Intranuclear membrane structure formations by CaaX-containing nuclear proteins

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Summary

The nuclear lamina is a protein meshwork lining the nucleoplasmic face of the nuclear envelope. Association of lamins with the inner nuclear membrane is mediated by specific modifications in the CaaX motif at their C-termini. B-type lamins are permanently isoprenylated whereas lamin A loses its modification by a lamin A-specific processing step after incorporation into the lamina. Lamins are differentially expressed during development and tissue differentiation. Here we show that an increased synthesis of lamins B1 and B2 in amphibian oocytes induces the formation of intranuclear membrane structures that form extensive arrays of stacked cisternae. These 'lamin membrane arrays' are attached to the inner nuclear membrane but are not continuous with it. Induction of this membrane proliferation depends on CaaX-specific posttranslational modification. Moreover, in transfected HeLa cells, chimeric GFP containing a nuclear localization signal and a C-terminal CaaX motif of N-Ras induces intranuclear membrane stacks that resemble those induced by lamins and ER-like cisternae that are induced in the cytoplasm upon increased synthesis of integral ER membrane proteins. Implications for the synthesis of CaaX-containing proteins are discussed and the difference from intranuclear fibrous lamina annulate lamellae formations is emphasized.

Key words: Intranuclear membranes, Lamins, CaaX motif, *Xenopus,* Oocytes, GFP

Introduction

Nuclear lamins belong to the class of intermediate filament proteins. They are the major structural components of the nuclear lamina, a protein meshwork associated with the inner nuclear membrane (Aebi et al., 1986) (for reviews, see Gruenbaum et al., 2003; Herrmann and Aebi, 2004). The nuclear lamina is an essential component of metazoan cells. It provides mechanical stability to the nuclear envelope and is involved in chromatin organization, DNA replication and anchoring of nuclear pore complexes (reviewed by Wilson et al., 2001). Several integral membrane proteins that interact with lamins and the complex interactions between these proteins and chromatin-associated proteins can influence lamin function and dynamics (Furukawa, 1999; Gant et al., 1999; Gotzmann and Foisner, 1999; Dechat et al., 2000; Holmer and Worman, 2001; Lee et al., 2001). The importance of lamin function is highlighted by the targeted disruption of the lamin A gene in mice (Sullivan et al., 1999; Alsheimer et al., 2004), RNAi experiments with *Caenorhabditis* (Liu et al., 2000), the study of a *Drosophila* mutant with reduced lamin Dmo activity (Lenz-Böhme et al., 1997) and by P element insertion (Guillemin et al., 2001). Specific mutations in the lamin A gene cause a wide range of heritable human diseases, collectively called laminopathies (for reviews see Burke and Stewart, 2002; Gruenbaum et al., 2003).

Targeting of lamins to the inner nuclear membrane depends on the presence of a nuclear localization signal (NLS) and posttranslational lipidation (for a review, see Nigg et al., 1992; Hofemeister et al., 2000; Maske et al., 2003). Most lamins contain a CaaX motif at their C-termini, which is the target of a series of posttranslational modifications, including isoprenylation, proteolytic trimming and carboxyl methylation. Other proteins that contain a CaaX motif include the Ras proteins and many other small G proteins, fungal mating pheromones and large G protein subunits. Large G proteins are geranyl-geranylated, whereas Ras proteins, the fungal mating pheromones and lamins are farnesylated (Zhang and Casey, 1996). In the majority of lamin proteins the terminal amino acid residue is a methionine (Döring and Stick, 1990; Hofemeister et al., 2002). The isoprene moiety is added via a stable thioether linkage to the CaaX cysteine. The subsequent endoproteolytic trimming and carboxyl methylation significantly increases the hydrophobicity of the C-termini of CaaX-modified proteins (Maske et al., 2003). For many of these proteins the importance of the modifications for certain functions has been shown. Lamins are the only nuclear isoprenylated proteins known to date.

