL testing solution. For wheat germ agglutinin (WGA) or antibody treatment, 2 μg WGA (Sigma) or 2 μg purified anti-Gal-3 polyclonal antibody was contained in the testing solution. The import reaction was done for 30 minutes at 30°C or 4°C, and the cells were then washed with ice-cold transport buffer and fixed with 4% paraformaldehyde/2% sucrose/PBS for 20 minutes at room temperature, followed by staining with Hoechst 33258 (Molecular Probes).

**In vitro binding assay.** We have followed the protocol as described previously (25).

**Results**

**Subcellular localization of wild-type and mutated Gal-3 EGFP-fused proteins.** To determine the region of Gal-3 necessary and sufficient for its nuclear localization in vivo, a variety of Gal-3...
deletion polypeptides were constructed and expressed in mammalian cells (Fig. 1A). The cDNAs were tagged with EGFP (see Materials and Methods) to enable the subcellular visualization of the proteins following transfection into COS-7 cells. To confirm the correct expression of the deletion mutants of EGFP-fused Gal-3, Western blotting of the transfected COS-7 cell lysates was done using antibodies directed against GFP and Gal-3 (Fig. 1B). All of the Gal-3 mutant plasmids expressed proteins of the predicted molecular weights as shown in the GFP Western blotting of Fig. 1B, I, with identical molecular weight bands revealed by the Western blotting of Gal-3, indicating correct expression of mutated/deleted Gal-3 polypeptides (Fig. 1B, II). Next, after

**Figure 1.** Subcellular localizations of wild-type or deletion derivatives of Gal-3 fused with monomer EGFP. A, schematic diagram of full-length human Gal-3 (aa 1-250) and the truncated mutants used for this study. The CRD of Gal-3 (aa 118-250) consists of two ε-pleated sheets: one of them contains six anti-parallel strands (S1-6, gray), whereas the other contains five strands (F1-5, gray). An α-helix (black) connects these ε-sheets. Right, relative nuclear localization ratio of each fusion protein. B, deletion derivatives of Gal-3 fused with EGFP identified by Western blotting of transiently transfected COS-7 cells. The proteins were probed with anti-polycional GFP (I) and anti-monoclonal Gal-3 (II) antibodies. Left, molecular standards (M), Lane 1, 1-250/G; lane 2, 1-245/G, lane 3, 1-240/G; lane 4, 1-222/G; lane 5, 1-200/G; lane 6, 1-175/G; lane 7, 1-150/G; lane 8, 1-135/G; lane 9, 1-115/G; lane 10, Δ136-238; lane 11, Δ230-238; lane 12, G/114-250; lane 13, x2GFP; lane 14, x1GFP. C, subcellular localization of wild-type and deletion derivatives of Gal-3 expressed as EGFP-fusion proteins in COS-7 cells. COS-7 cells were transiently transfected with the plasmids depicted in (A) and observed 24 hours after transfection by fluorescence microscopy. Each image is representative of the majority of the cells observed in several fields. Magnification, ×400. a to l, fluorescent signals of the cells expressing EGFP-labeled proteins; m to x, nuclear DNA stained with Hoechst 33258 of the cells in the same views as in (a-l), respectively.
establishing expression of the Gal-3 polypeptides, their subcellular distribution in COS-7 cells was analyzed under fluorescence microscopy 24 hours after transfection; following fixation and permeabilization, the cells were counterstained with Hoechst 33258 to clearly define the nuclear areas. A representative image of the subcellular localization for each mutant of Gal-3 in transfected COS-7 cells is depicted in Fig. 1C. Because some of the peptide expression patterns were heterogeneous, their distribution were categorized as follows: predominantly localized in the nucleus (N > C), equally localized throughout the nucleus and the cytoplasm (N = C), mostly localized in the cytoplasm, or spotted around the nucleus (N < C).

