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Distinct Regions Specify the Targeting of Otefin to the Nucleoplasmic Side of the Nuclear Envelope*

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Otefin is a 45-kDa nuclear envelope protein with no apparent homology to other known proteins. It includes a large hydrophilic domain, a single carboxyl-terminal hydrophobic sequence of 17 amino acids, and a high content of serine and threonine residues. Cytological labeling located otefin on the nucleoplasmic side of the nuclear envelope. Chemical extraction of nuclei from *Drosophila* embryos revealed that otefin is a peripheral protein whose association with the nuclear envelope is stronger than that of lamin. Deletion mutants of otefin were expressed in order to identify regions that direct otefin to the nuclear envelope. These experiments revealed that the hydrophobic sequence at the carboxyl terminus is essential for correct targeting to the nuclear envelope, whereas additional regions in the hydrophilic domain of otefin are required for its efficient targeting and stabilization in the nuclear envelope.

The nuclear envelope separates the nucleoplasm from the cytoplasm. It is a complex structure composed of outer and inner membranes that are separated by the perinuclear space. The two membranes are joined at the nuclear pore complexes. Underneath the inner membrane, there is a proteinaceous meshwork of intermediate filaments termed the nuclear lamina (for reviews, see Refs. 1–3). The inner and outer membranes of the nuclear envelope differ in their morphology and composition. Nevertheless, the two membranes are joined at the nuclear pore complexes. The way by which inner membrane proteins reach their target membrane and are retained there has been the subject of several recent studies. Isoprenylation of the C*AAX* motif in conjunction with a nuclear localization signal has been shown to be necessary in order to direct newly synthesized nuclear lamins to the inner nuclear envelope (4). The C*AAX* motif-mediated modifications, although necessary, are not sufficient for stable association of lamins with membranes (5). Therefore, additional factors such as inner membrane proteins must be involved in lamin-membrane association (6–8). At least two independent nucleoplasmic regions of LAP2 [1] are responsible for its nuclear envelope targeting, while the transmembrane domain of LAP2 mediates nonspecific membrane binding at the endoplasmic reticulum (9). Both the first transmembrane domain (10) and the amino-terminal domain of LBR (11, 12) mediate the targeting of LBR to the inner nuclear membrane. The highly charged amino-terminal domain of LBR can also direct cytosolic proteins to the nucleus and type II integral membrane proteins to the inner nuclear membrane (10). It was proposed that the mechanism for inner membrane targeting and retention of LBR and LAP2 involves lateral diffusion in the interconnected membranes of the endoplasmic reticulum and nuclear envelope and interaction with components of the nuclear lamina and chromatin (9, 12).

The *Drosophila* nuclear envelope protein otefin has a mobility on SDS-polyacrylamide gel electrophoresis consistent with a molecular mass of 53 kDa (13). The otefin gene encodes a putative primary translation product of 45 kDa with no apparent homology to known proteins. It is highly hydrophilic with a relatively high content of serine and threonine residues and a putative site for phosphorylation by Cdc2 kinase. The 17 carboxyl-terminal residues of the otefin protein are hydrophobic and resemble membrane-spanning domains of integral membrane proteins (14). Polyclonal antibodies raised against *Drosophila* otefin revealed conservation of mammalian nuclear envelope epitope(s) (14). Immunoelectron microscopy showed that otefin is associated with the nuclear envelope, yet it is excluded from the nuclear pores (13).

In this study, we have determined by immunogold electron microscopy that otefin is present underneath the inner nuclear membrane, facing the nucleoplasm. Extraction studies have demonstrated that in contrast to LAP1, LAP2, and LBR, otefin behaves as a peripheral protein. Expression in COS-7 cells of deletion mutants of otefin and of fusion constructs between otefin truncations and the *lacZ* gene coupled to a nuclear localization signal (NLS-*lacZ*) revealed that the COOH-terminal 17-amino acid hydrophobic sequence of otefin is essential for the targeting of otefin to the nuclear periphery. This sequence alone can target NLS-*lacZ* to the nuclear rim, albeit with low efficiency. Other sequences that are located between amino acids 173 and 372 of otefin are required for efficient targeting to the nuclear envelope, while sequences between amino acids 35 and 172 are required for stabilizing the interaction of otefin with the nuclear envelope.

MATERIALS AND METHODS

Antibodies—Anti-*Drosophila* lamin Dm mAb 611A3A6 and anti-*Drosophila* otefin mAb 618A2O7 have been described (13, 15). Polyclonal anti-*Drosophila* otefin antibodies were prepared by injecting 500 μg of otefin cDNA translation product into rabbits. Anti-β-galactosidase (Z378A) and anti-tubulin (T9026) mAbs were purchased from Promega and Sigma, respectively. Cy_3A-conjugated goat anti-rabbit IgG (H + L), Cy_3A-conjugated goat anti-mouse IgG, alkaline phosphatase-conjugated goat anti-rabbit IgG (H + L), and anti-rabbit IgG conjugated to 12-nm gold particles (catalog No. 111-205-144) were purchased from Jackson ImmunoResearch Laboratories, Inc. (West Grove, PA).

Vectors and Constructs—Vector pSP64-497–524.Z (16) is a eukaryotic expression vector carrying an SV40 enhancer, an α-globin pro-

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[1] The abbreviations used are: LAP, lamina-associated protein; LBR, lamin B receptor; NLS, nuclear localization signal of the glucocorticoid receptor (residues 497–524); mAb, monoclonal antibody; FCS, fetal calf serum; PBS, phosphate-buffered saline; TBS, Tris-buffered saline; TPCK, *N*-tosyl-L-phenylalanine chloromethyl ketone.

moter, NLS sequence of the glucocorticoid receptor (amino acids 497–524) fused 5′ to the *lacZ* sequences encoding the β-galactosidase protein, and polyadenylation sequences. pCA1038 (a kind gift from Dr. Nissim Benvenisty) is an expression vector containing the mouse phosphoglycerate kinase I promoter (17), a multiple cloning site, and polyadenylation sequences.

pCA1038-otefin was prepared by subjecting the *Eco*RI fragment containing the complete otefin cDNA (14) to end filling of the recessed 3′-ends with DNA polymerase I Klenow fragment and cloning it into the *Sma*I site of pCA1038. Δ388–406 was prepared by inserting a stop codon after amino acid 387 to pCA1038-otefin. Δ35–172 was prepared by digesting pCA1038-otefin with *Eag*I and *Hin*cII. Following end filling with a DNA polymerase I Klenow fragment, the construct was self-ligated to create a deletion between amino acids 34 and 173. Δ35–172,Δ388–406 was prepared by replacing the carboxyl terminus of Δ35–172 at the *Bgl*II site with the carboxyl terminus of Δ388–406.

