Regulated nuclear export of the homeodomain transcription factor Prospero

Zoya Demidenko1, Paul Badenhorst2, Tamara Jones1, Xiaolin Bi1 and Mark A. Mortin1,*

1Laboratory of Biochemistry, National Cancer Institute, National Institutes of Health, Bethesda, MD 20892 USA
2Laboratory of Molecular Cell Biology, National Cancer Institute, National Institutes of Health, Bethesda, MD 20892 USA
*Author for correspondence (e-mail: mortinn@mail.nih.gov)

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SUMMARY

Subcellular distribution of the Prospero protein is dynamically regulated during Drosophila embryonic nervous system development. Prospero is first detected in neuroblasts where it becomes cortically localized and tethered by the adapter protein, Miranda. After division, Prospero enters the nucleus of daughter ganglion mother cells where it functions as a transcription factor. We have isolated a mutation that removes the C-terminal 30 amino acids from the highly conserved 100 amino acid Prospero domain. Molecular dissection of the homeo- and Prospero domains, and expression of chimeric Prospero proteins in mammalian and insect cultured cells indicates that Prospero contains a nuclear export signal that is masked by the Prospero domain. Nuclear export of Prospero, which is sensitive to the drug leptomycin B, is mediated by Exportin. Mutation of the nuclear export signal-mask in Drosophila embryos prevents Prospero nuclear localization in ganglion mother cells. We propose that a combination of cortical tethering and regulated nuclear export controls Prospero subcellular distribution and function in all higher eukaryotes.

Key words: Leptomycin B, Neurogenesis, Nuclear export, Prospero, Subcellular localization, Transcription, Drosophila

INTRODUCTION

Regulated subcellular localization provides a convenient way to control the activity of transcription factors. A variety of environmental stresses and intercellular signals trigger changes in subcellular localization (reviewed by Turpin et al., 1999; Hood and Silver, 1999). For example, the Rel-domain protein NF-kB is tethered in the cytoplasm by association with its inhibitory partner IxB, which masks the NF-kB nuclear localization signal (NLS). IxB is targeted for degradation by a number of cellular stimuli resulting in release and nuclear entry of NF-kxB (reviewed by Baldwin, 1996).

It has also been shown that NF-kxB is actively exported from the nucleus (Harhaj and Sun, 1999). Nuclear export requires the presence of a nuclear export signal (NES), one class of which is typically a short leucine-rich sequence that directly interacts with CRM1/Exportin 1 (Stade et al., 1997; Fornerod et al., 1997; Fukuda et al., 1997). Selective partitioning between the cytoplasm and nucleus can be achieved by competition between NLS-driven nuclear entry and NES-driven export. For example, the yeast transcription factors Pho4 (Kaffman et al., 1998) and yeast AP1-like protein (Yan et al., 1998), and the Drosophila homeodomain transcription factor Extradenticle (Exd; Abu-Shaar et al., 1999; Berthelsen et al., 1999), all exhibit regulated nuclear export.

The prospero (pros) locus was originally identified because of its effects on the development of the Drosophila embryonic nervous system (Doe et al., 1991; Vaessin et al., 1991; Matsuzaki et al., 1992). In embryos, Prospero protein is first detected at high levels in neuroblasts. Typically, a neuroblast divides unequally to generate a ganglion mother cell (GMC), which divides once more to form the mature neurons and glia that constitute the central nervous system (CNS). In neuroblasts, Prospero is cytoplasmic and prior to division becomes associated with the basal cortex via attachment to the adapter protein, Miranda (Mir; Shen et al., 1997; Ikeshima-Kataoka et al., 1997), and thus is selectively partitioned to the GMC (Hirata et al., 1995; Spana and Doe, 1995). In GMCs, Prospero is exclusively nuclear. Nuclear entry of Prospero has been argued to establish GMC-specific transcription (Doe et al., 1991; Vaessin et al., 1991; Matsuzaki et al., 1992). Consistent with this, pros mutant embryos improperly express even-skipped (eve), fushi tarazu (ftz), engrafted (en), asense (ase) and deadpan (dpn). The Prospero DNA-binding site has been determined and a C-terminal fragment of Prospero can activate transcription from a promoter containing this element (Hassan et al., 1997).