Wild-type Gal-3 EGFP-fused protein (1-250/G) and its dimer protein (×2GFP) are randomly distributed between the nucleus and the cytoplasm, and the cytoplasmic compartments of >70% and >80% of transfected cells, respectively, whereas the fluorescent intensity of 1-250/G in the nucleus was clearly higher than the one of ×2GFP (Fig. 1A, a, b), indicating that Gal-3 translocated to the nucleus. In COS-7 cells transfected with a plasmid encoding 1-245/G, the nuclear fluorescent in the nucleus was decreased compared with 1-250/G and was localized in the cytoplasm in distinct patchy-like foci in ~23% of the transfected cells (Fig. 1G, c). Additional deletion of five amino acids (1-240/G) resulted in a complete abrogation nuclear staining associated with high intensity in a patchy pattern throughout the cytoplasm (Fig. 1C, d) typical for >95% of the transfected cells. In COS-7 cells transfected with the plasmid of 1-222/G, similar fluorescent speckles were observed in the perinuclear region of 99% of the transfected cells (Fig. 1C, e). However, the distribution expression pattern of the other deletion mutant proteins, e.g., 1-200/G, 1-175/G, 1-150/G, or 1-135/G, in which a partial CRD region of Gal-3 still remained, showed two opposite cellular distributions: one is the pattern of predominant nuclear localization and the other is entire cytoplasmic localization (Fig. 1C, f, g). On the other hand, the 1-115/G polypeptide, devoid of the CRD, was found to exhibit the highest affinity to the nucleus, but not the nucleoli (Fig. 1C, h), typical for ~85% of the transfected cells. Of note, the monomer EGFP (×1GFP) was found to be randomly diffusely distributed throughout the cell, including the nucleoli (Fig. 1C, i), implying that the first 115 amino acids of Gal-3 harbor a motif that regulates its nuclear transport. In contrast, deletion of complete NH₂-terminal region of Gal-3 and fusing it with EGFP (G/(114-250)) resulted in a peptide that distributed throughout the cell, including the nuclear area of >70% of the transfected cells (Fig. 1C, I), indicating that Gal-3 can be translocated into the nucleus without its NH₂-terminal region as described previously (22). These results may suggest that Gal-3 can be imported into the nucleus only with its COOH-terminal region (CRD). In particular, the last 10 amino acids (241-250) are required for its nuclear localization. Next, to address the possibility that the last 10 amino acids include the signal sequence for Gal-3 nuclear localization, we created two constructs for Gal-3 deletion mutants, in which the nucleotides for 136 to 238 and 230 to 238 amino acids of Gal-3 were deleted (Fig. 1A; Δ136-238, Δ230-238), and expressed these plasmids correspondingly. As shown in Fig. 1C, k, l, the expression patterns of these proteins were similar to that of 1-135/G and 1-222/G, respectively, suggesting that the terminal 12 amino acids were not involved in the nuclear transport of Gal-3.

It might be argued that the above results might be due to differences in the diffusion coefficient rather than active transport, because 1-250/G or ×2GFP (~60 kDa) polypeptides are small enough to be distributed throughout the nucleus and cytoplasm by passive diffusion (30–32). Therefore, to identify the region responsible for the NPC passage of Gal-3 protein, deletion derivatives of Gal-3 were constructed and fused with dimer or trimer of EGFP (×2GFP ×3GFP) as depicted in Fig. 2A. Western blotting of the transfected COS-7 cell lysates was done using antibodies to GFP (Fig. 2B, I) and Gal-3 (Fig. 2B, II). All Gal-3 mutant plasmids expressed proteins with the expected molecular mass (Fig. 2B, I, II). Thereafter, COS-7 and BT549 cells were transfected with these plasmids, and the distribution of the EGFP-fused Gal-3 deletion mutants was analyzed as described above. The representative pattern of each mutant in transfected COS-7 cells is shown in Fig. 2C.

