Shuttling of galectin-3 between the nucleus and cytoplasm

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Introduction

Galectin-3 is a member of a family of proteins, defined on the basis of structural analysis and binding specificity studies, that (1) bind β-galactosides, and (2) share significant sequence similarity in the carbohydrate-binding site (Barondes et al., 1994). On the basis of immunofluorescence and immunoelectron microscopy as well as quantitative immunoblotting of subcellular fractions, it has been documented that galectin-3 was localized in the nucleus and cytoplasm of cells (Moutsatsos et al., 1986; Hubert et al., 1995; Craig et al., 1995). Using a cell-free assay for the splicing of pre-mRNA, we have previously shown that galectin-3 is a required splicing factor (Dagher et al., 1995). More recently, we have found that galectin-3 interacts with Gemin4 (Park et al., 2001), a component of a macromolecular complex designated as the survival of motor neuron (SMN) complex (Charroux et al., 2000). Moreover, coimmunoprecipitation experiments demonstrated that galectin-3 is a bona fide member of the SMN complex. The SMN complex functions in both the nucleus and the cytoplasm. In the cytoplasm, the SMN complex mediates assembly of small nuclear ribonucleoprotein (snRNP) particles; in the nucleus, it delivers the snRNPs to the pre-mRNA during the early stages of spliceosome formation (Fischer et al., 1997; Pellizzoni et al., 1998).

Nucleocytoplasmic shuttling is typically defined as the repeated bidirectional movement of a protein across the nuclear membranes (Borer et al., 1989). The association of galectin-3 with the SMN complex raises the possibility that galectin-3 might perform related functions in both the nucleus and the cytoplasm and that it might shuttle between the two compartments. To test directly whether galectin-3 can shuttle, we monitored the localization of galectin-3 in two different types of heterokaryon systems: (1) human-mouse heterodikaryons, and (2) mouse–mouse heterodikaryons, in which the cell types contained a null mutation in the galectin-3 gene (Colnot et al., 1998). In the human-mouse heterodikaryons, we monitored the localization of human galectin-3 using a mouse monoclonal antibody that recognized human galectin-3 but not the mouse homolog. In the mouse–mouse heterodikaryons, we monitored the localization of mouse galectin-3 using a rat monoclonal antibody directed against galectin-3. The results obtained from both systems suggest that galectin-3 does shuttle between the nucleus and the cytoplasm.

Results

The monoclonal antibody NCL-GAL3 recognizes human galectin-3 but not the mouse homolog

The experimental strategy used to test for shuttling of galectin-3 is depicted in Figure 1. Heterokaryons were generated through fusion of two cell types: human LG-1 fibroblasts (cell X) and mouse 3T3 fibroblasts (cell Y). The presence of human galectin-3 in both nuclei of a heterodikaryon (path I) could arise due to nuclear import from three sources: (1) human galectin-3 in the cytoplasm, (2) newly synthesized human galectin-3, and (3) human galectin-3 from the human nucleus. The last of these three mechanisms requires export of human

Key words: galectins/nucleocytoplasmic transport/nuclear import/nuclear export/splicing factor
galectin-3 from the human nucleus into the cytoplasm of the heterodikaryon and subsequent import into the mouse nucleus. Therefore, if shuttling makes a significant contribution to the percentage of heterodikaryons exhibiting human galectin-3 staining in both nuclei, it should be sensitive to inhibition of nuclear export. On this basis, we expected the percentage of heterodikaryons showing human galectin-3 in both nuclei to be decreased and, correspondingly, the percentage of heterodikaryons showing galectin-3 in only one nucleus (path II) to be increased by inhibitors of nuclear export. This experimental scheme has two key requirements. First, we needed to be able to distinguish heterodikaryons (human–human and mouse–mouse cell hybrids) from homodikaryons (human–human and mouse–mouse cell hybrids). This was accomplished by tagging the two cell types with distinguishable microsphere beads prior to fusion (see the following). Second, we needed to be able to immunostain for human galectin-3 with an antibody that fails to recognize mouse galectin-3.

When lysates of human cells were subjected to immunoblotting with the mouse monoclonal antibody NCL-GAL3, a single polypeptide ($M_r \sim 29,000$) corresponding to the mobility of human galectin-3 was observed (Figure 2A, lanes 1 and 2). In contrast, no polypeptide band could be detected when NCL-GAL3 was used to blot a corresponding lysate derived from mouse 3T3 fibroblasts (Figure 2A, lane 3). The presence of galectin-3 in the mouse cell lysate was ascertained by blotting with anti-Mac-2, a rat monoclonal antibody directed against galectin-3 (Figure 2B, lane 3). The appearance of mouse galectin-3 at a higher molecular weight than human galectin-3 (Figure 2B, lanes 1–3) is consistent with the difference in the lengths of the respective polypeptide chains, as verified by cDNA sequencing. These results suggest that the mouse monoclonal antibody NCL-GAL3 recognizes human galectin-3 but not the mouse homolog.