Molecular dissection has revealed that Prospero contains four other functional regions (Fig. 1A). These include an asymmetric localization domain (ALD), which is the Miranda domain that targets Prospero to the basal cortex during neuroblast division (Hirata et al., 1995; Shen et al., 1997); a nuclear localization signal (NLS; Hirata et al., 1995); an atypical homeodomain (HD; Chu-Lagraff et al., 1991; Matsuzaki, et al., 1992); and a highly conserved domain with no previously described function, termed the Prospero domain (PD; Burglin, 1994).

We report the characterization of the novel pros88 allele, which produces a protein lacking the C-terminal 30 amino acids of the PD. This has allowed the function of the PD to be
elucidated. We demonstrate that Prospero contains a signal that mediates its nuclear export via the Exportin pathway. The PD acts to regulate or mask this export signal. In the absence of the masking motif, Prospero is constitutively exported and cannot function as a transcription factor. We propose that regulated nuclear export is one component of the mechanism that changes Prospero subcellular distribution during development from neuroblast to GMC and thus underlies the switch from neuroblast- to GMC-specific gene expression. The PD is highly conserved and regulated nuclear export of Prospero is observed in both insect and mammalian cells. This suggests that the control of Prospero function by regulated nuclear export is conserved in all higher eukaryotes.

**MATERIALS AND METHODS**

**Genomic cloning and sequencing**

A mutant l(3R)S8 chromosome was recovered during a selection for suppressors of a conditional lethal mutation in the largest subunit of RNA polymerase II, Rpl215K1 (Mortin, 1990). It was demonstrated that this chromosome carried two separable mutations, one responsible for the dominant suppressor phenotype and the other causing recessive lethality. The suppressor has been demonstrated to result from a mutation in the second largest subunit of RNA polymerase II, RplII40 (Kim et al., 1994; Krasnoselskaya et al., 1998). Genomic DNA was prepared from l(3R)S8 dead embryos or adults with the isogenic progenitor third chromosome. This DNA served as template for PCR (Pfu DNA polymerase, Stratagene) using sequence-specific primers spanning the pros gene (primer sequences for this and subsequent experiments are available upon request). Products were gel purified using a QIAquick Extraction Kit (Qiagen) and sequenced with an ABI Prism 310 Genetic Analyzer. DNA adjacent to the insertion element responsible for l(3)00609 was cloned using plasmid rescue (Bier et al., 1989) following restriction digestion with XbaI, ligation, transformation into bacteria and selection with Kanomycin.

**Mammalian expression system**

A PCR-based strategy was used to clone different fragments of the pros gene. These were restriction digested with either EcoRI or SmaI (5’) and XbaI (3’), and subcloned into the pM vector (Clontech) in frame with and downstream of the GAL4 DNA-binding domain; or with BamHI (5’) and XbaI (3’) and subcloned into pEYFP-Nuc (Clontech). Amino acids 1-147 of the GAL4 DNA-binding domain also have a nuclear localization signal (Silver et al., 1984). The plasmid pM3-VP16 (Clontech) has the VP16 transcription activation domain fused to the GAL4 DNA-binding domain and served as a positive control for activated transcription. The vector pEYFP-Nuc encodes an enhanced yellow-green variant of green fluorescent protein (GFP) fused to three NLs: this and all subclones in this vector were double digested with BglII and BamHI and ligated to produce variants lacking NLs.

CV-1 or COS cells were transfected using LipofectAMINE (GibcoBRL) with 2 µg of various combinations of plasmid DNAs. The pG5Luc reporter construct (Promega) with 5 GAL4 DNA-binding sites upstream of minimal TATA box expresses firefly luciferase upon activation. The pRL-TK vector (Promega) constitutively expresses Renilla luciferase in mammalian cells and serves as an internal control for transfection efficiency. A dual luciferase reporter assay system (Promega) was used to measure the different kinds of luciferase levels on a Monolight 2001. Prospero fusion proteins were excised from the pM vector and subcloned into the pRmHA-3 vector, which expresses the fusion proteins in transfected Drosophila SL2 cells following induction with CuSO4.