The expression of ×3GFP as well as ×4GFP was distributed in the cytoplasm (Fig. 2C, a, b). This pattern was typical for ~46% and 80% of the transfected COS-7 cells, respectively. The wild-type Gal-3 fused with ×2GFP (1-250/×2G), of which the molecular mass is almost same as ×3GFP, was localized throughout the cell, including the nuclear area in >67% of the transfected COS-7 cells (Fig. 2C, c, d), implying that Gal-3 was transported into the nucleus by an active transport system and not simply by a passive diffusion. The expression of wild-type Gal-3 fused with ×3EGFP (1-250/×3G) could be detected in the cytoplasm in a patchy vesicular pattern and weakly in the nucleus in ~88% of the transfected COS-7 cells (Fig. 2C, e), whereas the 1 to 115 amino acid peptide fused with ×3EGFP (1-115/×3G) was localized predominantly in the nucleus (Fig. 2C, f) of >30% of the transfected cells, supporting the suggestion of an active involvement of the NH₂-terminal region in Gal-3 nuclear transport. In contrast, a peptide constructed from the 114 to 250 amino acids of Gal-3 fused with ×3EGFP (114-250/×3G) remained entirely in the cytoplasm of transfected COS-7 cells (Fig. 2C, f). Similarly, the expression of 139 to 250 amino acid peptides of Gal-3 fused with ×3EGFP (139-250/×3G, 239-250/×3G) were diffused throughout the cytoplasm (Fig. 2C, g, h). These predominant cytoplasmic patterns are typical for ~50% of the transfected cells. However, the expression pattern of 139 to 193 amino acid peptide of Gal-3 fused with ×3EGFP (139-193/×3G) showed a nuclear localization (Fig. 2C, i) in 34% of the transfected cells. In the case of expression in BT549 cells, distribution patterns were similar to the results in COS-7 cells (not shown). These results indicate that Gal-3 is actively translocated into the nucleus.

Conjugation of SV40 T-antigen NLS can rescue the nuclear accumulation of impaired Gal-3. Gal-3 does not contain a consensus NLS, needed for the importin-mediated nuclear import pathway (18, 19). Previously, it was reported that Gal-3 may be localized in the nucleus following insertion into pCMV/myc/muc vector, which is designed for specific expression of the protein in the nucleus (13). Therefore, we questioned the nuclear translocation of impaired Gal-3, such as 1-222/G or 1-250/×3G, following conjugation to the classic NLS and constructed plasmids containing SV40 T-antigen NLS sequence as depicted in Fig. 3A, transfected them, and followed their expression in COS-7 cells. Western blotting revealed that all transfected COS-7 cell lysates used expressed proteins with the predicted molecular mass (Fig. 3B). A representative expression pattern of the proteins in transfected COS-7 cells is depicted in Fig. 3C and the relative proportion of the distinct subcellular localization was calculated and plotted in Fig. 3D.

As expected, the expression of ×2GFP with SV40NLS (NLS+×2GFP) was exclusively restricted to the nucleus (Fig. 2C, a), in >95% of the transfected cells (Fig. 2D), indicating that this fusion protein contains a bona fide NLS and may be used in...
confidence to analyze the Gal-3 peptides. The expression of all Gal-3 derivatives fused with dimer or trimer of EGFP, A, schematic diagram of full-length human Gal-3 (aa 1-250) and the deletion mutants used for this study. Right, relative nuclear localization ratio of each fusion protein. B, deletion derivatives of Gal-3 fused with dimer or trimer of EGFP identified by Western blotting of transiently transfected COS-7 cell lysates. The proteins were probed with anti-polycional GFP (I) and anti-polycional Gal-3 (II) antibodies. Left, molecular standards (M). Lane 1, ×3GFP; lane 2, ×4GFP; lane 3, 1-250/×2G; lane 4, 1-250/×3G; lane 5, 1-115/×3G; lane 6, 139-250/×3G; lane 7, 239-250/×3G; lane 8, 114-250/×3G; lane 9, 139-193/×3G. C, subcellular localization of wild-type and deletion derivatives of Gal-3 expressed as EGFP-fusion proteins in COS-7 cells. COS-7 cells were transiently transfected with the plasmids depicted in (A) and observed 24 hours after transfection by fluorescence microscopy. Each image is representative of the majority of the cells observed in several fields. Magnification, ×400. a to i, fluorescent signals of the cells expressing EGFP-labeled proteins; j to r, nuclear DNA stained with Hoechst 33342 of the cells in the same views as in (a-i), respectively.