NLS-lacZ-Otefin Fusions—The construction of fusions between otefin cDNA and the *lacZ* gene was performed in a pT7-7 vector in which the *Nde*I and *Eco*RI restriction sites were destroyed. The NLS-*lacZ* region was then cloned into the *Bam*HI site of pT7-7. The NLS-*lacZ*-otefin cDNA fusions (see below) were subcloned into the *Bam*HI restriction site, replacing the normal *lacZ* gene in pSP64-497–524.Z with the NLS-*lacZ*-otefin fusions.

To prepare NLS-*lacZ*-otefin, the AAAATG translation start sequence in otefin cDNA was polymerase chain reaction-mutated to the *Nde*I site CATATG. The *Nde*I-*Eco*RI fragment of otefin cDNA, which contains the complete open reading frame, was cloned into the *Nde*I and *Eco*RI sites at the 3′-end of the *lacZ* coding sequences. This created an in-frame fusion between *lacZ* and otefin. NLS-*lacZ*-Δ1–33 was prepared by inserting the *Eag*I-*Eco*RI fragment of otefin cDNA, encoding amino acids 34–406, into the *Eco*RI site of the *lacZ* gene. Both the *Eag*I-*Eco*RI fragment of otefin and the *Eco*RI site in *lacZ* were subjected to end filling of the recessed 3′-ends prior to the ligation. NLS-*lacZ*-Δ372–406 was prepared by digesting NLS-*lacZ*-otefin with *Stu*I and *Eco*RI. The digested construct was subjected to end filling of the recessed 3′-ends and self-ligated. NLS-*lacZ*-Δ1–371 was prepared by digesting otefin cDNA with *Stu*I and *Eco*RI. The *Stu*I-*Eco*RI fragment, encoding amino acids 1–371, was cloned into the *Nde*I site of the *lacZ* gene. Both the *Stu*I-*Eco*RI fragment of otefin and the *Nde*I site in *lacZ* were subjected to end filling of the recessed 3′-ends prior to the ligation.

Cell Culture and Transfection—COS-7 cells (American Type Culture Collection CRL1651, SV40-transformed CV-1 cells) were grown in Dulbecco's modified Eagle's medium containing 10% FCS, 40 mM glutamine, 200 units/ml penicillin, and 0.2 mg/ml streptomycin (Beit Haemek, Israel). For transfection, 5×10^5 cells were plated on 10 × 10-mm coverslips (prewashed with 70% ethanol and treated with poly-L-lysine (Sigma)) in a six-well plate and grown overnight. Lipofection mixtures were prepared by vortexing 1 ml of Opti-MEM (Life Technologies, Inc.) and 15 μl of Lipofectin transfection reagent (Boehringer Mannheim catalog No. 1202375) in polystyrene tubes. 5 μg of supercoiled plasmid DNA (prepared with a Qiagen plasmid kit, QIAGEN GmbH, Hilden, Germany) were then added and mixed gently for 10 min with the lipofection mixture. Following overnight incubation, the cells were washed once with Opti-MEM, and the lipofection mixture was then added to the cells. After a 5-h incubation, 1 ml of Dulbecco's modified Eagle's medium supplemented with 20% FCS was added to the culture. After 24 h, the transfection medium was replaced with fresh Dulbecco's modified Eagle's medium containing 10% FCS. Following an additional 24 h, the cells were fixed and stained for immunofluorescence analysis.

Immunogold Localization of Otefin in Drosophila Nuclei—Drosophila embryos (0–7 h old) were dechorionated and homogenized in buffer NM (250 mM sucrose, 2.5 mM $MgCl_2$, 50 mM KCl, 1 mM dithiothreitol, 10 μM leupeptin, 10 μg/ml aprotinin). Nuclei were separated by centrifugation of the homogenate at 10,000 × *g* for 10 min at 4 °C. The isolated nuclei were washed at 4 °C with PBS, fixed for 30 min at room temperature in PBS containing 4.0% paraformaldehyde and 0.1% glutaraldehyde, and washed with PBS containing 1% bovine serum albumin and 0.1% Triton X-100 (PBSBT). The nuclei were then incubated for 16 h at 4 °C with polyclonal anti-*Drosophila* otefin antibody diluted in PBSBT, washed three times for 1 h each with PBSBT, and then incubated for 2 h at room temperature with 12-nm colloidal gold-conjugated goat anti-rabbit IgG diluted 1:25 in PBSBT. Nuclei were washed for 2 h with PBSBT and then post-fixed with PBS containing 2% formaldehyde and 2% glutaraldehyde for 30 min at room temperature. Following a wash with PBS, nuclei were washed for 16 h at 4 °C with 100 mM sodium cacodylate (pH 7.5) and then fixed for 90 min at room temperature with 1% OsO_4 in 100 mM sodium cacodylate. Samples were dehydrated, embedded, sectioned, stained, and viewed as described previously (13).

Immunolocalization of Otefin in Transfected Cells—Transfected cells were grown to ~80% confluence on 10 × 10-mm coverslips, washed once with PBS, fixed with 100% methanol for 10 min at 4 °C, washed again with PBS, and fixed again for 30 min at 20 °C with 4% formaldehyde in PBS containing 0.1% Triton X-100 (PBSTR). Cells were then washed three times for 30 min: first with PBS, then with PBSTR, and then with PBSTR containing 2% FCS. These washes were followed by a single 10-min wash with PBSTR containing 5% low fat milk. Cells were then incubated overnight at 4 °C with mAb 618A2O7 or mAb Z387A diluted in PBS. Following incubation with the antibody, the cells were washed three times for 30 min each with PBSTR and were stained for 2 h with Cy_3A-conjugated goat anti-mouse IgG in PBS containing 1% FCS. The cells were then washed three times for 30 min with PBSTR and stained with 1 μg/ml 4′,6-diamidino-2-phenylindole in PBS. Coverslips were mounted with 2% (w/v) *n*-propyl gallate in 50% glycerol/PBS (1:1) and viewed with a Leitz epifluoresence microscope using a 63×/NA = 1.4 Planapo objective lens. To better localize the immunofluorescence signal, the same cells were also viewed with a Bio-Rad confocal microscope (see below).