**Immunohistochemistry**

Transfected CV-1, COS and SL2 cells were fixed in 4% formaldehyde for 10 minutes at room temperature followed by three washes in PBS. Cells were then treated with 5 µg/ml RNase I (Stratagene) for 1 hour and washed three times in PBS. Blocking was performed with 3% normal serum for 1 hour, followed by a 2 minute wash in PBT and three washes with PBS. Primary antibodies were incubated overnight at 4°C. Cells were then washed three times in PBS and incubated with secondary antibodies and propidium iodide (5 µg/ml) for 1 hour at room temperature. After three washes in PBS, the cells were mounted in vectashield (Vector Laboratories). Antibodies to Prospero (MRIA; Spana and Doe, 1995) were used at a dilution of 1:10. Anti-GAL4 (Clontech) was used at 0.5 µg/ml. Conjugated FITC-donkey (or goat) anti-mouse secondary antibodies (Jackson Immunoresearch Laboratories) were used at a dilution of 1:200. Cells were examined on a Biorad MRC 1024 laser confocal microscope. To assess the subcellular localization of Prospero fusion proteins, multiple optical sections were examined focusing through cells to distinguish among nuclear, nuclear and cytoplasmic, and cytoplasmic localization.

Eggs were collected from pros5/MITM6b, Scr-lacZ and pros1490/TM6b, Scr-lacZ flies, and the resulting embryos were fixed in 4% formaldehyde and processed as described elsewhere (Harsh et al., 1993). Propidium iodide staining was performed by treating embryos with 5 µg/ml RNase I (Stratagene) for 2 hours at room temperature, followed by washing in PBT and standard blocking. During incubation with secondary antibodies, 10 ng/ml of propidium iodide was included. Primary antibodies used were Prospero (MRIA, Spana and Doe, 1995) used at a dilution of 1:4, BP102 (Seeger et al., 1993) used at a dilution of 1:50, 22C10 (Fujita et al., 1982) used at a dilution of 1:50, and β-galactosidase (Cappell) used at a dilution of 1:1000. Secondary antibodies used were FITC- or rhodamine-conjugated donkey anti-rabbit or anti-mouse, and Cy5-conjugated donkey anti-mouse (Jackson Immunoresearch Laboratories). Homozygous mutant embryos were determined by their lack of β-galactosidase staining from the transgene on the TM6b, Scr-lacZ balancer chromosome.

**RESULTS**

**Cloning and Identification of l(3R)S8 as Prospero**

The l(3R)S8 locus was originally recovered during a selection for suppressors of a conditional lethal mutation in the largest subunit of RNA polymerase II, Rpl215K1 (Mortin, 1990). It was demonstrated that this locus resided between the genes curled and stripe (Mortin, 1990). When we screened a set of P-element-induced recessive lethal lines (Cooley et al., 1988; Bier et al., 1989) that map to the same genetic interval, we identified one, l(3)00609, that failed to complement l(3R)S8. Genomic DNA adjacent to the P-element insertion of l(3)00609 was cloned by plasmid rescue (Bier et al., 1989) and its sequence revealed that the P-element had inserted approximately 0.2 kb upstream of the pros transcription start site, indicating that l(3)00609 is an allele of pros, hereafter called pros00609.

To confirm that l(3R)S8 is an allele of pros, we used PCR to clone and sequence pros genomic DNA from the l(3R)S8 chromosome and its isogenic progenitor chromosome (Kim et al., 1994). A schematic diagram of the Prospero protein is shown with its functional domains indicated (Fig. 1A). We sequenced a region corresponding to the entire transcription unit of pros, including all exons and across the intron/exon boundaries, from both the mutant and parental isogenic chromosomes. A single nucleotide difference was found between the two chromosomes. It is located near the 3’ end of
the pros gene, converting the tryptophan codon at amino acid 1378 into a stop codon (TGG→TGA; Fig. 1B). The resulting mutant Prospero protein lacks its C-terminal 30 amino acids, which resides in a 100 amino acid motif, known as the Prospero domain. This domain is highly conserved amongst Prospero proteins from Drosophila to mammals (Fig. 1C). This is the first reported mutation in the PD and has allowed us to determine its function.

