A hydrophilic lamin-binding domain from the *Drosophila* YA protein can target proteins to the nuclear envelope

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Summary

The nuclear lamina provides an architectural framework for the nuclear envelope and an attachment site for interphase chromatin. In *Drosophila* eggs and early embryos its major constituent, lamin Dm_0, interacts with a lamina protein called YA. When the lamin-interaction region of YA is deleted, YA still enters nuclei but fails to localize to nuclear envelopes, suggesting that lamin interaction targets YA to the nuclear envelope. Here, we show that C-terminal lamin-interacting region of YA is sufficient to target the heterologous soluble protein GFP-NLS to the nuclear periphery in *Drosophila* tissue culture cells. Yeast two-hybrid analysis and transient transfection assays further defined this domain: residues 556-696 of YA are sufficient for both lamin Dm_0 interaction and the targeting of GFP-NLS to the nuclear periphery. This region of YA is hydrophilic and lacks any transmembrane domain or known membrane-targeting motifs. We propose that the localization of YA to the nuclear lamina involves interaction with polymerized lamin Dm_0 mediated by the lamin-targeting domain of YA. This hydrophilic YA domain might provide a useful molecular tool for targeting heterologous non-membrane-associated proteins to the nuclear envelope.

Key words: Nuclear lamina, Targeting, Yeast two-hybrid, *Drosophila* cell transfection, GFP fusion

Introduction

The nuclear envelope of animal cells is composed of two bilayer membranes (the inner and outer nuclear membranes), nuclear pore complexes, which allow for nucleocytoplasmic transport, and a nuclear lamina, the proteinaceous layer that underlies the inner nuclear membrane. Each of these subcompartments contains characteristic proteins that must be targeted to the nucleus and localized to the correct layer of the envelope in the correct orientation (Dreger et al., 2001; Gruenbaum et al., 2000; Wilson et al., 2001).

Lamins, the major constituents of the nuclear lamina, are intermediate filament proteins whose fibers support the nuclear envelope and provide attachment sites for interphase chromatin (Stuurman et al., 1998; Taniura et al., 1995). Most eukaryotes contain multiple species of these hydrophilic coiled-coil proteins, which can usually be subdivided into two groups: lamin A/C proteins found primarily in differentiated cells, and lamin B proteins found in all cell types examined, except for sperm in at least some organisms (Liu et al., 1997; Moir and Spann, 2001; Moir et al., 2000; Riemer et al., 1995). Recent evidence suggests that the nuclear lamina functions, in part, as a scaffold onto which a number of macromolecules are hung. Examples include inner membrane proteins on the cytoplasmic side of the nuclear envelope and chromatin and chromatin-binding proteins on the nucleoplasmic side (Holmer and Worman, 2001). This suggests that lamins can target proteins to the nuclear periphery, either directly or by preferential retention.

Protein targeting to the nuclear periphery is thought to occur after the protein has entered the nucleus (Nakielny and Dreyfuss, 1999). Targeting to the nuclear envelope by lamins and integral nuclear membrane proteins is due to lipid modification motifs or transmembrane domains in these proteins (Gruenbaum et al., 2000; Holaska et al., 2002). Lamins A and B possess a lipid modification motif (CAAX) at their C-terminus that can be isoprenylated, allowing insertion into membranes and targeting to the nuclear periphery (Hofemeister et al., 2000; Holtz et al., 1989; Kitten and Nigg, 1991). Transmembrane domains are essential for nuclear periphery targeting of integral proteins to the inner nuclear membrane, such as the lamin B-receptor (Soullam and Worman, 1995), MAN1 (Lin et al., 2000; Wu et al., 2002), emerin (Fairley et al., 1999), nurim (Rolls et al., 1999) and nesprins (Zhang et al., 2001). Localization of these proteins can be explained by a 'diffusion-retention' model (Ellenberg et al., 1997). These proteins are translated on ER (endoplasmic reticulum)-bound ribosomes and are inserted into the ER membrane. They diffuse within that membrane, entering the inner nuclear membrane compartment. Presence of these proteins at the nuclear periphery is often further stabilized by interaction with lamin (e.g. Furukawa et al., 1998; Vaughan et al., 2001), and chromatin and/or additional DNA bridging proteins such as BAF (Holmer and Worman, 2001; Vlcek et al., 2001).