This conclusion was substantiated by immunofluorescence staining of fixed and permeabilized cells. NCL-GAL3 yielded strong staining in HeLa cells but not in 3T3 cells (Figure 3A and C). Again, the presence of galectin-3 in both cell types was verified by staining with anti-Mac-2 (Figure 3E and G). In these experiments, we also ascertained that the secondary antibodies used for these and all subsequent immunofluorescence studies, fluorescein isothiocyanate (FITC)-conjugated goat anti-mouse IgG, and FITC-conjugated goat anti-rat IgG, yielded negligible staining in human and mouse cells when used in the absence of any primary antibodies (Figure 3B, D, F, and H).

**Bead tagging distinguishes heterodikaryons from homodikaryons**

Fusion of human and mouse cells was expected to produce three types of cell hybrids: human–human, mouse–mouse, and human–mouse. To distinguish the three types and focus only on human–mouse fusions, we tagged our cells with microsphere styrene beads (~2 µm in diameter) prior to fusion. Human LG-1 fibroblasts were tagged with nonfluorescent (black) beads, whereas mouse 3T3 fibroblasts were tagged with green fluorescent beads. Tagged cells were then cocultured and fused as depicted in Figure 1. Several hours postfusion, the cells were fixed and immunostained with NCL-GAL3 and FITC-goat anti-mouse IgG to determine the localization of human galectin-3 and the nuclei were stained with propidium iodide (PI).

Monokaryons were immediately distinguished from di- or polykaryons by the number of nuclei as observed by PI fluorescence (not shown). As shown in Figure 4A–C, human–human homodikaryons were indicated by the presence of only nonfluorescent beads in the cytoplasm. Similarly, mouse–mouse homodikaryons were indicated by the presence of only fluorescent beads in the

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**Fig. 1.** Schematic illustrating the use of heterokaryons to study shuttling. Heterokaryons were generated through fusion of two cell types, for example: human LG-1 (cell X) and mouse 3T3 fibroblasts (cell Y). The presence of human galectin-3 in both nuclei of a heterodikaryon (path I) could be due to three mechanisms: (1) human galectin-3 originally in the cytoplasm of LG-1 cells; (2) newly synthesized human galectin-3; and (3) galectin-3 originally from the human nucleus. Mechanism (3) requires that human galectin-3 first gets exported from the human nucleus into the cytoplasm of the heterodikaryon and subsequently gets imported into the mouse nucleus. Path II depicts a heterodikaryon in which only one of the nuclei contains human galectin-3. This general scheme was also applied to the second cell pair in our test of galectin-3 shuttling: mouse 3T3 fibroblasts expressing galectin-3 (cell X) and MEF Gal-3 –/– cells not expressing galectin-3 (cell Y).

**Fig. 2.** Western blotting for galectin-3 in lysates of human HeLa cells, human LG-1 fibroblasts, and mouse 3T3 fibroblasts. Lane 1: human HeLa lysate. Lane 2: human LG-1 lysate. Lane 3: mouse 3T3 lysate. 50 µg of total protein was loaded in each lane. (A) Galectin-3 was detected using the mouse monoclonal antibody NCL-GAL3 (21 ng/ml) plus HRP-goat anti-mouse Ig. (B) Galectin-3 was detected using the rat monoclonal antibody anti-Mac-2 (125 ng/ml) plus HRP-goat anti-rat immunoglobulin. The mobilities of molecular weight standards are indicated.
cytoplasm (Figure 4D–F). In contrast, the presence of both fluorescent and nonfluorescent beads within a dinucleated cell indicated a human–mouse heterodikaryon (Figure 4G–I). Thus, the bead-tagging method proved to be a reliable technique for recognizing heterodikaryons.