The PD is required for Prospero function in vivo

Examination of pros$^{S8}$ mutant embryos showed that the PD is required for Prospero activity. Homozygous pros$^{S8}$ embryos die and show mutant nervous system phenotypes identical to existing pros loss-of-function mutants (Fig. 2). In particular, the CNS of pros$^{S8}$ embryos is highly disrupted. By the end of embryonic development, axons of the CNS of wild-type flies adopt a highly regular structure. A ladder-like array that corresponds to the longitudinal connectives and anterior and posterior commissures can be seen in wild-type embryos, when highlighted by the antibody BP102 (Fig. 2A). In pros$^{S8}$ mutants this regular array is not observed. The anterior and posterior commissures fail to separate and the longitudinal connectives do not form (arrowhead and arrows, respectively, Fig. 2B). Similar disruption of axons is noted when the antibody 22C10 is used (data not shown). An identical mutant phenotype is observed in embryos homozygous for the null allele pros$^{B149}$ (Fig. 2C).

The peripheral nervous system of pros$^{S8}$ embryos also is disrupted. As had been noted with other pros loss-of-function mutations, axon pathfinding defects are observed. While the correct number of sensory organs are generated in their proper position in both pros$^{S8}$ and pros$^{B149}$ embryos, axons from these sensilla often fail to innervate the CNS and display pathfinding defects (asterisks and arrowheads, respectively; Fig. 2D-F). We conclude that mutant phenotypes caused by pros$^{S8}$ resemble loss-of-function pros mutations.
Transcription regulation by Prospero requires an intact PD

Mutations of pros alter expression of genes in the developing nervous system, suggesting that Prospero acts as a transcription factor (Doe et al., 1991; Vaessen et al., 1991; Matsuzaki et al., 1992). Certainly, the C terminus of Prospero from amino acids 1172-1407, including the PD, can activate transcription of a reporter gene in transfected cells (Hassan et al., 1997). To explore whether the PD plays a role in transcription activation by Prospero, we tested a series of Prospero deletions as fusion constructs, for their ability to influence transcription of a reporter gene in transfected cells.

We used a heterologous mammalian expression system to dissect the function of the C-terminal region of Prospero. We fused the first 147 amino of the GAL4 DNA-binding domain, which should direct nuclear localization (Silver et al., 1984), to fragments of Prospero. Four sets of Prospero fusion proteins were constructed. Each set represents one of a progression of N-terminal deletions of Prospero (I-IV). Within each set, constructs possessing the full C-terminus of Prospero (A), a deletion of the C-terminal 30 amino acids, mimicking the prosS8 allele (B) and a deletion of both the HD and PD (C, except for set IV, which only contains the PD) were generated (Fig. 3A). For sets I-III, we tested both the short and long isoforms (Fig. 1A); however, we only present data obtained for the short isoform as our results were similar with both forms.

The constructs shown in Fig. 3A were co-transfected along with an inducible firefly luciferase reporter gene construct, under the control of five tandem GAL4 DNA-binding sites, and a constitutively expressed Renilla luciferase reporter gene construct. Relative fiery to Renilla luciferase activity was determined for each construct (Fig. 3B). None of the first set of N-terminal deletion constructs activated transcription of a reporter gene, relative to the GAL4-DBD, which is itself a weak transcriptional activator. In fact, constructs IA and IC appear to repress transcription relative to the GAL4-DBD (Fig. 3B). A further deletion of 123 amino acids from the N terminus results in weak transcriptional activation by construct IIA, containing the full C terminus. This activation is abrogated by deletion of the C-terminal 30 amino acids, construct IIB (Fig. 3B).

A further deletion of 161 amino acids from the N terminus of Prospero resulted in increased activation. Construct IIIA is most similar in length to the fragment of Prospero that was previously shown to activate transcription (Hassan et al., 1997). When fused to the GAL4-DBD, we see a fourfold activation relative to the GAL4-DBD alone. Deletion of the C-terminal 30 amino acids again abrogates activation. Surprisingly, a further deletion of 129 amino acids from the N terminus of the PD restores activation (Fig. 3B). This demonstrates that the HD and PD are not required for activation in the presence of an independent DNA-binding motif. The region in construct IIC, amino acids 1098 to 1219 (which overlaps the activation domain identified by Hassan et al., 1997) most likely includes a transcription activation domain.