Very little is known, about how hydrophilic non-membrane-

associated proteins are targeted to, and retained on, the nucleoplasmic side of the nuclear envelope. The *Drosophila* YA (Young Arrest) protein is an excellent test case to investigate this question. YA is an essential, maternally provided, nuclear lamina protein found in *Drosophila* oocytes, eggs and early embryos (Lin and Wolfner, 1991; Lopez et al., 1994). This entirely hydrophilic protein, with no discernible lipid modification or membrane-targeting motifs, is required for fertilized eggs to enter the mitotic cleavage phase of early development. YA binds to chromatin (Lopez and Wolfner, 1997; Yu and Wolfner, 2002) and localizes to the nuclear laminas of cleavage-stage embryos in a cell-cycle-dependent manner (Lin et al., 1991). YA and lamin Dm_0 [the B-type lamin of *Drosophila* and the only lamin present in embryos of this stage (Harel et al., 1989; Riemer et al., 1995)] dissociate from the nuclear periphery at metaphase. Lamin Dm_0 reassembles at the nuclear periphery at telophase; YA first appears at the nuclear periphery at the start of the next interphase (Lin and Wolfner, 1991). Nuclear envelope localization of YA appears to be necessary for its function since mutant YA proteins that enter nuclei, but do not localize to the nuclear periphery, do not rescue the null *Ya* phenotype (Liu and Wolfner, 1998).

To address how YA attains its subnuclear location, we searched for YA-interacting proteins using a yeast two-hybrid assay (Goldberg et al., 1998). Lamin Dm_0 was identified as a partner of YA in this screen. Only full-length lamin interacted with YA, suggesting that YA binds only to polymerized lamin. The C-terminal 190 amino acids (residues 506-696) of YA were sufficient for a two-hybrid interaction with lamin Dm_0 (Goldberg et al., 1998). Deletion of this region from YA prevented nuclear peripheral targeting of YA in *Drosophila*, although the deleted YA still entered nuclei (Liu and Wolfner, 1998).

These data, coupled with the cell cycle kinetics of YA relative to those of lamin Dm_0, led us to propose that the interaction of YA with the polymerized lamin network that is assembled at the nuclear periphery targets YA to the nuclear periphery (Goldberg et al., 1998). We test this hypothesis by narrowing down the lamin-interaction domain of YA using two-hybrid analysis and testing whether this region of YA can target a heterologous, non-membrane protein to the nuclear lamina. We identify a hydrophilic domain capable of interaction with lamin that targets a heterologous soluble protein to the *Drosophila* nuclear periphery. The lamin-interaction domain could be useful for targeting proteins to the nucleoplasmic side of the nuclear envelope.

Materials and Methods

Yeast two-hybrid analysis

We used the Matchmaker yeast two-hybrid system (Clontech), with C-terminal sequences from YA cloned into the DNA-binding domain vector pGBT9, and the lamin-pGAD424 construct as described previously (Goldberg et al., 1998). Yeast cells co-transformed with pGBT9 and pGAD424 derivatives were grown on Trp^- Leu^- synthetic medium and tested for interaction using β-galactosidase filter assays and ONPG liquid assays as described previously (Goldberg et al., 1998). The presence of YA fusion proteins of the correct size was confirmed by western blotting for yeast carrying the fusions to YA regions 506-696, 556-696 and 607-696.

Plasmid constructions

Constructs containing regions of the C-terminal domain of YA were generated by PCR by using gene-specific primers containing engineered *Eco*RI sites (primer sequences will be provided upon request). The amplified products were cloned into *Eco*RI site of pGBT9 and their sequences verified (Cornell Bioresource Center, Ithaca NY).