Localization of human galectin-3 to both nuclei of human–mouse heterodikaryons was partially dependent on de novo protein synthesis

Figure 4 also serves to illustrate how an individual nucleus was scored for the presence of human galectin-3. In the mouse 3T3 homodikaryon (Figure 4E), both nuclei yielded fluorescence intensity values of less than 250. Based on our quantitative scoring criteria (detailed in Materials and methods), these nuclei would be negative in terms of reactivity with the NCL-GAL3 antibody (i.e., no human galectin-3). In the human LG-1 homodikaryon (Figure 4B), the two nuclei yielded fluorescence intensity values of 1700 and 1800, far surpassing our threshold value of 300 for scoring positive in terms of human galectin-3. In the human–mouse heterodikaryon (Figure 4H), the two nuclei yielded values of 760 and 900. Thus, human galectin-3 was observed in both nuclei of this heterodikaryon, indicating that it was redistributed into the mouse nucleus.

To assess the contribution of newly synthesized human galectin-3 in supplying the mouse nucleus, cycloheximide (CHX) was employed to block de novo protein synthesis (Agrwal et al., 1989; Borer et al., 1989). CHX appeared to have two effects on the human–mouse heterodikaryons. First, it decreased the overall fluorescence intensity due to NCL-GAL3 staining, presumably because the drug inhibited de novo synthesis of human galectin-3. For example, in human–mouse heterodikaryons treated with CHX (Figure 5E), the values of fluorescence intensity for the two nuclei were 450 and 530, reflecting a further decrease in fluorescence beyond the dilution of human galectin-3 into the two nuclei of the heterodikaryon (described for Figure 4H, Figure 5B). Nevertheless, both of the nuclei of this heterodikaryon (Figure 5E) were above the threshold value of 300 and therefore were scored positive for human galectin-3, indicating that there was still transport into the mouse nucleus.

Second, CHX also decreased the percentage of heterodikaryons exhibiting human galectin-3 in both nuclei. In the absence of CHX (Figure 5A–C), the fraction of heterodikaryons exhibiting human galectin-3 in both nuclei 9 h postfusion was 72% (58 out of 81 heterodikaryons counted). In the presence of CHX (Figure 5D–F), the corresponding value was 50% (59 out of 119 heterodikaryons counted). Thus, although de novo synthesized human galectin-3 contributed to the localization of the protein in both nuclei of human–mouse heterodikaryons, at least half of the heterodikaryons still exhibited human galectin-3 in both nuclei even in the presence of CHX. We interpret these results to indicate that newly synthesized human galectin-3 was not the only source for the mouse nucleus in the heterodikaryon. Rather, it appears that the majority of human galectin-3 supplying the mouse nucleus came from either the human nuclear pool or the human cytoplasmic pool.

Localization of human galectin-3 to both nuclei of human–mouse heterodikaryons was dependent on nuclear export

Using digitonin-permeabilized 3T3 fibroblasts, we had previously shown that galectin-3 is rapidly and selectively exported from the nucleus and that this export was sensitive to inhibition by leptomycin B (LMB) (Tsay et al., 1999). Therefore, to distinguish between the contributions of the nuclear pool and the cytoplasmic pool of human galectin-3, LMB was employed to block nuclear export of galectin-3. LMB binds and inactivates
the chromosome region maintenance (CRM1) nuclear export receptor (Nishi et al., 1994), which recognizes the leucine-rich nuclear export signal. In Figure 5, panels G and H show a representative human–mouse heterodikaryon treated with CHX and LMB.

Nine hours after fusion, approximately 50% of heterodikaryons treated with CHX alone exhibited human galectin-3 in both nuclei (59 out of 119 heterodikaryons counted, Table I). In the presence of CHX and LMB, the corresponding value was 19% (23 out of 120 heterodikaryons counted, Table I). These results suggest that the cytoplasmic pool of human galectin-3 was not the only source of the protein for the mouse nucleus. Rather, human galectin-3 in the human nucleus must also contribute to supplying the mouse nucleus, because inhibition of export of human galectin-3 from the human nucleus concomitantly reduced the proportion of heterodikaryons exhibiting human galectin-3 in both nuclei. On this basis, we conclude that at least some of the human galectin-3 appearing in the mouse nucleus of a heterodikaryon had to first exit the human nucleus into the common cytoplasm followed by entry into the mouse nucleus, thus fulfilling the definition of shuttling.

**Mouse galectin-3 shuttles in 3T3-MEF Gal-3 −/− heterodikaryons**

In previous studies (Colnot et al., 1998), the generation of mice carrying a null mutation of the galectin-3 gene was reported. This provided the opportunity to corroborate our test of galectin-3 shuttling in another system. We isolated fibroblasts from embryos of this strain of mice, designated mouse embryo fibroblast (MEF) Gal-3 −/−.