Triton X-100 extraction of cells was performed by incubation of the cells prior to fixation with 1% Triton X-100 for 10 min at 4 °C, followed by a brief wash with PBS, after which the cells were fixed and treated as described above.

Confocal Microscopy—A Bio-Rad MRC-1024 confocal scanhead coupled to a Zeiss Axiovert 135M inverted microscope was used to acquire images of the stained cells. A 63×/NA = 1.4 objective was used. Excitation light was provided by a 100-microwatt air-cooled argon ion laser run in the multiline mode. The 514-nm line of the laser was used for excitation and was selected with an interference filter. The emission filter was a D580/32 (32 band pass centered about 580 nm). The confocal iris diameter was between 1.3 and 3.5 mm, with the larger opening used for weaker signals. Vertical resolution was between 0.5 and 1 μm, depending on the iris setting. Two to four images were averaged in order to reduce point noise. Images of 512 × 512 pixels were acquired using a hardware zoom of 3–10 (0.106–0.032 μm/pixel). When needed, a 3 × 3 median filter was used to remove point noise from the digital images.

Immunoelectron Microscopy Localization of NLS-lacZ-Otefin in Transfected Cells—Labeling was with horseradish peroxidase-conjugated antibodies, and visualization was with the chromogen 3,3′-diaminobenzidine tetrahydrochloride and H_2O_2 since they allowed the detection of the few cells that expressed the construct with a light microscope before visualization with an electron microscope. COS-7 cells were transfected with NLS-*lacZ*-otefin. After 48 h, the cells were collected; washed with PBS; and fixed for 30 min at room temperature in PBS (pH 7.4) containing 4% paraformaldehyde, 0.2% picric acid, and 0.05% glutaraldehyde. Subsequently, the cells were washed five times with PBS and twice with TBS. The fixed and washed cells were incubated for 30 min with 0.25% sodium borohydride in water; washed eight times with TBS; and then incubated with blocking solution containing 0.05% Triton X-100, 2% egg albumin, 0.5% glycine, 0.5% lysine, and 0.9% NaCl in 0.5 M Tris-HCl (pH 7.4). Cells were then incubated for 72 h at 4 °C in 4 μg/ml mAb Z378A (anti-β-galactosidase) in TBS containing 0.05% Triton X-100 and 1% egg albumin. The cells were washed eight times with TBS and incubated for 3 h at room temperature with biotinylated secondary anti-mouse antibody. The cells were then incubated for 2 h in avidin-biotinylated horseradish peroxidase (Vectastain, Elite ABC kit, Vector Laboratories, Inc.) and washed eight times with TBS. The horseradish peroxidase was visualized using the chromogen 3,3′-diaminobenzidine tetrahydrochloride and 0.02% H_2O_2. The cells were washed eight times with TBS and embedded in 2% low melting point agarose. The cells were then post-fixed for 1 h at room temperature in 0.1 M cacodylate buffer containing 1% OsO_4 and 1.5% $K_3Fe(CN)_6$. After brief washes with 0.1 M cacodylate, the cells were dehydrated in ascending concentrations of ethanol and infiltrated with epoxy resin. Embedding, sectioning, staining, and viewing were as described (13).

Extraction of Nuclei—Nuclei were prepared according to Adra *et al.* (17) by thawing frozen 0–15-h-old *Drosophila* (Canton S) embryos on ice and homogenizing them in 10 volumes of buffer D (10 mM Tris-HCl (pH 8), 5 mM $MgCl_2$, 1.3 M sucrose). The homogenate was filtered through a 125-μm nylon mesh overlaid above a cushion of buffer D and centrifuged at 10,000 × *g* for 10 min at 4 °C. The supernatant was removed, and the nuclear pellet was washed twice with buffer B (20 mM Tris-HCl (pH 7.5), 50 mM NaCl, 5 mM $MgCl_2$, 1 mM phenylmethylsulfonyl fluoride, 100 μM leupeptin, 100 mM TPCK) containing 40% glycerol. The nuclei were resuspended to a concentration equivalent to 2 μl of packed embryos/μl of buffer and frozen in liquid N_2. For chemical extraction,

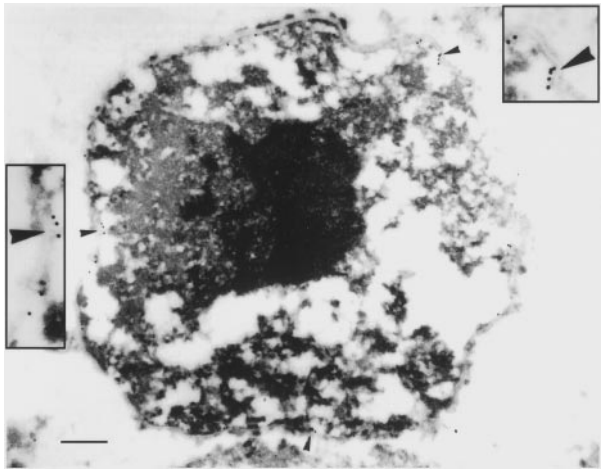

FIG. 1. **Immunoelectron microscopy localization of otefin in *Drosophila* embryonic nuclei.** *Drosophila* embryonic nuclei were incubated with polyclonal anti-otefin antibodies, followed by an incubation with goat anti-rabbit antibody conjugated to 12-nm gold particles. *Arrowheads* mark selected areas in the inner nuclear membrane where gold particles are seen. *Bar* = 30 nm.