Two functional domains of Prospero regulate nuclear export

The PD alone did not activate transcription as revealed by the set IV constructs (Fig. 3B). However, although the PD does not appear to contain transcription-stimulating activity, it does play a crucial role in the functioning of Prospero. Deletion of the C-terminal 30 amino acids always abrogates activation by Prospero when both the HD and the remainder of the PD are present in the protein. We postulated that the PD might regulate either the subcellular localization or stability of the Prospero protein. To examine this, constructs were transfected into the mammalian cell line CV-1 and assayed for the subcellular localization and abundance of the fusion proteins (Fig. 4). Immunohistochemistry using the Prospero antibody MR1A (Spana and Doe, 1995) shows the subcellular distribution of the fusion proteins in transfected CV-1 cells. All of the constructs that contain an intact C terminus are expressed highly and are nuclear localized (Fig. 4, constructs IA-IIIA). The IV constructs lack the epitope recognized by M1RA.

In contrast, after deletion of the C-terminal 30 amino acids, the fusion proteins become restricted primarily to the cytoplasm (Fig. 4, constructs IB-IIIB). Note that constructs IB and IIB contain both the Prospero and GAL4 nuclear localization signals while construct IIB contains only the GAL4 NLS. The abundance of Prospero protein does not appear to be affected as the A and B constructs were treated identically. Similar results were obtained in COS and
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**Drosophila** SL2 cells (data not shown). Further deletion of both the HD and PD restored nuclear localization of the fusion proteins (Fig. 4, constructs IC-IIIC) again with little or no effect on abundance.

The changes in subcellular distribution of Prospero caused by the progressive C-terminal deletions were informative. They indicated that two previously undescribed domains, which regulate Prospero subcellular distribution, are present in the C terminus. One corresponded to a NES and/or cytoplasmic tether, and the second to a region that inhibits or masks this export signal/tether. The initial deletion derivatives allowed the export signal/tether to be mapped roughly to the Prospero HD and the N-terminal 70 amino acids of the PD. This signal could overcome a functional NLS (constructs III and IV have an NLS from GAL4 while sets I and II also have the Prospero NLS) and cause Prospero derivatives to be restricted to the cytoplasm as long as the C-terminal 30 amino acids of the PD were removed. The C-terminal 30 amino acids include the second domain: the regulatory or masking region that blocks the nuclear export and/or tethering signal.

**Nuclear export of Prospero by Exportin**

To distinguish whether the C-terminal portion of Prospero, including the HD and PD, functions as a nuclear export signal or a cytoplasmic tether we made use of the inhibitor leptomycin B (LMB). LMB has been shown to inhibit exclusively the Exportin nuclear export system (Fukuda et al., 1997; Kudo et al., 1997). The HD and PD include several conserved leucine residues; however, they do not match other leucine-rich NESs (Henderson and Eleftheriou, 2000). If deregulated nuclear export drives the truncated Prospero constructs lacking the C-terminal 30 amino acids into the cytoplasm, treatment with LMB would block re-localization. To test this, we conducted transfection experiments followed by either mock or LMB treatment. Constructs IIA and IIIA were predominantly nuclear in transfected CV-1 cells (Fig. 4, constructs IIA, IIIA). In contrast, constructs IIB and IIIB, which lack the C-terminal 30 amino acids, were primarily cytoplasmic in mock treated CV-1 cells (Fig. 5A), even though these constructs contain one or more functional NLSs (Fig. 3A). Treatment with LMB caused relocation of these Prospero fusion proteins into the nucleus of the transfected CV-1 cells (Fig. 5B). Similar results were obtained in COS cells. (Fig. 5C,D). These inhibitor studies indicate that the region of Prospero, including the HD and first 70 amino acids of the PD, is acting as a nuclear export signal that functions via the Exportin pathway.

**Refinement of the NES and NES-mask regions**

Additional deletion constructs were tested to delimit more precisely the NES and its mask (Fig. 6). Internal deletions of the PD showed that the entire domain is required for masking. Constructs lacking amino acids1378/1407 (Fig. 6A, construct...
IB), 1340/78 or 1308/39 are all localized to the cytoplasm (Fig. 6A).

To define the NES more precisely we made a further series of terminal deletions of Prospero (Fig. 6B, constructs ID, IIIE, IIIF). Each of these deletion constructs is exported from the nucleus. However, both the short and long isoforms of the terminal deletion IIIC are nuclear (Fig. 6B), demonstrating that the NES requires at least some of the amino acids between 1252 and 1265 as deletion of residues 1252/1265 abrogates NES function but deletion of residues 1266/1281 or 1282/1307 has no effect. (C) The 28 amino acids from the short isoform of 1215/1271 act as an NES when fused to GFP (pEYFP-1215/71) as they cause exclusion of the fusion protein from the nucleus. Addition of the PD relocates the GFP fusion protein (pEYFP-1215/1407) to the nucleus, confirming its ability to mask an NES.