For S2 cell transfections, the GFP-coding sequence was amplified from eGFP-N1 (Clontech) using gene-specific primers with *Kpn*I and *Pst*I overhangs. The SV40 T-antigen nuclear localization signal (NLS) sequence (Makkerh et al., 1996) was synthesized as oligonucleotides with *Pst*I and *Eco*RI overhangs and annealed. The digested PCR product and oligonucleotides were ligated into pBS KS(+) (Stratagene) and verified by sequencing. GFP-NLS was then cloned into the pMT V5 B vector (Invitrogen) to generate pMT GFP-NLS. The metallothionein promoter in this construct allows inducible expression in cultured *Drosophila* cells (Bunch et al., 1988). *Eco*RI fragments from the YA C-terminal deletions in pGBT9 (see above) were cloned into the *Eco*RI site of pMT GFP-NLS to generate pMT GFP-NLS-YA constructs.

Nuclear envelope targeting assay

18 μg of pMT GFP-NLS (control) or pMT GFP-NLS-YA constructs was transfected into S2 cells using the calcium phosphate method (Di Nocera and Dawid, 1983); (*Drosophila* Expression System, Invitrogen). The transfected cells were induced to express GFP-NLS or GFP-NLS-YA with 0.5 mM $CuSO_4$ per well. 24 hours later, the cells were harvested, fixed and processed as described by Sangoram et al. (Sangoram et al., 1998). Typically, 30-40% of the transfected cells expressed the construct, although the levels of expression varied between cells. Representative fields of cells were imaged and analyzed. Endogenous lamin Dm_0 in the transfected S2 cells was detected by staining with affinity-purified rabbit polyclonal lamin Dm_0 antibody, followed by rhodamine-conjugated goat α-rabbit IgG secondary antibody (Jackson ImmunoResearch Laboratories, West Grove, PA). Actin was detected by staining with AlexaFluor-594-coupled phalloidin (Molecular Probes, Eugene, OR) following the manufacturer's protocol. DNA in fixed cells was stained using either DAPI (0.1 μg/100 μl) or propidium iodide (1 μg/ml).

Immunofluorescence imaging and confocal microscopy

The fixed, stained and mounted S2 cells were imaged using an Olympus BX-50 fluorescence/DIC microscope fitted with a high resolution Pentamax cooled CCD camera (Princeton Instruments) and equipped with digital microscopy image analysis software (Metamorph, Universal Imaging Corporation, PA). For further resolution, a Bio-Rad MRC-600 attached to a Zeiss Axiovert 10-inverted microscope was used to image single optical sections of transfected and antibody stained cells at 63× or 100× magnification. Signals from GFP and red (propidium iodide, rhodamine or Alexa Fluor 594) channels were collected using a Z-series to determine colocalization. The acquired images were processed using Confocal Assistant software (BioRad) and images were assembled using Photoshop (Adobe).

Results and Discussion

The C-terminal domain of YA targets a heterologous protein to the nuclear periphery in transfected *Drosophila* S2 cells

To determine whether the C-terminal 190 amino acids of YA, which interact with lamin Dm_0 (Goldberg et al., 1998), are sufficient to target a protein to the nuclear periphery, we examined the subcellular distribution of a fluorescent reporter