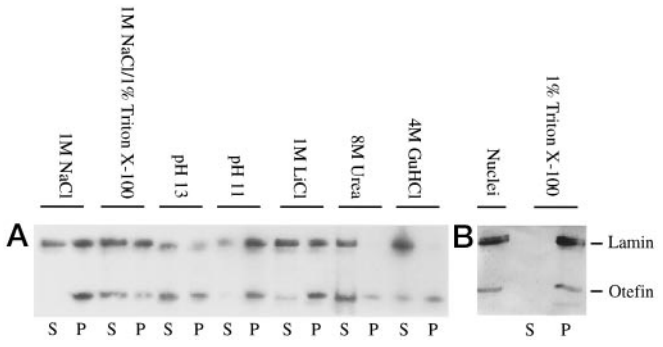

FIG. 2. **Solubility properties identify otefin as a peripheral protein.** *Drosophila* embryonic nuclei (0–15 h old) were digested for 15 min in buffer B containing 1 μg/ml DNase I and 1 μg/ml RNase A at 23 °C. *A*, digested nuclei were extracted in buffer B containing 1 M NaCl; buffer B containing 1% Triton X-100 and 1 M NaCl; buffer B at pH 13 (without Tris); buffer B at pH 11 (without Tris); buffer B containing 1 M LiCl; 8 M urea; or 4 M guanidine HCl (*GuHCl*). *B*, digested nuclei (control) were extracted in buffer B containing 1% Triton X-100. Following extraction, the residual pellets (*P*) and the supernatants (*S*) were separated by centrifugation, and proteins were separated by 10% SDS-polyacrylamide gel electrophoresis and analyzed by immunoblotting using mAb 618A2O7 (anti-otefin) and mAb 611A3A6 (anti-lamin Dm). The positions of otefin and lamin Dm are indicated.

nuclei were thawed on ice, washed once in buffer B, and digested for 15 min with 1 μg/ml DNase I and 1 μg/ml RNase A at 23 °C. The nuclear pellet was further extracted for 15 min at 4 °C in 4 volumes of buffer B supplemented with the extraction reagent. Extraction with high pH was performed in buffer B devoid of Tris-HCl, the pH of which was adjusted with NaOH.

After extraction, the residual nuclear pellet was separated from the supernatant by centrifugation at 14,000 × *g*. The supernatant was centrifuged once more for 10 min at 150,000 × *g*. From each fraction, amounts equivalent to 5 μl of packed embryos were subjected to protein blot analysis using specific mAbs. Colorimetric detection of alkaline phosphatase activity was according to McGrady (19).

RESULTS

Otefin Is Localized to the Nucleoplasmic Side of the Nuclear Envelope—To reveal the exact orientation of otefin within the nuclear envelope, polyclonal anti-otefin antibodies were raised against the otefin cDNA translation product. These were used to refine the localization of otefin in *Drosophila* embryonic nuclei by immunogold electron microscopy. As shown in Fig. 1, otefin was localized on the nucleoplasmic side of the inner nuclear membrane. Although the labeling of otefin in the current experiments was highly specific, the immunolabeling was weaker than in a previous study in which, due to the sensitivity of the available epitope to fixatives such as glutaraldehyde, lighter fixation conditions and 5-nm gold particles were used (13).

Otefin Is Peripherally Associated with the Nuclear Membrane—Extraction with detergents, salt, and chaotropic reagents was employed in order to study the mode of the association of otefin with the nuclear envelope (20). *Drosophila* embryonic nuclei were isolated, digested with DNase I and RNase A, and extracted with buffer B containing different reagents. The supernatant and pellet were then separated and analyzed by protein blot analysis using mAb 618A2O7 (anti-*Drosophila* otefin) and mAb 611A3A6 (anti-*Drosophila* lamin Dm) (12, 14). Extraction with 8 M urea, with 4 M guanidine HCl, or with buffer B at pH 13 resulted in extraction of both otefin and lamin Dm (Fig. 2*A*). These data show that the association of otefin with the inner nuclear membrane is peripheral and different from that of other known integral membrane proteins (20). Otefin remained associated mostly with the nuclear envelope after washing with buffer B containing 1 M NaCl, 1 M LiCl, or 3 M NaBr (data not shown) or after washing with buffer B at pH 11. Under the same conditions, a fraction of the lamin Dm molecules were extracted (Fig. 2*A*). These data reveal that association of otefin with the nuclear membrane is stronger than that of lamin Dm. It was interesting to note that both otefin and lamin remained associated with the nuclear envelope after a 1% Triton X-100 extraction (Fig. 2*B*), which suggests that both otefin and lamin are present in a protein complex that is stable to extraction with Triton. Both lamin and otefin were solubilized with 1% Triton X-100 combined with 1 M NaCl (Fig. 2*A*).

Otefin Localization to the Nuclear Membrane Requires the Hydrophobic Sequence at Its Carboxyl Terminus—Wild-type and truncated otefin forms were visualized following transient transfection into COS-7 cells in order to analyze sequences in otefin that participate in its targeting to the nuclear periphery (Fig. 3*A*). Expression of the otefin constructs was detected in ~1% (pCA1038) or 10% (pSP64-497–524.Z) of the transfected cells. COS-7 transfection with wild-type otefin labeled the nuclear rim in all otefin-expressing cells. In some transfected cells, otefin was also localized to the cytoplasm (Fig. 4*C* and Table I). In *Drosophila* Schneider cells, otefin was resistant to extraction with 1% Triton X-100 as judged by protein blot (Fig. 2*B*) and immunofluorescence (Fig. 4, *A* and *B*) analysis. Similarly, otefin remained associated with the nuclear rim in ~60% of the otefin-expressing COS-7 cells following extraction with Triton X-100, while most of the cytoplasmic staining disappeared (Fig. 4*D* and Table I). The remaining cytoplasmic staining appeared punctated probably due to aggregation of the protein at these sites.

In contrast to wild-type otefin, transfection with the Δ388–406 construct, which lacks the hydrophobic sequence, or with the Δ35–172,Δ388–406 construct, which lacks amino acids 35–172 and 388–406, resulted in the exclusive localization of the proteins to the nucleoplasm (Fig. 5, *A* and *C*). The lack of nuclear rim localization of the Δ388–406 and Δ35–172,Δ388–406 proteins shows that the last 17 amino acids of otefin are essential for directing otefin to the nuclear envelope. The Δ388–406 and Δ35–172,Δ388–406 proteins were completely soluble following a Triton X-100 extraction (Table I).