To confirm the absence of the galectin-3 polypeptide in MEF Gal-3 −/− cells, we blotted cell lysate from MEF Gal-3 −/− cells with the anti-Mac-2 antibody. We did not detect any polypeptide in the lane containing MEF Gal-3 −/− lysate (Figure 6A, lane 2). In contrast, immunoblotting with anti-Mac-2 yielded a band of the same mobility as recombinant galectin-3 (Figure 6A, lane 1) in lysates from MEF Gal-1 −/− cells (MEF cells containing a null mutation in the Gal-1 gene) (Poirier and Robertson, 1993),
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MEF wild-type (WT) cells (without any mutations), and mouse 3T3 cells (Figure 6A, lanes 3–5). These results indicated that the MEF Gal-3 –/– cells did indeed lack the galectin-3 polypeptide. This conclusion is substantiated by immunofluorescence staining of fixed and permeabilized cells. Anti-Mac-2 stained mouse 3T3 cells but not MEF Gal-3 –/– cells (Figure 6B, panels A and C). In these experiments, we also ascertained that the secondary antibody FITC-conjugated goat anti-rat IgG did not react nonspecifically with fixed and permeabilized MEF Gal-3 –/– cells (Figure 6B, panel D) when used in the absence of anti-Mac-2.

The experimental scheme of Figure 1 was used to study galectin-3 shuttling in 3T3-MEF Gal-3 –/– heterodikaryons. In this case, 3T3 cells served as the source of galectin-3 (cell X) and were tagged with nonfluorescent (black) beads. The MEF Gal-3 –/– cells served as the recipient of galectin-3 (cell Y) and were tagged with fluorescent green beads. The localization of galectin-3 in these heterodikaryons was monitored by immunofluorescence using anti-Mac-2. The results of shuttling assays conducted in 3T3-MEF Gal-3 –/– heterodikaryons were generally similar to those obtained from the assays in human–mouse heterodikaryons. Four hours after fusion, 97% (47 out of 48) of heterodikaryons treated with CHX prior to fusion exhibited galectin-3 in both nuclei. In the presence of CHX and LMB, the corresponding value was 18% (16 out of 89 heterodikaryons counted, Table I). In Figure 7, panels A–C show a typical 3T3-MEF Gal-3 –/– heterodikaryon treated with CHX, and panels D–F show a typical 3T3-MEF Gal-3 –/– heterodikaryon treated with CHX and LMB. These results suggest

Table I. Percent of heterodikaryons showing staining for galectin-3 in both nuclei

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Harvest time</th>
<th>CHX</th>
<th>CHX + LMB</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>9</td>
<td>50% (59/119)</td>
<td>19% (23/120)</td>
</tr>
<tr>
<td>B</td>
<td>4</td>
<td>97% (47/48)</td>
<td>18% (16/89)</td>
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Experiment A: Human–mouse fusion. Mouse monoclonal antibody NCL-GAL3 used to detect human galectin-3. Experiment B: 3T3-MEF Gal-3 –/– fusion. Rat monoclonal antibody anti-Mac-2 used to detect mouse galectin-3.

Fig. 5. Effect of CHX and LMB on localization of human galectin-3 in human–mouse heterodikaryons. Heterodikaryons were generated and immunostained as described in the legend to Figure 4. (A–C) Human–mouse heterodikaryons incubated in the absence of CHX and LMB. (D–F) Human–mouse heterodikaryons incubated in the presence of LMB (2 ng/ml; 3.8 nM) and CHX (10 µg/ml) prior to fusion. (B, E, and H) FITC fluorescence (green), showing localization of human galectin-3. Note the staining for human galectin-3 in both nuclei in B and E. In contrast, note the absence of human galectin-3 staining in one of the nuclei in H and the accentuated staining in the other nucleus. (C, F, and I) PI fluorescence (red), showing the location of both nuclei in each heterodikaryon. (A, D, and G) Dual fluorescence showing FITC and PI. Note the colocalization of human galectin-3 staining with the PI fluorescence of both nuclei in A and D. In contrast, note the absence of human galectin-3 staining in one of the nuclei in G. Bar, 10 µm.
that the localization of galectin-3 to both nuclei of 3T3-MEF Gal-3 –/– heterokaryons was independent of protein synthesis but dependent on nuclear export of galectin-3.