Fig. 1. Intracellular distribution of GFP-NLS and GFP-NLS-YA (506-696) fusion in *Drosophila* S2 cells. Fluorescence images of S2 cells transfected with GFP-NLS or GFP-NLS-YA (506-696). A-C shows a representative cell expressing GFP-NLS. (A) DAPI staining to mark the nucleus. (B) GFP fluorescence in nucleus and cytoplasm (indicated by *). (C) Overlay of A and B. D-G shows a representative field of cells expressing GFP-NLS-YA (506-696). (D) DAPI staining of nuclei (arrowhead indicates a transfected cell). (E) GFP fluorescence from the expressed fusion. (F) Overlay of panels D and E. (G) Higher magnification view of transfected cells expressing GFP-NLS-YA (506-696) showing GFP fluorescence in the nuclear periphery surrounding DNA (red). Bar, 10 μm.
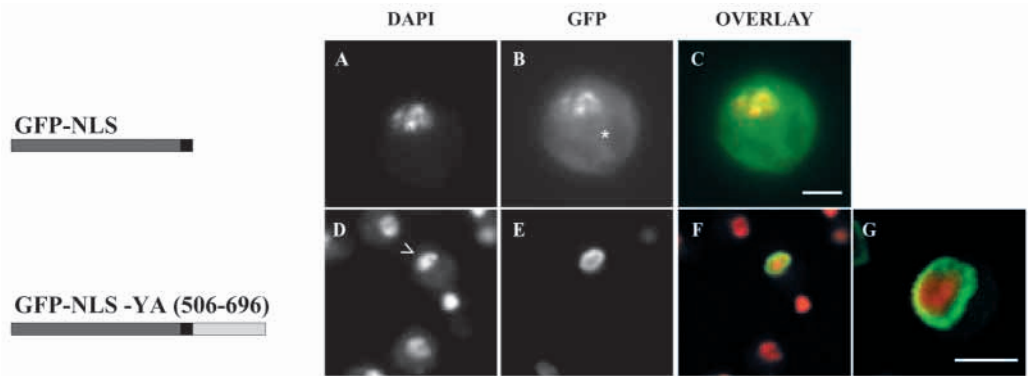
protein fused to this domain. The reporter, GFP-NLS, contains GFP for detection and the SV40 T-antigen's nuclear localization sequence (NLS) to permit entry into nuclei (Makkerh et al., 1996). We transfected GFP-NLS-YA constructs into *Drosophila* S2 cells. These cells correctly target wild-type YA expressed during transient transfection [data not shown; expressed YA is seen in the nucleoplasm and nuclear periphery, as previously reported for wild-type YA transiently transfected into *Drosophila* Kc cells (Lopez et al., 1994)]. S2 cells do not contain endogenous YA (data not shown) that compete with the expressed fusion protein for binding with endogenous molecular partners.

Control GFP-NLS protein expressed in S2 cells is detected in both nuclei and cytoplasm, but appears slightly enriched in the former compartment, presumably owing to targeting by its NLS (Fig. 1A-C). Its fluorescence was noticeable throughout the nucleus, including the nuclear periphery, and the cytoplasm (Fig. 1B,C). We believe that the presence of the fusion protein in the cytoplasm, despite the presence of an NLS, is due to passive diffusion through the nuclear pores, as the predicted size of GFP-NLS, 27.4 kDa, is below the 30 kDa size limit for passive diffusion (Keminer and Peters, 1999). Analogous observations of GFP distribution in transfected CHO-K1 cells indicate that GFP on its own does not show preferential retention in any intracellular compartment (Broers et al., 1999).

Fusion of amino acids 506-696 of YA to GFP-NLS targets the fusion protein preferentially to the nuclear periphery (Fig. 1D-G, Fig. 2A). GFP fluorescence was exclusively nuclear, and within that compartment primarily circumferential around the DNA; this was particularly evident in cells expressing high levels of the reporter (Fig. 1F). These results show that the C-terminal region of YA (residues 506-696), the region that interacts with lamin Dm$_0$ in the yeast two-hybrid system, is sufficient to target a heterologous protein to the *Drosophila* nuclear periphery. The location of the GFP-NLS-YA fusion in transfected S2 cells was nearly identical to that of wild-type (untagged) YA expressed in transfected Kc cells (Lopez et al., 1994). Interestingly, preliminary experiments using mammalian HEK cells (S.S.M., unpublished) suggest that the same YA domain fused to GFP-NLS is not sufficient to target the reporter protein to the mammalian nuclear periphery. This observation suggests that the targeting ability of YA 506-696 is not species-general but instead may require a conformation

or sequence of the lamina (or lamin) present in the *Drosophila* nuclear envelope.

Dissection of the lamin-binding domain of YA

To further define the lamin-interaction domain of YA, we tested deletions of YA (506-696) for their ability to interact with lamin Dm$_0$ in the yeast two-hybrid system (Table 1). The interaction was assessed by using the ability of cells carrying the YA deletion in pGBT9 and lamin in pGAD424 to grow in the absence of histidine and by using β-galactosidase reporter activity. Lamin interaction was not impaired by deleting 50 amino acids from the N-terminus of the 190-residue YA region (YA 556-696). Deletion of up to 50 additional amino acids from the N-terminus of the lamin-binding domain (YA 581-696 and YA 607-696) still allowed significant interaction with lamin. These data indicate that the C-terminal 140 amino acids (YA 556-696), and possibly the C-terminal 90 residues (YA 607-696), are sufficient to interact with lamin.