The PD was able to mask nuclear export of the GFP fusion protein (Figs 5G, 6C). Addition of the region 1215/1407 to GFP restores the ubiquitous distribution observed using GFP alone. Thus the PD is sufficient to act as an NES mask in isolation from other regions of Prospero.

prosS8 alters subcellular localization in embryos

The prosS8 allele causes a mutant phenotype indistinguishable from loss-of-function pros alleles (Fig. 2). Our results using tissue culture have suggested that the reason for this mutant phenotype is that deletion of the C-terminal 30 amino acids affects the subcellular distribution of Prospero protein. We performed immunofluorescence on prosS8 embryos using antibodies that recognize Prospero. We failed to detect protein when we used antibodies that had been raised against the extreme C terminus of the protein (Matsuzaki et al., 1992), predicted to be deleted in prosS8 mutant embryos (data not shown). However, we were able to detect Prospero protein using antibodies raised against the central region of the protein (MR1A, Spana and Doe 1995). This confirms that Prospero protein synthesized from the prosS8 allele is in fact truncated. When we examined homozygous prosS8 embryos, Prospero protein was detected in its normal pattern in the cytoplasm of neuroblasts. In contrast, Prospero was not detected in the nuclei of daughter ganglion mother cells but was incorrectly localized to the cytoplasm (see arrows and arrowheads, respectively; Fig. 7). We conclude that removal of the C-terminal 30 amino acids results in mislocalization of Prospero to the cytoplasm of GMCs and that this causes the loss-of-function phenotype.
DISCUSSION

The current study identifies the Exportin nuclear export system as an important regulator of the homeodomain transcription factor Prospero. We demonstrate that two regions of Prospero control nuclear export, a nuclear export signal and an NES-masking region. These domains were identified during an initial analysis of regions required for transcription regulation by Prospero. A functional dissection of Prospero expressed in mammalian cells confirmed that it contains an independent transcription activation domain (Fig. 3). The activation domain identified in this study overlaps one reported elsewhere (Hassan et al., 1997). Possible targets for transcriptional control by Prospero include eve and ftz, which are not expressed in GMCs of pros mutants (Doe et al., 1991; Vaessin et al., 1991).

Control of subcellular distribution of Prospero

The subcellular distribution of Prospero is dynamically regulated during development of the Drosophila CNS and PNS (Doe et al., 1991; Vaessin et al., 1991; Matsuzaki et al., 1992). The nervous system derives from specialized precursor cells called neuroblasts that have delaminated from the neuroectodermal layer. Neuroblasts divide asymmetrically a variable number of times to bud off GMCs, which in turn divide terminally to form two mature neurons each. Although Prospero contains a canonical NLS, in neuroblasts it is localized to the cell cortex. Nuclear Prospero is only detected in the GMCs. After formation of mature neurons, Prospero is no longer detected.

Previous investigations have shown that Prospero contains a motif that can tether it to the cortex, the asymmetric localization domain (Ikeshima-Kataoka et al., 1997; Shen et al., 1997). Localization to the cortex requires the cytoskeletal adapter protein Miranda. Miranda is expressed only in neuroblasts and not in GMCs. Consequently, it has been proposed that the subcellular distribution of Prospero is controlled simply by the presence or absence of the cortical tether protein Miranda (Ikeshima-Kataoka et al., 1997; Shen et al., 1997; Schuld et al., 1998). However, we show that in addition to the ALD and the canonical NLS, Prospero contains a region that acts as a nuclear export signal. We propose that competition between NLS-driven nuclear import and NES-directed export plays an additional role in regulating Prospero subcellular localization.