**Discussion**

The key findings of this study include: (1) Human galectin-3 was observed in both nuclei of human–mouse heterokaryons, and mouse galectin-3 was observed in both nuclei of 3T3-MEF Gal-3 –/– heterokaryons. In both cases, galectin-3 had been imported into recipient nuclei that previously lacked it. (2) The addition of CHX caused a reduction of approximately 20% in the fraction of human–mouse heterokaryons showing human galectin-3 in both nuclei. However, 50% of heterokaryons still showed staining for human galectin-3 in both nuclei. (3) The addition of CHX and LMB caused a further reduction of approximately 30% in the fraction of human–mouse heterokaryons showing human galectin-3 staining in both nuclei. In the 3T3-MEF Gal-3 –/– heterokaryons, the addition of CHX and LMB caused a reduction of approximately 79% in

**Fig. 6.** (Top panel) Western blotting for galectin-3 in lysates of MEF Gal-3 –/– fibroblasts, MEF Gal-1 –/– fibroblasts, MEF WT fibroblasts, and mouse 3T3 fibroblasts. Galectin-3 was detected using anti-Mac-2 (125 ng/ml) and HRP-conjugated goat anti-rat immunoglobulin. Lane 1: recombinant mouse galectin-3, 50 ng. Lane 2: MEF Gal-3 –/– lysate, 6.5 µg total protein. Lane 3: MEF Gal-1 –/– lysate, 6.5 µg total protein. Lane 4: MEF WT lysate, 6.5 µg total protein. Lane 5: mouse 3T3 lysate, 6.5 µg total protein. (Panels A–D) Immunofluorescence staining for galectin-3 in fixed and permeabilized mouse 3T3 fibroblasts and MEF Gal-3 –/– fibroblasts. Cells were prepared as described in the legend to Figure 3. Galectin-3 was detected with anti-Mac-2 (25 µg/ml) and FITC-conjugated goat anti-rat immunoglobulin. Panels A and B: mouse 3T3 cells. Panels C and D: bead-tagged MEF Gal-3 –/– cells. Panels A and C: anti-Mac-2 and FITC-conjugated goat anti-rat immunoglobulin. Panels B and D: FITC-conjugated goat anti-rat immunoglobulin. Bar, 10 µm.

**Fig. 7.** Effect of CHX and LMB on localization of galectin-3 in mouse 3T3-MEF Gal-3 –/– heterokaryons. Mouse 3T3 fibroblasts were tagged with nonfluorescent (black) beads, and MEF Gal-3 –/– fibroblasts were tagged with green fluorescent beads. Following bead-tagging, both cell types were co-cultured and fused. Fused cells were then fixed, permeabilized, and immunostained for galectin-3 using anti-Mac-2 (25 µg/ml) and FITC-conjugated goat anti-rat immunoglobulin. (A–C) 3T3-MEF Gal-3 –/– heterokaryons incubated in the presence of CHX (10 µg/ml) prior to fusion. (D–F) 3T3-MEF Gal-3 –/– heterokaryons incubated in the presence of LMB (2 ng/ml; 3.8 nM) and CHX (10 µg/ml) prior to fusion. (B and E) FITC fluorescence (green), showing localization of galectin-3. Note galectin-3 staining in both nuclei in B. In contrast, note the absence of galectin-3 staining in one of the nuclei in panel E. (C and F) PI fluorescence (red), showing the location of both nuclei in each heterokaryon. (A and D) dual fluorescence showing FITC and PI. Note the colocalization of galectin-3 staining with the PI fluorescence in A. In contrast, note the absence of galectin-3 staining in one of the nuclei in D. Bar, 10 µm.
the fraction of heterodikaryons showing galectin-3 staining in both nuclei. We conclude from these results that galectin-3 indeed undergoes nucleocytoplasmic shuttling. Our previous documentation of nuclear export of galectin-3, carried out in a permeabilized cell system, demonstrated that galectin-3 fulfilled a necessary condition for shuttling (Tsay et al., 1999). We have now directly demonstrated shuttling of galectin-3 in an in vivo assay.

Interspecies heterokaryons have been used to demonstrate nucleocytoplasmic shuttling of several other proteins, including nucleolin and B23/No38 (Borer et al., 1989), hnRNP A1 (Piñol-Roma and Dreyfuss, 1992), the hdm2 oncoprotein (Roth et al., 1998), and the 2A7 antigen (Levasseur-Paulin and Julien, 1999). However, these proteins differ from galectin-3 in that they are typically most prominent in the nucleus, whereas galectin-3 typically exhibits both nuclear and cytoplasmic localization. In the case of a predominantly nuclear protein, two pools of protein could supply a recipient nucleus in a heterokaryon assay: the nuclear pool and the newly synthesized pool. In contrast, when the protein is also present in the cytoplasm, as is the case for galectin-3, then the cytoplasmic pool represents a third pool that can also supply the recipient nucleus.