Table 1. Interactions between lamin and various C-terminal deletion constructs of YA as measured by yeast two-hybrid assay
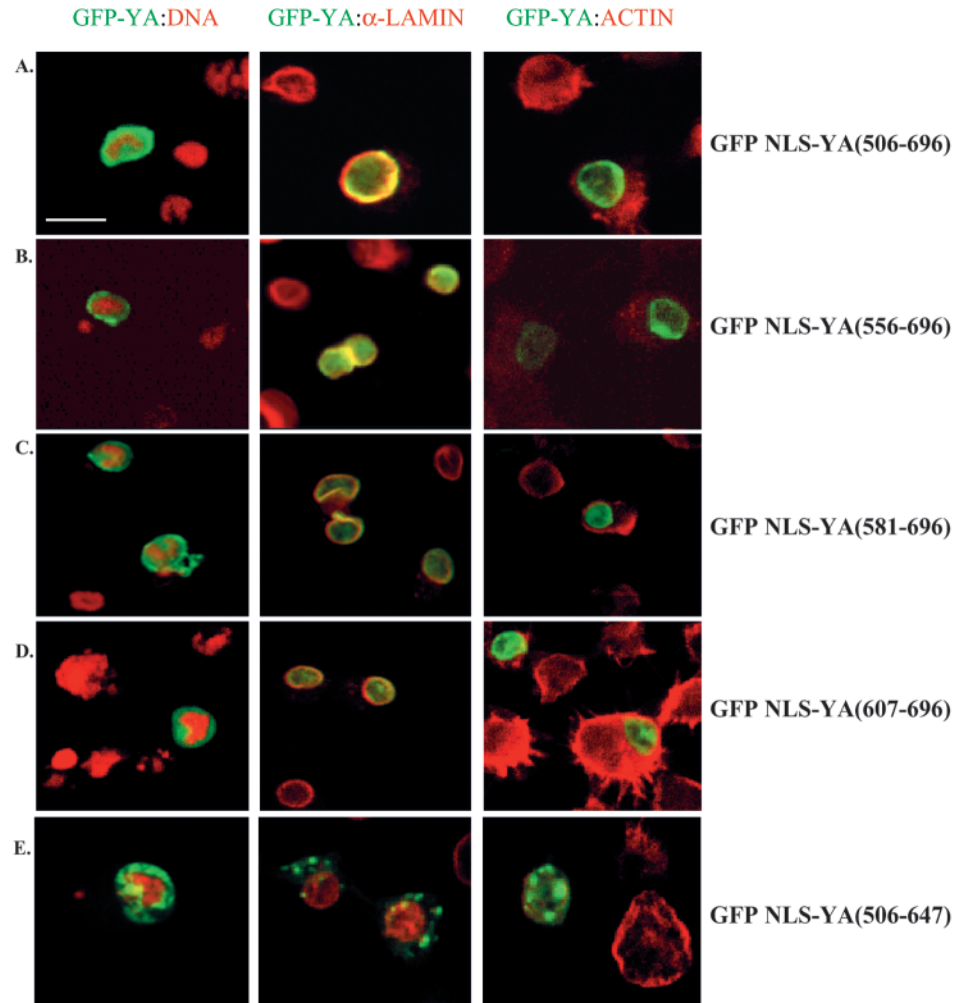
AD construct	BD construct	Growth without histidine*	β-Gal filter assay[†]
Lamin	YA 506-696	+++	+
Lamin	YA 556-696	+++	+
Lamin	YA 581-696	++	+
Lamin	YA 607-696	++	(+)
Lamin	YA 506-647	–	–
Lamin	YA 506-671	–	–
Lamin	YA 581-671	–	–

AD and BD represent the pGAD424 and pGBT9 two-hybrid vectors, respectively. The amino acids of each C-terminal YA deletion are indicated in the BD construct; these regions were fused in-frame to pGBT9 as described in Materials and Methods. None of the fusion constructs was able to promote transcription of the reporter genes when cotransformed with the other empty vector into yeast hosts (data not shown).