Nuclear import of Gal-3 in digitonin-permeabilized cells. To provide a direct evidence for the Gal-3 nuclear import pathway of Gal-3, we have adopted the digitonin-permeabilized, cell-free transport assay (27, 28), a methodology commonly used to establish the mechanism of nuclear import of nucleoproteins like β-catenin, importin-β, and mitogen-activated protein kinase (MAPK; refs. 25,
Initially, we tested whether the in vitro assay system would accurately function when using well-characterized substrates, such as GFP-fused SV40 T-antigen NLS polypeptides (GST/NLS/GFP) or importin-β (GFP/imp-β; refs. 25, 26, 29, 33). In addition, monomer EGFP was used as a positive control substrate and fluorescence-conjugated bovine serum albumin (F/BSA) was used as a negative control substrate for passive diffusion (25, 26). The complete in vitro assay system consisted of digitonin-permeabilized HeLa cells, cytosol extracts of HeLa cells ("cytosol"), and the ATP-regenerating system ("ATP"). Digitonin-permeabilized HeLa cells were incubated in the testing solution (10 μL), including the import substrate with or without cytosol, ATP, and WGA, an inhibitor of molecules passing through the NPC, for 30 minutes at 30°C. At the end of incubation, cells were fixed and stained with Hoechst 33258 to define the nuclear areas.

As reported previously (26, 33), GST/NLS/GFP can be imported into the nucleus under these conditions (Fig. 4A, a), whereas either depletion of cytosol or the addition of WGA abrogates its nuclear accumulation (Fig. 4A, g, m) and confirmed that GFP/imp-β can be translocated into the nucleus irrespective of cytosol depletion, and can be inhibited by the addition of WGA (Fig. 4A, h, n). We also confirmed that the addition of cytosol did not stimulate but rather inhibit import of GFP/imp-β as reported previously (Fig. 4A, b). GFP can migrate into the nucleus even when WGA was added in the testing solution (Fig. 4A, c, i, o), as reported previously (25). F/BSA was not fully imported into the nucleus under any of the tested conditions (Fig. 4A, f, l). Thus, the in vitro nuclear assay system used here mimics those reported previously (25, 26, 29, 33). Thereafter, EGFP-fused Gal-3 (Gal-3/GFP) and fluorescence-labeled Gal-3 (F/Gal-3) were tested in this in vitro nuclear import assay. However, both Gal-3/GFP and F/Gal-3 accumulated on the cell surface with or without cytosol and never migrated into the nucleus under these conditions (Fig. 4A, d, e, j, k). This accumulation of exogenous Gal-3 on the cell surface implies that...
the binding of Gal-3 to the cell surface glycoprotein receptors inhibit its free diffusion. Thus, we added a specific Gal-3 sugar-binding inhibitor, i.e., lactose, to the reaction mixture (50 mmol/L) and repeated the *in vitro* assay. In this modified condition, both GFP/Gal-3 and F/Gal-3 were able to effectively migrate into the nucleus (Fig. 4B, c, d), whereas the addition of the control sugar, i.e., sucrose (50 mmol/L), did not change the distribution pattern (Fig. 4B, a, b). Moreover, GFP/Gal-3 could not efficiently accumulate in the nucleus without cytosol (Fig. 4B, g) and its nuclear accumulation was completely inhibited by addition of WGA (Fig. 4B, i), whereas F/Gal-3 could freely migrate into the nucleus without cytosol even when WGA was added in the testing solution (Fig. 4B, h, j). These results suggest that wild-type Gal-3 can either migrate into the nucleus by passive diffusion while maintaining an active transport mechanism for its NPC passage. Because F/Gal-3 can migrate into the nucleus in the presence of WGA (Fig. 4B, j), it is reasonable to assume that wild-type Gal-3 can enter the nucleus, in part, by passive diffusion. To confirm this, we examined whether F/Gal-3 and Gal-3/GFP enter the nucleus by performing the *in vitro* assay at 4°C, whereby the active nuclear import pathway is...
completely inhibited (26, 33), and added 50 mmol/L lactose to the reaction mixture that was incubated at 4°C for 30 minutes. GFP was used as a positive control and GFP/import-β was used as a negative control. Consistent with the result of the WGA inhibition, F/Gal-3 (Fig. 4C, c) and GFP (Fig. 4C, a) were able to diffuse into the nucleus at 4°C, whereas Gal-3/GFP (Fig. 4C, b) and GFP/import-β did not (data not shown). This nuclear accumulation of F/Gal-3 is effectively blocked by purified polyclonal antibody against Gal-3 (Fig. 4C, d), thus supporting the finding that F/Gal-3 can enter the nucleus by passive diffusion, attesting to the specificity of the experimental system used here and suggesting that endogenous Gal-3 can migrate into the nucleus, in part, by passive diffusion.