The presence of a cytoplasmic pool of galectin-3 complicated our analysis of galectin-3 shuttling in that we had to differentiate between the contributions of cytoplasmic galectin-3 and galectin-3 that was exported from the nucleus. Therefore, we employed the antibiotic LMB to block nuclear export of galectin-3. By conducting shuttling assays in the presence of CHX (inhibiting protein synthesis), as well as in the presence of CHX and LMB (inhibiting protein synthesis and nuclear export), we were able to assess the contribution of each of the three pools of galectin-3 to the recipient nuclei in our heterokaryons. Indeed, our data from the human–mouse heterokaryon assays suggest that all three pools did in fact contribute to supplying the mouse nucleus in roughly equal proportions.

The effect of LMB on the nuclear versus cytoplasmic distribution of galectin-3 has been documented for both of the cell types used in our human–mouse heterokaryon fusions. Incubation of live, intact mouse 3T3 fibroblasts in the presence of LMB (3.8 nM) resulted in the accumulation of galectin-3 in the nucleus, as revealed by accentuation of the nuclear staining (Tsay et al., 1999). At a higher concentration of LMB (15.2 nM), galectin-3 was found predominantly (if not exclusively) in the nucleus. Similar results were obtained for human LG-1 cells; incubation with LMB (15.2 nM) resulted in the accumulation of galectin-3 in the nucleus, as revealed by the almost exclusively nuclear staining (Openo et al., 2000). These results indicate that the effect of LMB on decreasing the appearance of galectin-3 in both nuclei of heterokaryons must be due to its inhibitory effect on the export of galectin-3 from the donor nucleus, rather than any secondary effects of the drug on nuclear import.

Other shuttling proteins that appear to bear functional similarity to galectin-3 are nucleolin and hnRNP A1. Both of these proteins are associated with RNA processing, and both are believed to leave the nucleus in association with their substrate RNA molecule. Nucleolin imports ribosomal proteins from the cytoplasm into the nucleus and then coordinates binding of the ribosomal proteins to the nascent rRNA transcript (Ghisolfi-Nieto et al., 1996; Ginisty et al., 1998). The resulting complex may mediate processing of the transcript or stimulate cleavage reactions, after which nucleolin exports assembled ribosomal subunits out of the nucleus and deposits them in the cytoplasm (Ghisolfi-Nieto et al., 1996). hnRNP A1 mediates nuclear export of mRNA from the nucleus (Piñol-Roma and Dreyfuss, 1992). Pre-mRNAs are bound by hnRNP A1 in the nucleus, and on completion of splicing, the nuclear export signal in hnRNP A1 directs the export of the hnRNP A1-mRNA complex to the cytoplasm (Michael et al., 1995; Pollard et al., 1996). Once in the cytoplasm, hnRNP A1 dissociates from the mRNA and returns to the nucleus to repeat the cycle.

The significance of trafficking of galectin-3 between the nuclear and cytoplasmic compartments is not fully understood. We have recently identified galectin-3 as a component of a macromolecular complex, designated as the SMN complex (Park et al., 2001). Like previous immunofluorescence and ultrastructural studies on galectin-3 (Moutsatsos et al., 1986; Hubert et al., 1995), the SMN complex is found in both the cytoplasm and the nucleus (Dietz, 1998; Matera and Frey, 1998). In the cytoplasm, the SMN complex is associated with the core proteins of snRNPs and is involved in the biogenesis of the snRNP particles (Fischer et al., 1997; Mattaj, 1998). In the nucleus, the SMN complex is found in discrete nuclear bodies called gems (gemini of coiled bodies) and it is required for supplying snRNPs to the H-complex during spliceosome assembly (Pellizzzone et al., 1998; Meister et al., 2000). This H-complex juncture is also where galectin-3 appears to be required for splicing, as demonstrated by the effect of galectin depletion on the accumulation of intermediates during the assembly of splicing complexes (Dagher et al., 1995).

We propose that galectin-3 might initially associate with Gemin4 in the cytoplasm, possibly during the course of snRNP biogenesis. When the SMN complex is imported into the nucleus, galectin-3 may be taken along by way of its interaction with Gemin4. Once in the nucleus, galectin-3 may participate with the SMN complex in the assembly of the spliceosome. Last, galectin-3 may be exported from the nucleus, in association with Gemin4 and possibly other members of the SMN complex, to repeat the cycle of snRNP biogenesis and delivery.

Materials and methods

Cell culture and reagents

NIH mouse 3T3 fibroblasts were obtained from the American Type Culture Collection and cultured in minimal essential Eagle’s medium, supplemented with 0.2 mM L-aspartic acid, 0.2 mM L-serine, 1 mM sodium pyruvate, 100 U/ml penicillin, and 0.1 µg/ml streptomycin (MEM-ASP) plus 10% calf serum at 37°C and 5% CO₂.