*+++ (strong), ++ (modest), + (weak) refers to the ability of yeast strains carrying the GAL1-*HIS*$_3$ reporter gene and the AD and BD plasmids to grow on plates lacking histidine.

[†]β-Gal activity of yeast grown with glucose as the carbon source as determined by X-Gal analysis of cells immobilized on filters. + indicates blue, (+), pale blue and –, white color of yeast in the X-Gal filter assay.

Fig. 2. Nuclear envelope localization and targeting of GFP-NLS-YA constructs in transfected S2 cells. Confocal images of S2 cells that express the listed GFP-NLS-YA constructs. GFP fluorescence was visualized in fixed, permeabilized cells at 24 hours post-induction. The location of GFP fluorescence was determined relative to that of DNA (left panels, propidium iodide staining), lamin Dm_0 (middle panels, stained with rabbit α-lamin) or actin (to visualize the cytoplasm; right panels, stained with AlexaFluor-594-coupled phalloidin). Bar, 10 μm.

GFP-YA:DNA   GFP-YA:α-LAMIN   GFP-YA:ACTIN

A.   GFP NLS-YA(506-696)
B.   GFP NLS-YA(556-696)
C.   GFP NLS-YA(581-696)
D.   GFP NLS-YA(607-696)
E.   GFP NLS-YA(506-647)

Deletion of amino acids 506-696 from the C-terminus of YA abolished the interaction with lamin in the two-hybrid system (YA 506-647, YA 506-671 and YA 581-671; Table 1). This suggests that residues 671-696 of YA are necessary for optimal lamin interaction and/or for proper folding (see below). In summary, residues 607-696 of YA are sufficient for interaction with lamin Dm0, although additional residues N-terminal to this region (residues 556-606) may strengthen the association.

Subcellular localization of GFP-NLS-YA deletions in transfected S2 cells

We next tested the same subregions of YA's C-terminus of YA for their ability to target GFP-NLS fusions to the nuclear periphery in transfected *Drosophila* S2 cells. Nuclear envelope targeting is retained by the N-terminal deletions that interact with lamin Dm_0 in the two-hybrid system (YA 556-696, YA 581-696, and YA 607-696) (Fig. 2B-D). Although these GFP-NLS-YA fusions are targeted to the nuclear periphery, there is also nucleoplasmic staining. Because this distribution is like that seen with full-length YA expressed in Kc cells (Lopez et al., 1994), we believe that it reflects binding of YA sequences to a minor population of lamin Dm_0 found by P. Fisher and colleagues to reside in the interior of the *Drosophila* nucleus (P. Fisher, personal communication) or to chromatin (Yu and Wolfner, 2002) or saturation of YA binding sites owing to high level expression of the transfected DNA. Lamin-GFP fusions have also been reported to localize to the interior of the nucleus (in addition to the nuclear periphery) in transfected mammalian cells (Broers et al., 1999).

To test whether lamin-interacting sequences at the C-terminus of the 190 amino acid domain are essential for targeting, we carried out similar experiments on GFP-NLS-YA 506-647, 506-671, and 581-671. Although in each case the protein was present in transfected cells, it was distributed in a punctate fashion in the cytoplasm and failed to translocate into the nucleus (for example, see Fig. 2E). A likely explanation for

this apparent lack of nuclear entry by these NLS-containing fusion proteins is that the C-terminal truncations of the YA domain cause misfolding of the fusion protein. This would also explain why these deletions fail to interact with lamin in the two-hybrid system. Secondary structure analysis of amino acids in the lamin-interaction domain (506-696) predicts a strong propensity to form α-helices and turns (Chou and Fasman, 1978a; Chou and Fasman, 1978b; Garnier et al., 1978). Disrupting this region could cause a conformation unsuitable for proper folding of the fusion protein. In particular, amino-acid residues from YA 647 to 696 include a number of charged residues (38%) that could form hydrogen bonds or participate in electrostatic interactions. Such interactions are believed to stabilize the intrahelical ion pairing and contribute to higher-order multimolecular interactions in the case of intermediate filament proteins (Letai and Fuchs, 1995). Future studies using site-directed mutagenesis can shed light on the role of this region, and of the charged amino acids within it, in mediating the interaction of YA with polymerized lamin Dm_0.