**NH2-terminal region of Gal-3 has active nuclear transport capacity without ATP and any soluble factors.** Because Gal-3/GFP could enter the nucleus *in vitro* in the presence of lactose (Fig. 4B, c), whereas its nuclear migration was inhibited by WGA that binds to the nucleoporin in the NPC and thus inhibits passages through the NPC (Fig. 4B, i) or at 4°C (Fig. 4C, b), it is highly unlikely that it migrates into the nucleus by a passive diffusion. The results obtained from the transfection experiments using Gal-3 deletion mutants fused with EGFP indicate that the domain(s) responsible for Gal-3 protein nuclear localization is in part in the NH2-terminal half (1-115) and in part on its core COOH-terminal region (139-193). Therefore, 1 to 115 amino acids of Gal-3 fused with ×2EGFP (1-115/×2GFP) and 139 to 193 amino acids fused with GST and EGFP (GST/139-193/GFP) were constructed and used as import substrates using the *in vitro* transport assay.

As shown in Fig. 5, the 1-115/×2GFP protein was evenly distributed in the nucleus with or without cytosol and/or ATP in the reaction mixture (Fig. 5A-C), whereas its nuclear accumulation was inhibited by the addition of WGA (Fig. 5D). However, the GST/139-193/GFP protein, under any solution condition, could not be detected in any significant level in the nucleus and was predominantly confined to the cytoplasmic compartment (Fig. 5E-H). It should also be noted that nuclear accumulation of GST/139-193/GFP protein was partially observed in the presence of cytosol and sucrose (control; Fig. 5E), and was not detected in the presence of lactose (Fig. 5K), suggesting that Gal-3 may also translocate into the nucleus by carrier protein system through its β-galactoside binding domain. The 1-115/×2GFP protein was imported to the nucleus more effectively compared with the GST/139-193/GFP protein; therefore, we may conclude that the NH2-terminal region of Gal-3 protein is significantly involved in the nuclear translocation of Gal-3.

Because the NH2-terminal regulation of Gal-3 migration into the nucleus is independent of cytosol and ATP, similarly to the importin-β and β-catenin nuclear import pathway (29, 33, 34), we questioned whether the active transport pathway of Gal-3 is, in part, dependent on a similar nuclear import pathway of importin-β. To address this, we have done competitor experiments in the digitonin-permeabilized cells using cytosol, which contains low (1 mg/mL) or high (10 mg/mL) concentrated soluble cytoplasmic proteins, or recombinant importin-β protein fused to GST (2 μmol/L). As shown in Fig. 6A, GFP/import-β nuclear accumulation was significantly inhibited by the addition of a high protein concentration (Fig. 6A, c) or a nontagged recombinant importin-β (Fig. 6A, d). However, neither Gal-3/GFP nor 1-115/×2GFP nuclear accumulation were inhibited by the addition of cytosol or excess importin-β protein (Fig. 6A, e-h, i-l), suggesting that the Gal-3 transversing through the NPC passage is distinct from that of importin-β. Next, we examined whether Gal-3 could directly interact with an FG repeat motif of nucleoporins and considered as common docking sites for proteins like importin-β during passage though the NPC (25, 35). To address this, we have constructed a fusion protein, GST, and an FG repeat containing fragment of human nucleoporin CAN/Nup214(FG-repeat) as previously described (25), and did GST pull-down assay followed by Western blotting of GFP. As shown in Fig. 6B, GFP/import-β complexed with FG repeat, but did not bind to GST (CTRL), consistent with the previous report (25) indicating that this FG

![Figure 5.](image-url)
repeat motif was properly expressed and importin-β can interact with this motif. In contrast, neither Gal-3/GFP nor 1-115/C22GFP could bind to FG repeat (Fig. 6B, CTRL). We also confirmed the same results by Western blotting of importin-β and Gal-3 (data not shown), suggesting that Gal-3 nuclear accumulation is not saturable and its nuclear import pathway is independent on an FG repeat region of the nucleoporins.