The nature of the Prospero NES

A series of C-terminal truncations and internal deletions of the Prospero protein allowed us to restrict a Prospero NES to the first helix of the Prospero homeodomain. Fusion of a short 28 amino acid peptide including this region to GFP causes nuclear exclusion (Fig. 5E), confirming that it is sufficient to act as an NES. Recently, a large number of proteins have been identified that contain sequences that have the ability to act as NESs. Usually, these motifs interact with a shutting receptor Crm1 (Fornerod et al., 1997; Stade et al., 1997), which binds Ran GTPase. This complex interacts with nuclear pore components, allowing translocation across the nuclear envelope. Diagnostic features of Crm1-dependent NESs are their sensitivity to the antibiotic leptomycin B (Fukuda et al., 1997; Kudo et al., 1997) and the fact that they often contain the consensus amino acid sequence LX(1-3)LX(2-3)LXL (Henderson and Eleftheriou, 2000). Based on its sensitivity to LMB, the NES we have identified should be Crm1 dependent. The sequence, however, is not typical of the existing consensus sequence. It is possible that the existing consensus is too restrictive. Certainly, export of cyclin D1 has been shown to be LMB sensitive, yet deletion of the only sequences matching this NES consensus fails to block nuclear export (Alt et al., 2000).

An interesting feature of the NES we have identified is that it localizes to the putative DNA-binding domain of Prospero. This would imply that DNA binding and interaction with the export machinery might be mutually exclusive. The transcription factor Stat1 is also subject to controlled nuclear export (Begitt et al., 2000; Mowen and David et al., 2000). Stat1 contains an NES within its DNA-binding domain (McBride et al., 2000). Downregulation of both proteins could occur by simultaneous inhibition of DNA binding and exposure or activation of an NES.

Control of the Prospero NES

We have shown that the C-terminal PD plays an important role in controlling the Prospero NES by inhibiting or masking its function. In the absence of the PD, the Prospero NES is constitutively active and can override the NLS present in Prospero (Fig. 7) or in fusion proteins (Figs 4-6). When the full PD is present, however, the NES is masked or inhibited and the NLS can dominate, restricting Prospero to the nucleus.

A precedent for such NES-masking is provided by the homeodomain transcription factor Exd, which (despite the presence of an NLS) is exported from the nucleus unless its NES is masked by its partner protein Homothorax (Abu-Shaar et al., 1999; Berthelsen et al., 1999). Exd is expressed throughout the Drosophila embryo but enters the nucleus only of cells expressing Hth. In cells lacking Hth the competition between NLS and NES functions favors export. Regulation of the subcellular distribution of another protein, Hdac5, is reminiscent of the Prospero domain/NES interaction, as a partial deletion of the HDAC domain causes nuclear to cytoplasmic re-localization, presumably through the constitutive activity of a more N-terminally localized NESs (McKinsey et al., 2000).

At least two mechanisms could account for the masking of the Prospero NES by the PD. First, the masking region may interact directly with the NES to block association with the nuclear export receptor. Alternatively, the PD may serve to recruit a co-factor analogous to Hth. As the motif is able to block nuclear export in both mammalian and Drosophila cells, it must be highly conserved and is likely to represent a regulatory mechanism common to all higher eukaryotes.

The ability of the PD to inhibit the Prospero NES does not of itself indicate how differential nuclear export of Prospero is achieved. It does, however, indicate that alterations in export occur by regulating activity of the PD. The PD masking region contains numerous potential phosphorylation sites. Biochemical analysis has revealed that Prospero is more highly phosphorylated when cytoplasmic than nuclear (Srinivasan et al., 1998). Phosphorylation status has been implicated in the function of the NESs found in other proteins such as ternary complex factor, Net (Ducret et al., 1999), the yeast protein Far1p (Blondel et al., 1999), Cyclin D1 (Alt et al., 2000) and Hdac5 (McKinsey et al., 2000). The transition in Prospero...
subcellular distribution may be driven by similar kinase cascades acting to modify the masking domain.

By regulating the activity of the NES at different stages of neuronal development it is possible to switch Prospero distribution from cytoplasmic to nuclear and back. In neuroblasts, an active NES will cause the rapid cycling of Prospero protein, which escapes interaction with Miranda and enters the nucleus, back into the cytoplasm. When the neuroblast buds off a GMC, concomitant inactivation of the Prospero NES and degradation of Miranda in the daughter GMC, allows Prospero to localize to the nucleus. After division of the GMC to produce two daughter neurons, reactivation of the NES could allow Prospero to be exported. Thus, regulated nuclear entry of the Prospero transcription factor could control the transcription programs characteristic of each cell type.

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