The human fibroblast strain, designated LG-1 (Morgan et al., 1991), was a gift from Drs. J.J. McCormick and V.M. Maher at Michigan State University. LG-1 cells were used through passage 20 and were serially passaged at a split ratio of 1:4 as described previously (Openo et al., 2000) in MEM-ASP supplemented with 10% fetal calf serum at 37°C and 5% CO₂.

Primary MEFs were derived from 129 WT strain (MEF WT), galectin-1 null mutant strain (MEF Gal-1 –/–) (Poirier and Robertson, 1993) and galectin-3 null mutant strain (MEF Gal-3 –/–) (Colnot et al., 1998) using the procedure described by Hogan et al. (1994). MEF WT, MEF Gal-1 –/–, and MEF Gal-3 –/– cells

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were cultured in Dulbecco's modified Eagle medium with 4.5 g/L glucose, supplemented with 44 mM sodium bicarbonate, 100 U/ml penicillin, 0.1 μg/ml streptomycin, 50 μg/ml gentamicin sulfate, plus 10% calf serum at 37°C and 5% CO2. Cells were passaged serially at split ratios of 1:5 or 1:10.

The rat monoclonal antibody anti-Mac-2 recognizes an epitope in the amino-terminus of galectin-3 (Ho and Springer, 1982). The mouse monoclonal antibody NCL-GAL3 was purchased from Novocastra Laboratories (UK). FITC-conjugated goat anti-rat IgG was obtained from Boehringer Mannheim, and FITC-conjugated goat anti-mouse IgG was obtained from Santa Cruz Biotech. Microsphere styrene beads were obtained from Polysciences.

**Bead tagging and polyethylene glycol–mediated cell fusion**

Human LG-1 and mouse 3T3 fibroblasts were seeded separately in 100 × 20 mm tissue culture plates at 5 × 104 cells/cm², and allowed to attach for approximately 6 h. Microsphere styrene beads were then added to the culture medium at a concentration of 250 beads/cell and incubated overnight. Plates were then rinsed with Versene (140 mM NaCl, 2.68 mM KCl, 10 mM Na2HPO4, 1.47 mM KH2PO4, 0.68 mM ethylenediamine tetra-acetic acid, 0.15% phenol red, pH 7.2), trypsinized, and resuspended separately. The concentration of each cell suspension was determined, and both cell types were seeded together, each at a density of 3.9 × 103 cells/cm², into 35-mm dishes containing sterile glass coverslips. Cocultured cells were then incubated overnight. CHX (Sigma) was added at 10 μg/ml in MEM-ASP 1 h prior to fusion, removed during fusion, and returned after fusion. LMB was a gift from Dr. Minoru Yoshida (University of Tokyo), and was added at 2 ng/ml (3.8 nM) in MEM-ASP 1 h prior to fusion, removed during fusion, and returned after fusion.

Polyethylene glycol-1000 (Fluka Chemical) was melted and diluted to 45% (v/v) with warmed serum-free MEM-ASP media, then filtered through a 0.22-μm Millex syringe driven filter unit (Millipore). Cocultured LG-1 and NIH 3T3 cells were rinsed in warm phosphate buffered saline (PBS; 140 mM NaCl, 2.68 mM KCl, 10 mM Na2HPO4, 1.47 mM KH2PO4, pH 7.4), and treated with 45% polyethylene glycol-1000 for 55 s. The medium was promptly removed, and the cells were rinsed gently three times in warmed PBS. Cells were then incubated with MEM-ASP containing 10% fetal calf serum until immunostaining.

**Immunostaining and fluorescence microscopy**

Cells were prepared for fluorescence microscopy following fixation with 4% paraformaldehyde and permeabilization with 0.2% Triton X-100 (Tsay et al., 1999). Human-mouse fusions were stained with NCL-GAL3 (2.1 μg/ml in PBS containing 0.2% gelatin) for 1 h at room temperature, then washed three times with T-TBS (10 mM Tris, pH 7.5, 0.5 M NaCl, 0.05% Tween 20) on an orbital shaker at room temperature. The coverslips were then stained with FITC-goat anti-mouse IgG (in PBS containing 0.2% gelatin) for 30 min at room temperature. This and all subsequent steps were carried out in the dimmest light possible. The coverslips were washed with T-TBS, stained with PI (32 μg/ml in PBS containing 0.2% gelatin) for 30 min at room temperature, and rinsed again in T-TBS. The coverslips were then inverted onto clean microscope slides with a drop of Perma-Fluor (Lipshaw Immunon) and allowed to dry in a darkened environment overnight at room temperature. 3T3-MEF Gal-3–/– fusions were treated similarly except they were stained with anti-Mac-2 (25 μg/ml), followed by FITC-conjugated goat anti-rat IgG.