Nuclear export of Gal-3. The above data show that Gal-3 possesses the ability to migrate into the nucleus constitutively through at least two pathways. However, Gal-3 is not exclusively localized in the nucleus due to Gal-3 nuclear export system (5, 20). This export mechanism was better characterized using the digitonin-permeabilized cells and it was speculated that this export was regulated by NES in the COOH-terminal region of Gal-3 (Gal-3 NES; Fig. 7A; ref. 20). Therefore, we questioned whether this leucine-rich motif could actually serve as a typical NES. To address this, we constructed an expression vector of C22EGFP fused with Gal-3 NES or p53NES2 (Fig. 7A), expressed them in the COS-7 cells, and followed their nuclear accumulation with or without LMB, which inhibits the interaction between NES and CRM1 export receptor (36, 37). In addition, we examined the nuclear accumulation of Gal-3/GFP and ×2GFP with or without LMB as well. To confirm the correct expression of those proteins, we did Western blotting of GFP using COS-7 cell lysates transfected with the plasmids as shown in Fig. 7B. Thereafter, LMB treatment was done to the transiently transfected COS-7 cells at 30 ng/mL (4 hours at 37°C) and to cells were incubated with DMSO vehicle, control. At the end of the incubation, cells were processed for fluorescence microscopy analysis. The representative images are shown in Fig. 7C and the relative proportion of distinct subcellular localization in each plasmid transfected COS-7 cells is depicted in Fig. 7D. The expression pattern of ×2GFP was not affected by the LMB treatment, and the proteins were distributed throughout the cell (Fig. 7C, a, e and d, f), whereas the expression of 1-250/G was effectively accumulated in the nucleus following LMB treatment (Fig. 7C, b, f and d). This result is consistent with previous reports (20). Meanwhile, the expression of Gal-3NES+ ×2GFP as well as p53NES2 ×2GFP was restricted to the nucleus with LMB treatment compared with no treatment (Fig. 7C, c, g, d, h and D). These results suggest that Gal-3 NES can play a role in the nuclear export of Gal-3 as a bona fide NES and this export system can also regulate the nuclear localization of Gal-3 protein in vivo.

Discussion
Gal-3 plays a central role in diverse biological functions, including regulation of cell adhesion, proliferation, differentiation, angiogenesis, antiapoptotic activity, and RNA processing activity in juxtaposition with its interacting ligands that specify function (2, 5, 38). In the nucleus, Gal-3 is thought to play a role in pre-mRNA splicing (39), and regulation of gene expression of cyclin D1 and c-myc (15, 16). Therefore, the unveiling the mechanism of Gal-3...
shuttling to and from the nucleus is very significant as it might be developed as a therapeutic modality to inhibit and/or regulate gene expression related to cancer. To date, the mechanism of Gal-3 nuclear import is not implicit, due, in part, to the fact that Gal-3 lacks a consensus NLS and the obtained information are based mainly on indirect evidences. Several reports have identified the domain(s) sequences of Gal-3 for its nuclear localization by transfection techniques (21–23). Previously, a role for the NH₂-terminal region of Gal-3 for its nuclear localization was suggested (21), whereas others have argued that its NH₂ terminus is not involved for its nuclear localization (22), and that the last 10 amino acids of the COOH-terminal region of Gal-3 are responsible for its nuclear transport properties (23). These contradictory findings may be due to the lack of a direct experimental evidence for the Gal-3 nuclear import mechanism and whether it results from passive diffusion or active transport. Thus, we examined the cellular distribution of various deletion mutants of human Gal-3 by transfection to check the essential domain for its nuclear localization in vivo, and did in vitro nuclear import assay necessary to direct evidence for Gal-3 nuclear import mechanism(s). In vivo, the last 10 amino acid deletion (1-240/G) dramatically abrogated the nuclear localization of Gal-3 as described recently (23), but a complete CRD region–deleted Gal-3 (1-115/G, 1-115/×3GFP), in turn, was predominantly localized in the nucleus. However, it