The localization of the Δ35–172 protein was similar to that of the normal protein (Fig. 5*B*), but was significantly less stable to extraction with Triton X-100. Only ~20% of the cells that expressed the Δ35–172 protein remained stained following extraction with Triton X-100 (Table I), and the level of labeling in these cells was significantly reduced (data not shown). The

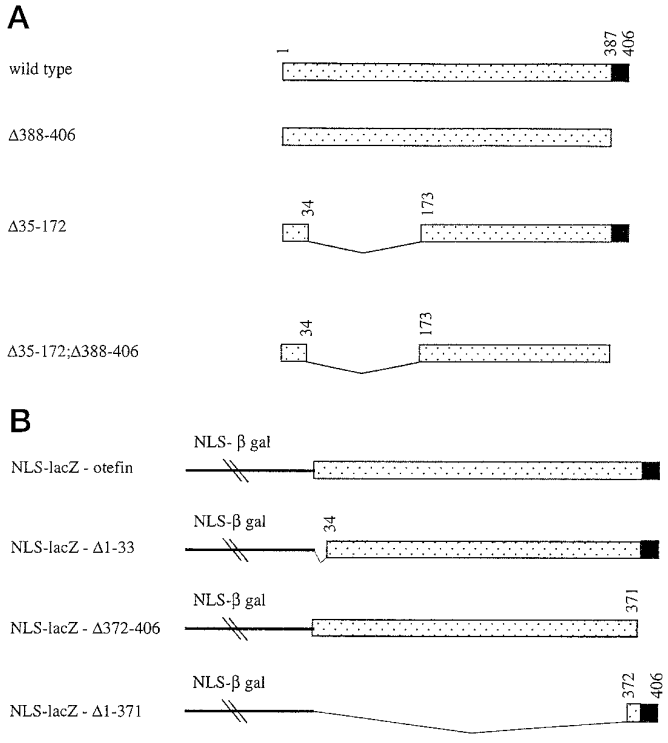

FIG. 3. **Schematic diagrams of proteins expressed in the transfection experiments.** Shown are schematic diagrams of the constructs. The different sequences are presented as a *broken line* (NLS-β-galactosidase (*NLS-β gal*)), a *dotted bar* (the hydrophilic sequences in otefin), and a *solid bar* (the hydrophobic sequence in otefin). *A*, native and truncated otefin mutants: full-length otefin (wild type), truncation of the hydrophobic sequence (Δ388–406), truncation of amino acids 35–172 (Δ35–172), and truncation of both the hydrophobic sequence and amino acids 35–172 (Δ35–172,Δ388–406). Constructs for these proteins were prepared for transfection in the pCA1038 vector. *B*, fusion proteins between NLS-β-galactosidase and native or truncated forms of otefin: full-length otefin (NLS-*lacZ*-otefin), otefin truncated at amino acids 1–33 (NLS-*lacZ*-Δ1–33), otefin truncated at amino acids 372–406 (NLS-*lacZ*-Δ372–406), and otefin truncated at amino acids 1–371 (NLS-*lacZ*-Δ1–371). Constructs for these proteins were prepared for transfection in the pSP64-497–524.Z vector. The amino terminus of each protein is shown on the left.

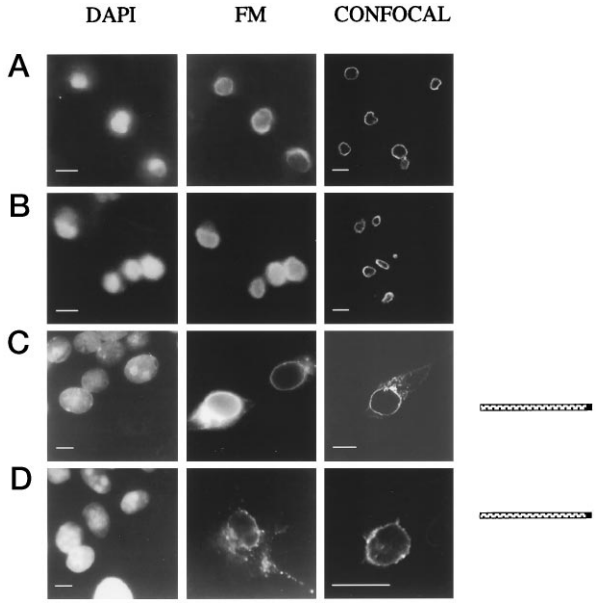

FIG. 4. **Cellular localization of the otefin protein in Schneider and COS-7 cells by immunofluorescence.** Cells were viewed using a Leitz fluorescence microscope for 4′,6-diamidino-2-phenylindole (*DAPI*) and Cy_3A (*FM*) staining. Cy_3A staining was also viewed with a Bio-Rad confocal microscope (*CONFOCAL*). *A*, Schneider cells were fixed with methanol and 4% formaldehyde and permeabilized with 0.1% Triton X-100. The cells were than incubated with mAb 618A2O7 (anti-otefin) and with Cy_3A-conjugated goat anti-mouse IgG, followed by staining with 4′,6-diamidino-2-phenylindole. Antibody staining was localized to the nuclear rim. *B*, Schneider cells were extracted with 1% Triton X-100 for 10 min at 4 °C and then fixed and stained as described for *A*. Following the extraction with Triton X-100, staining remained in the nuclear rim. *C*, COS-7 cells were Lipofectin-transfected with full-length otefin in the pCA1038 vector. After 24 h, the transfection medium was replaced with fresh Dulbecco's modified Eagle's medium containing 10% FCS. Following an additional 24 h, the cells were fixed and stained as described for *A*. Staining was localized to the nuclear rim and the cytoplasm (please note the cytoplasmic staining in the left cell in the *FM* image). *D*, the transfected COS-7 cells were washed with 1% Triton X-100 for 10 min at 4 °C, followed by fixation and staining as describe for *C*. Staining remained in the nuclear rim. In some cells, some aggregates remained in the cytoplasm as shown in the *FM* images. Cells with relatively high levels of cytoplasmic staining are presented in *D*. *Bars* = 5 μm (*A* and *B*) and 10 μm (*C* and *D*). ~1% of the transfected cells expressed otefin. mAb 618A2O7 (anti-otefin) did not recognize a native otefin in mammalian cells, as indicated by cells that do not express *Drosophila* otefin (*C* and *D*).

extraction of Δ35–172 with Triton X-100 demonstrated that although the amino-terminal portion of the hydrophilic domain has little or no effect on the cellular localization of otefin, it does play a role, when present together with the hydrophobic sequence, in stabilizing the interaction of otefin at the nuclear periphery.