Fluorescent cells were viewed using an Insight Plus laser scanning confocal microscope (Meridian Instruments). The maximum and minimum fluorescence intensities of each nucleus were determined using the image analysis subroutine. By viewing over 100 images, including human and mouse monokaryons as well as homodikaryons and heterodikaryons, we found that a nucleus devoid of human galectin-3 (and therefore, not stained by NCL-GAL3) yielded a maximum fluorescence of 250 (arbitrary units). In contrast, a nucleus containing human galectin-3 always yielded a fluorescence intensity of 300 or greater. On this basis, (1) a nucleus whose maximum fluorescence intensity was 250 or lower was scored negative for the presence of human galectin-3, and (2) a nucleus whose minimum fluorescence intensity was 300 or higher was scored positive for the presence of human galectin-3.

**Preparation of cell lysates and immunoblotting**

Cells were grown to near confluence in 100 × 20 mm tissue culture plates, rinsed once with ice-cold PBS, and scraped into 4 ml PBS with a rubber policeman. Scraped cells were pooled into 15-ml tubes, centrifuged at 1470 × g for 10 min at 4°C, and the supernatant was decanted. The cells were resuspended in 1 ml ice-cold PBS, transferred to Eppendorf tubes, and pelleted in a microfuge at 3406 × g for 2 min at 4°C. The supernatant was aspirated and the cells were resuspended in 150 μl of 10 mM Tris, pH 7.4, then incubated on ice for 10 min. The cell suspension was then sonicated eight times (15 s each) and stored at −20°C.

The amount of protein in each sample was quantitated by the Bradford method (Bradford, 1976) using Coomassie protein assay reagent (Pierce). Equal amounts of protein from each lysate were separated on a 12.5% sodium dodecyl sulfate–polyacrylamide gel and then transferred electrophoretically to a nitrocellulose membrane in a buffer containing 25 mM Tris, 193 mM glycine, and 10% methanol (pH 8.3). The membrane was blocked overnight at room temperature in T-TBS containing 10% nonfat dehydrated milk, then rinsed three times briefly in T-TBS and blocked with a 1:1000 dilution of goat anti-rabbit IgG in T-TBS containing 1% nonfat dehydrated milk. Following blocking, the membrane was rinsed with T-TBS.

In immunoblots employing NCL-GAL3, the membrane was incubated with NCL-GAL3 (21 ng/ml) in T-TBS containing 1% nonfat dehydrated milk for 1 h at room temperature. Following incubation with NCL-GAL3, the membrane was rinsed with T-TBS and incubated with horseradish peroxidase (HRP)–conjugated goat anti-mouse IgG in T-TBS containing 1% non-fat dehydrated milk for 1 h at room temperature. The membrane was then rinsed with T-TBS, and the proteins were visualized using the Renaissance western blot chemiluminescence reagents (New England Nuclear Life Science Products, Boston, MA). Immunoblots employing anti-Mac-2 were carried out in a similar fashion except that anti-Mac-2 was used at 125 ng/ml, and HRP-conjugated goat anti-rat IgG was used as the secondary antibody.
Acknowledgments

This work was supported by a grant (GM-38740) from the U.S. National Institutes of Health, a grant (MCB 97–23615) from the U.S. National Science Foundation, and a grant (5751) from Association de la Recherche contre le Cancer. We thank Kyle Oreno for his initial characterization of the specificity of the NCL-GAL3 antibody for human galectin-3. We thank Dr. Minoru Yoshida, University of Tokyo, for his generous gift of leptomycin B, and Drs. J.J. McCormick and V.M. Maher for their generous gift of LG-1 cells.

Abbreviations

CHX, cycloheximide; FITC, fluorescein isothiocyanate; HRP, horseradish peroxidase; LMB, leptomycin B; MEM-ASP, minimal essential Eagle’s medium with L-aspartic acid, L-serine, sodium pyruvate, penicillin, and streptomycin; PBS, phosphate buffered saline; PI, propidium iodide; SMN, survival of motor neuron protein; snRNP, small nuclear ribonucleoprotein; T-TBS, Tris-buffered saline containing 0.05% Tween 20; WT, wild-type.

References


Shuttling of galectin-3


Shuttling of galectin-3