Figure 7. Nuclear export of Gal-3. A, schematic diagram of p53 NES2 and Gal-3 NES, which is inserted into the plasmid of ×2GFP used in this study. B, detection of NES containing derivatives fused with EGFP identified by Western blotting of transiently transfected COS-7 cell lysates. The proteins were probed with anti-polyclonal GFP. Left, molecular standards (M). Lane 1, 1-250/G; lane 2, ×2GFP; lane 3, Gal-3 NES+/×2GFP; lane 4, p53 NES2+/×2GFP. C, COS-7 cells were transiently transfected with the plasmids encoding ×2GFP, 1-250/G, Gal-3 NES+/×2GFP, and p53 NES2+/×2GFP for 24 hours, and examined the subcellular localization of EGFP expression with (a-h) or without (a-d) treatment with 30 ng/mL LMB for 4 hours. Each image is representative of the majority of the cells observed in several fields. Magnification, ×400. a to h, fluorescent signals of the cells expressing EGFP-labeled proteins; i to p, nuclear DNA stained with Hoechst33258 of the cells observed in the same views as in (a-h), respectively. D, expression patterns for the EGFP fusion proteins with or without LMB treatment were scored for over 300 cells in two or three separate experiments. Cells with a predominant expression in the nucleus (N > C), an even distribution both in the cytoplasm and the nucleus (N = C), or a predominant cytoplasmic expression (N < C) were scored, respectively. Columns, mean; bars, SD.
should be noted that all these results could not directly provide the nuclear import mechanism and the observed result is the balance between cytoplasmic anchorage, nuclear import, nuclear retention, and nuclear export (23). Therefore, an in vitro nuclear import assay to directly assess the nuclear import mechanism of Gal-3 was used and constructed Gal-3 protein that was conjugated with fluorescent material (F/Gal-3).

Gal-3 translocates into the nucleus by an active nuclear import mechanism as shown here, and the NH₂-terminal domain of Gal-3 protein plays a significant role in its NPC passage; this domain contains a glycine, tyrosine, and proline repeat motif and is critical for molecule self-association (40). Independently, it has been reported that NPC has a similar repeat motif, such as an FG repeat or a WD repeat, that are used for the interaction with the nucleoproteins for their NPC passage (41, 42). We cannot exclude the possibility of another Gal-3 nuclear import pathway, through its carbohydrate-binding capacity, because the results from the in vivo and the in vitro experiments suggest that 139 to 193 amino acid motif of the human Gal-3 could be translocated into the nucleus by a soluble factor (Figs. 2C, i and 5F).

Here, we revealed at least two mechanisms responsible for the nuclear import of Gal-3 similarly to other proteins. RCC1, the Ran exchange factor, uses two distinct nuclear import mechanisms (43). MAPK uses at least three distinct pathways: passive diffusion as a monomer, active transport as a dimer, and an NPC-mediated pathway (25, 44). Correspondingly, Gal-3 might be imported into the nucleus by passive diffusion as a monomer, and it is also possible for several Gal-3 to be imported by active transport as a dimer (40) or a pentamer formation (45).

It is crucial to point out that 1-240/G or 1-250/C, i.e., and 5E, which contain the NH₂-terminal region promoting the nuclear migration in vitro. Concerning these phenomena, it would be worth mentioning the speculations by Davidson et al. (23), who have reported and discussed the importance of the COOH-terminal region of murine Gal-3 for nuclear localization (46). The CRD region of Gal-3 is composed of two β-pleated sheets associated in a sandwich-like arrangement, one of which contains five antiparallel strands (F1-5) and the other contains six antiparallel strands (S1-6). They speculated that the deletion of the last 10 amino acids, which compose the F1 strand, could unravel the β-sandwich structure, followed by the impaired nuclear localization (23). However, it should be pointed out that the crystal structure of whole Gal-3 is not available and reliance on part of the structure might be misleading. Based on the result presented here, we suggest that this impaired nuclear accumulation could be caused due to the protein aggregation following the disruption of the β-sandwich structure because the expression of defective Gal-3 showed a patchy vesicular pattern and is spotted in the perinuclear region (Figs. 1C, d, e and 2C, d). This alteration should be more inducible and rapid compared with the nuclear import efficiency of Gal-3, but not to the nuclear import capacity of a typical NLS because SV40 T-antigen NLS could effectively rescue the nuclear accumulation of impaired Gal-3 (Fig. 3C, D).

To conclude, we provide here the first direct evidence for the nuclear transport of Gal-3 and show that it is regulated by complicated mechanisms of balance between cytoplasmic anchorage, nuclear import (passive diffusion and active), nuclear retention, and nuclear export.

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References

28. Melchior F, Sweet DJ, Gerace. Analysis of Ran/TC4

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Galectin-3 and Nuclear Import


Characterization of the Nuclear Import Pathways of Galectin-3

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