Domains in Otefin That Are Required for the Efficient Targeting of NLS-lacZ to the Nuclear Membrane—To further analyze the role of specific sequences in targeting otefin to the nuclear periphery, a series of constructs was prepared in which the otefin sequences were fused in frame to the 3′-end of the open reading frame of the *lacZ* gene in the pSP64-497–524.Z vector (Fig. 3*B*). These constructs were transiently transfected into COS-7 cells. A comparison of the results that were obtained with the different constructs is presented in Table I.

When the pSP64-497–524.Z vector encoding an NLS-β-galactosidase protein was transiently transfected into COS-7 cells, the expressed protein was localized in the nucleoplasm as judged by indirect immunofluorescence (Fig. 6*D*). In ~70% of the expressing cells, low levels of cytoplasmic staining were also observed, perhaps due to the high expression levels in these cells. In contrast, the NLS-β-galactosidase protein fused to amino acids 1–406 of otefin was detected mainly in the nuclear periphery (Fig. 6*A*). In some cells, reticular cytoplasmic staining was also observed. Both anti-β-galactosidase (Fig. 6*A*) and anti-otefin (618A2O7; data not shown) mAbs demonstrated similar behavior. Extraction of NLS-*lacZ*-otefin with Triton X-100 resulted in punctated perinuclear staining, similar to the pattern observed in COS-7 cells transfected with otefin (compare Fig. 4*D* with Fig. 6*A*).

The location of the NLS-β-galactosidase-otefin fusion protein was further determined with electron microscopy immunolocalization using anti-β-galactosidase mAb and horseradish peroxidase-conjugated anti-rabbit antibodies. Horseradish peroxidase staining appeared under the inner membrane of the nuclear envelope of the *lac Z*-expressing cells (Fig. 7, *A–C*), but not in cells that do not express the fusion protein (Fig. 7*D*). In addition to the inner nuclear membrane staining, horseradish peroxidase staining was also observed in the outer nuclear membrane and in the cytoplasm of many cells.

The NLS-β-galactosidase-Δ372–406 protein, which lacks the hydrophobic sequence, showed a nucleoplasmic localization, similar to that of the NLS-β-galactosidase protein alone (Fig. 6*E*). While confirming the importance of the hydrophobic sequence in directing otefin to the nuclear periphery, these findings imply that the hydrophilic sequences of otefin are not sufficient for promoting localization to the nuclear envelope.

TABLE I

Summary of the results obtained following transfection of COS-7 cells with the different wild-type and truncated otefin constructs

The location of the expressed protein within the cell is indicated as follows: N, nuclear; NE, nuclear envelope; and Cyt, cytoplasm. The different immunofluorescence intensities were estimated qualitatively and are indicated as follows: ++, high intensity; +, medium intensity; +/−, low intensity; and +/− −, intensities close to background levels. The percent resistance to Triton X-100 was measured as the fraction of expressing cells in the culture following treatment with Triton X-100 divided by the fraction prior to treatment with Triton X-100. Low immunofluorescence intensity (+/−) was probably not detected following extraction with Triton X-100. The fractions were calculated from three different experiments. Please note the 13% background level for the NLS-*lacZ* constructs.

Construct	Location						Resistance to Triton X-100
	After transfection			After Triton X-100			
	N	NE	Cyt	N	NE	Cyt	
							%
Otefin	−	++	+	−	++	+/−	60
Δ35–172	−	++	+	−	++	+/−	20
Δ388–406	++	−	−	+/− −	−	−	0
Δ35–172,Δ388–406	++	−	−	+/− −	−	−	0
NLS-*lacZ*	++	−	+/− −	+/− −	−	−	13
NLS-*lacZ*-otefin	−	++	+/−	−	++	+/− −	77
NLS-*lacZ*-Δ1–33	−	++	+/−	−	++	+/− −	ND[a]
NLS-*lacZ*-Δ374–406	++	−	−	+/− −	−	−	16
NLS-*lacZ*-Δ1–373	+	+	+	−	+	−	35

[a] ND, not determined.

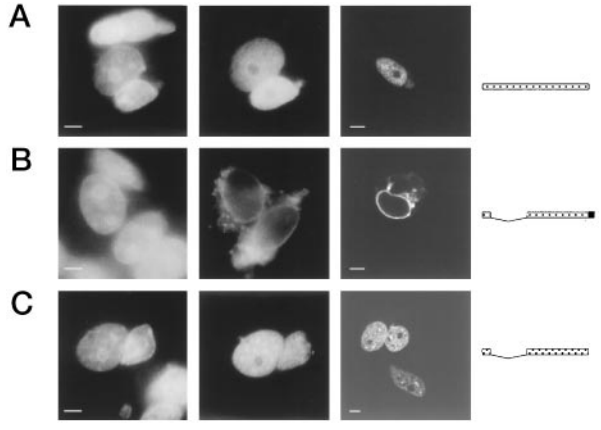

FIG. 5. **Cellular localization of truncated otefin proteins in COS-7 cells.** Transfection and staining of the different constructs were as described for Fig. 4. *A*, transfection with the Δ388–406 construct resulted in nuclear staining. *B*, transfection with the Δ35–172 construct resulted in nuclear rim staining and lower levels of cytoplasmic staining as compared with wild-type otefin. *C*, transfection with the double-deletion Δ388–406,Δ35–172 construct showed a pattern of staining similar to that of the Δ388–406 construct. *Bars* = 5 μm. The schematic diagrams (on the right) are described in the legend to Fig. 3. *DAPI*, 4′,6-diamidino-2-phenylindole staining; *FM*, Cy_3A staining; *CONFOCAL*, Cy_3A staining viewed with a Bio-Rad confocal microscope.