Our results, summarized in Fig. 3, favor a model in which YA is targeted to, and/or retained at, the nuclear periphery by interaction with the polymerized lamin network that has already assembled at this location. This targeting mechanism, for a largely hydrophilic protein, differs from mechanisms

Fig. 3. Summary of nuclear envelope localization and lamin interaction of C-terminal deletions of YA. Regions of the YA C-terminus (light gray box) fused in frame with the GFP coding sequence (dark gray box) and SV40 T-Ag NLS (black box) tested for nuclear periphery location (Figs 1 and 2) or in the yeast two-hybrid system (Table 1) are shown. Nuclear envelope localizations of GFP-NLS-YA fusions are: +, colocalization with lamin; –, presence of the fusion in both nucleus and cytoplasm with no preferential peripheral staining of the nucleus; 'misfolded', inability of the fusion protein to enter the nucleus. Interaction of the various deletion constructs with lamin Dm_0 in the yeast two-hybrid assay: +, strong interaction; (+), weak interaction; –, no detectable interaction.

	Nuclear envelope localization	Interaction in yeast two-hybrid assay
506-696	+	+
556-696	+	+
581-696	+	+
607-696	+	+
506-647	Misfolded	-
GFP-NLS	-	N.D.

reported for nuclear periphery targeting of proteins that insert into the nuclear membranes in the course of their targeting (Ashery-Padan et al., 1997; Ellenberg et al., 1997; Foisner and Gerace, 1993; Hofemeister et al., 2000; Soullam and Worman, 1995; Worman et al., 1990; Wu et al., 2002). For one of those proteins, mammalian LAP2, and for a heterologous transmembrane reporter protein, a lamin-binding domain was suggested to stabilize targeting to the nuclear periphery by preventing the protein's diffusion from the inner nuclear membrane compartment (Furukawa et al., 1998). Our results indicate that binding to lamin Dm_0 can also target proteins to the *Drosophila* nuclear periphery from the nucleoplasmic side of the envelope without requiring a membrane tether. The requirement of lamin A for the presence of lamin C [a lamin variant that lacks the C-terminal CAAX motif (Lin and Worman, 1993)] at the mammalian nuclear periphery is consistent with use of an analogous mechanism to target hydrophilic proteins to the nuclear envelope of vertebrate cells (Pugh et al., 1997; Vaughan et al., 2001).

The cell cycle dynamics of YA are consistent with this model. Lamin Dm_0 is present in the nuclei of all cells in which YA is in the nuclear envelope (Lin et al., 1991; Lin and Wolfner, 1991; Liu et al., 1995; Lopez et al., 1994). At metaphase, the two proteins disappear from the nuclear periphery simultaneously, as one would expect if the presence of YA at the nuclear periphery depended on the presence of polymerized lamin. After anaphase, both proteins reappear at the nuclear periphery, but lamin Dm_0 is detected there before YA reappears (Lin and Wolfner, 1991), consistent with the idea that lamin polymerizes to form a scaffold to which YA becomes attached. Our model is further supported by the finding that the YA-lamin interaction in yeast is abolished if either the head or tail domain of lamin is deleted, suggesting that YA interacts with a multimeric form of lamin Dm_0 (Goldberg et al., 1998).

The 90 amino-acid region of YA that we have defined here also provides a useful tool for further studies of nuclear structure and function in the *Drosophila* model system. It could be used to bring to the nuclear envelope hydrophilic proteins that are not normally found at the nuclear periphery, for studies of nuclear assembly, replication or transcriptional silencing of chromatin (Andrulis et al., 1998). It would also be interesting to see if this domain can compensate for loss of a nuclear targeting domain from nuclear envelope proteins that normally use other mechanisms to target to this cellular compartment.

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