To examine whether the hydrophobic sequence is sufficient for nuclear envelope localization, COS-7 cells were transfected with NLS-*lacZ*-Δ1–371, encoding the NLS-β-galactosidase protein fused to amino acids 372–406. This fusion protein had a complex localization pattern. In ~60% of the expressing cells, staining of the nuclear rim was observed (Fig. 6*C*). In addition, in all of the expressing cells, nonhomogeneous cytoplasmic labeling of unidentified blobs was detected. In ~40% of the cells, nucleoplasmic staining was also observed. Triton X-100 extracted most of the cytoplasmic and nuclear background, while the nuclear rim staining remained in ~20% of the expressing cells (Table I), apparently reflecting the fraction of this fusion protein that stably interacts with component(s) of the nuclear envelope (Fig. 6*C*). It should be noted, however, that the estimation of the percent of extractability by Triton X-100 was qualitative; following extraction with Triton X-100, cells that expressed low levels of the truncated protein could have been missed. Together, these data indicate that while low efficiency targeting to the nuclear periphery of NLS-containing proteins is obtained with the hydrophobic sequence of otefin alone, high efficiency targeting requires additional sequences in otefin.

Removal of the first 33 amino acids in otefin (NLS-*lacZ*-Δ1–33) resulted in a localization pattern similar to that of the complete otefin (Fig. 6*B*). Extraction of these cells with Triton X-100 resulted in punctated nuclear rim staining, similar to NLS-*lacZ*-otefin (Table I). These data, together with the transfection experiments, allow the identification of three domains in otefin that are important for directing otefin to the nuclear envelope. The hydrophobic sequence at the carboxyl terminus is essential for the sorting of otefin to the nuclear envelope, while the domain containing amino acids 35–172 is required to confer a stable protein complex in the nuclear envelope. Additional sequences at the hydrophilic domain play a role in the efficient targeting of otefin to the nuclear periphery.

DISCUSSION

Otefin Association with the Nuclear Envelope—In this study, we used polyclonal anti-otefin antibody and the immunogold electron microscopy technique to show that in the nuclear envelope, otefin is facing the nucleoplasm. These findings add to our previous study (13), in which otefin was localized by mAb to the nuclear envelope, but its topology within the nuclear envelope could not be determined.

The solubility properties identify otefin as a peripheral protein of the nuclear envelope. A major operational criterion for this classification was its extraction with 8 M urea, with 4 M guanidine HCl, or with buffer B at pH 13. Therefore, the topology of the association of otefin is different from that of LAP1, LAP2, and LBR, which are all type II integral membrane proteins (21–23). The only other known peripheral proteins of the inner nuclear membrane are the lamins. The association of otefin with the nuclear membrane was found to be stronger than that of lamins since it remained resistant to extractions with high salt or high pH (pH 11), while lamin was solubilized. Otefin also remained associated with the nuclear envelope following extraction with Triton X-100. Hence, otefin maintained its association with the insoluble lamin fraction, similar to LAP1, LAP2, and LBR (23).

There are several indications that otefin may also interact *in*

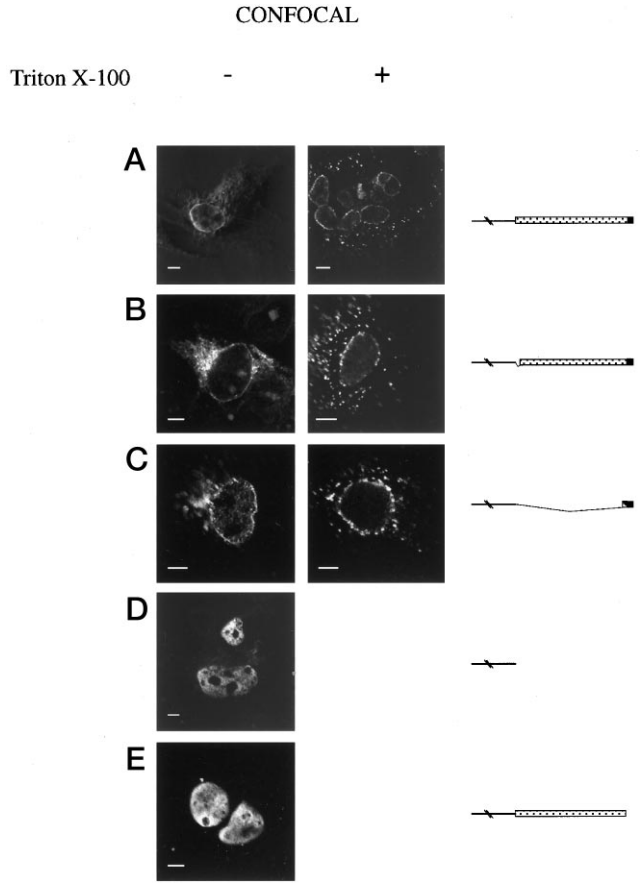

FIG. 6. **Cellular localization of fusion proteins between β-galactosidase and wild-type or truncated forms of otefin as observed by confocal microscopy.** COS-7 cells were transfected with the following constructs: *A*, NLS-*lacZ*-otefin; *B*, NLS-*lacZ*-Δ1–33; *C*, NLS-*lacZ*-Δ1–371; *D*, NLS-*lacZ* (pSP64-497–524.Z vector); *E*, NLS-*lacZ*-Δ372–406. Transfection and staining of the different constructs were similar to those described for Fig. 4, except that mAb Z378A (anti-β-galactosidase) was used as the primary antibody. Because only background levels were observed following extraction of NLS-*lacZ*-Δ372–406 and NLS-*lacZ* with Triton X-100, these results are not shown. Staining with mAb 618A2O7 (anti-otefin) resulted in similar results (data not shown). The schematic diagrams (on the right) are described in the legend to Fig. 3. *Bars* = 10 μm.

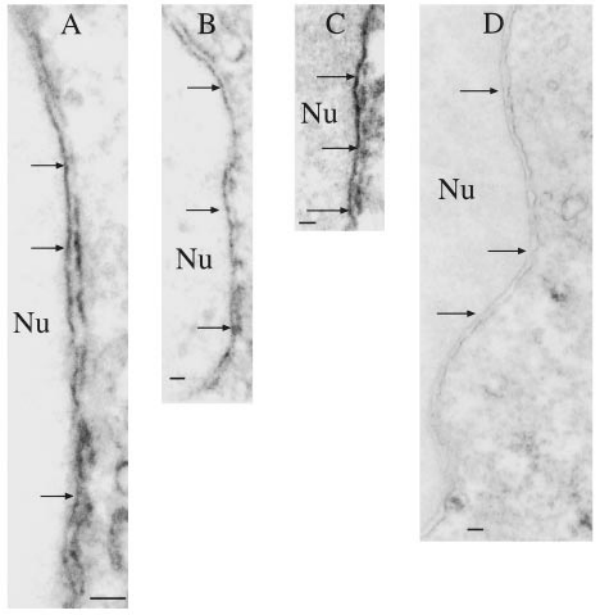

FIG. 7. **Immunoelectron localization of NLS-β-galactosidase-otefin to the nuclear envelope following transfection into COS-7 cells.** COS-7 cells were transfected with NLS-*lacZ*-otefin, and detection was by horseradish peroxidase staining. Examples of staining from three different cells expressing otefin are shown (*A–C*). *Arrows* point toward the inner nuclear membrane, and the nuclear side is indicated (*Nu*). Each panel shows a section of a different nucleus. *D* shows the nuclear envelope of a cell that did not express the fusion protein. *Bars* = 50 nm (*A*) and 62 nm (*B–D*).

vivo with the nuclear lamina and/or chromatin. These indications include the peripheral nucleoplasmic localization of both otefin and lamin; the resistance of otefin to extraction with Triton X-100, similar to lamin; the finding that in early embryos, otefin remains associated with the spindle envelope during mitosis, like lamin Dm_{mit} (13); and the finding that in the maternal pool, otefin is associated with the same membrane vesicle fractions as lamin Dm_{mit}. These results motivate further studies to determine if such interactions exist.

Sequences That Are Required for the Targeting of Otefin to the Nuclear Envelope—The data presented here indicate that as in *Drosophila* nuclei, *Drosophila* otefin interacts with factor(s) that are present only at the nuclear periphery of the COS-7 cells to form a Triton-stable complex. In a fraction of the otefin-expressing cells, some of the label was found associated with the cytoplasm. This cytoplasmic association was largely solubilized with Triton X-100 and therefore did not involve the formation of Triton-stable complexes.

The hydrophilic domain of otefin was not sufficient for the targeting of proteins to the nuclear envelope. The localization of otefin to the nuclear envelope depended absolutely on the COOH-terminal hydrophobic sequence. Removal of the short hydrophobic sequence of otefin resulted in the nuclear localization of the Δ388–406 and NLS-*lacZ*-Δ372–406 proteins, which is in line with our previous suggestion that otefin has putative NLS sequences (14). The hydrophobic sequence of otefin and its neighboring 11 amino acids had, by itself, only limited capability for directing the NLS-β-galactosidase protein to the nuclear envelope. Other sequences in otefin were required for the efficient localization of otefin to the nuclear envelope and for its interaction with other nuclear envelope proteins. Since the Δ35–172 and NLS-*lacZ*-Δ1–33 proteins had mainly a nuclear rim localization, similar to wild-type otefin, efficient targeting to the inner membranes requires the hydrophobic sequence together with hydrophilic sequences between amino acids 173 and 389.

Removal of amino acids 35–172 from otefin did not affect the localization of otefin. However, the expressed construct became sensitive to extraction with Triton X-100. Although this region in otefin may be involved in the interaction of otefin with other factors that are present at the nuclear envelope, we cannot exclude the possibility that the sensitivity to Triton X-100 is due to misfolding of the mutant protein. It is interesting to note that this region in otefin contains a conserved site for Cdc2 kinase phosphorylation (SPKK) that may be involved in the regulation of the formation of a complex *in vivo*.

Based on these observations, an attractive model for the topology of otefin is that it is attached to the inner nuclear membrane by its hydrophobic sequence. This attachment is stabilized by integral membrane proteins. Treatment with guanidine HCl, urea, or pH 13 disrupts these protein-protein interactions and allows the extraction of otefin from the protein complex. Further study is required to determine whether the known integral membrane proteins of the nuclear envelope are involved in the formation of an otefin-containing complex.

Mechanisms for Targeting Proteins to the Nuclear Periphery—It is possible that otefin is directed to the nuclear envelope by entering the nucleus with the aid of its NLS(s), followed by a hydrophobic sequence-dependent sorting to the

nuclear envelope and retention by other nuclear envelope proteins. This mechanism of targeting resembles the mechanism by which lamins are localized to the nuclear envelope. However, in the case of lamins, this association with the nuclear envelope requires a post-translational modification resulting in the addition of an isoprenyl group to the C*AAX* motif at the carboxyl terminus (24). In a previous report, it was noted that the stretch of amino acids 74–81 in otefin has homology to known NLSs (14). In the present study, we show that deletion of this region and the carboxyl-terminal domain does not abolish nuclear targeting. The ability of the deleted otefin to reach the nucleus can be explained either by the presence of a different functional NLS or by a passive diffusion of this ~30-kDa protein into the nucleus and retention by binding to nuclear ligands. The location of NLSs in otefin is currently under investigation.

Since otefin was found to be associated with membrane vesicles in the maternal pool, sorting and retention at the nuclear envelope could also occur at the end of mitosis, when vesicles attach to chromatin and fuse to form the nuclear envelope. In that case, the precise localization of otefin within the inner nuclear membrane would require direct or indirect interactions of otefin with other nuclear envelope proteins or with proteins that are present on the surface of the chromatin.

Another mechanism suggested for the transport of type II integral membrane proteins to the inner nuclear membrane includes insertion of the protein into endoplasmic reticulum membranes, followed by lateral diffusion through the endoplasmic reticulum network and accumulation at the inner membrane by binding interactions (9, 12). Like otefin, which is a peripheral protein, the targeting of these integral membrane proteins involves more than one region in the protein. However, the ability of the large-size fusions of NLS-β-galactosidase-otefin (~170 kDa) to reach the inner nuclear membrane suggests that in the COS-7 cells, they did not enter the nuclear envelope by lateral diffusion. Nevertheless, since otefin is probably small enough for lateral diffusion, we cannot rule out the possibility that *in vivo*, a fraction of otefin is targeted to the inner membrane by association with other inner membrane proteins at the endoplasmic reticulum.

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