Vertebrates synthesize, in cell type-specific patterns, a variety of lamins encoded by separate genes or generated by differential RNA splicing (e.g. Fisher et al., 1986; Döring and Stick, 1990; Furukawa and Hotta, 1993; Furukawa et al., 1994; Machiels et al., 1996). Based on their domain structure two types of lamins, A and B, can be distinguished. The larger A lamins comprise about 100 additional amino acid residues in their tail domain that are encoded by an extra exon (Stick, 1992; Lin and Worman, 1993). Although B lamins are

permanently prenylated, A lamins lose their isoprene moiety soon after incorporation into the lamina by endoproteolytic processing at their C-termini (Weber et al., 1989). This processing step is essential for lamin A function. In Zmpste24 metalloproteinase-deficient mice defective lamin A processing leads to severe abnormalities that are similar to those observed in lamin A gene knockout mice and in defects observed in humans suffering from congenital laminopathies (Pendás et al., 2002). Lamin C and lamin C2, two splice variants of the mammalian lamin A, lack a CaaX motif and are never prenylated. Although lamins A, B1 and B2 are found in all classes of vertebrates, the additional lamin LIII, a B-type lamin, is only detected in amphibians and fish (Stick and Krohne, 1982; Yamagushi et al., 2001; Hofemeister et al., 2002). Lamins B1 and B2 genes are, in general, constitutively expressed in somatic cells, whereas synthesis of A-type lamins is developmentally regulated (Lehner et al., 1987; Stewart and Burke, 1987; Wolin et al., 1987; Röber et al., 1989). Lamin A is absent in early embryonic cells and appears asynchronously in certain cell types of various tissues (Stewart and Burke, 1987). Germ cells and cells in early embryonic development show a particularly complex pattern of lamin synthesis that has been analyzed in detail in amphibian oocytes and early embryos (Benavente et al., 1985; Stick and Hausen, 1985; Furukawa and Hotta, 1993; Furukawa et al., 1994; Yamaguchi et al., 2001; Hofemeister et al., 2002).

In amphibian oocytes the organization of the lamina appears remarkable regular: 10 nm filaments form a near-tetragonal lattice, in which the nuclear pore complexes are embedded (Aebi et al., 1986). Lamin LIII is the predominant lamin of amphibian oocytes, eggs and cleavage embryos (Stick and Hausen, 1985). In contrast to birds, which synthesize lamins A, B1 and B2 in diplotene oocytes (Lehner et al., 1987), lamins B1 and B2 are very minor components in amphibian oocytes and lamin A is completely absent from these cells (Wolin et al., 1987; Lourim et al., 1996). The specific expression of the lamin LIII gene in fish and amphibian oocytes and early embryos has been interpreted as an adaptation to the particular type of oogenesis and early development found in these organisms (Hausen et al., 1985). The giant oocyte nucleus, the ‘germinal vesicle’ (GV), stockpiles large amounts of nuclear proteins, including lamin LIII. These maternal proteins serve as a protein pool for the generation of embryonic nuclei during the rapid cleavage divisions up to the 5000-10,000 cell stage (Hausen and Riebesell, 1991). Synthesis of lamins B1 and B2 starts at midblastula and gastrula, respectively, concomitant with but independent of the onset of embryonic transcription and the lengthening of the cell cycle (Newport and Kirschner, 1982a; Newport and Kirschner, 1982b; Stick and Hausen, 1985).

In attempts to study the architecture and functional significance of complex lamina structures we followed the ectopic expression of special lamin genes and the synthesis of certain lamin mutants in *Xenopus laevis* oocytes to alter experimentally the composition of the nuclear lamina in vivo. We chose amphibian oocytes as the cellular system because of their accessibility to direct experimental manipulation and structural analysis. Surprisingly, we observed that the synthesis of lamins B1 and/or B2 induces the formation of a novel kind of intranuclear membrane structure and that this induction of membrane proliferation critically depends on CaaX modifications. Moreover, transfection experiments using genes encoding chimeric CaaX proteins indicate that induction of such intranuclear membranes might be a general property of CaaX-containing proteins.

Materials and Methods

Isolation and manipulation of oocytes

Xenopus laevis oocyte techniques were as described (Firmbach-Kraft and Stick, 1993; Firmbach-Kraft and Stick, 1995). 30-75 nl of the appropriate RNA in water was injected per oocyte. Oocytes were maintained at 18°C.

Plasmids and plasmid construction

Plasmids encoding *Xenopus laevis* lamin B1 (LI) (GenBank accession number X06344), Flag epitope-tagged lamin LIII (accession number X13169), chimeric GFP NLS-MT-GFP-N-Ras and NLS-MT-GFP have been described (Ralle et al., 1999; Hofemeister et al., 2000). A cDNA clone in vector pBs (Stratagene, La Jolla, CA) encoding *Xenopus laevis* lamin B2 (LII) (accession number X54099) was kindly provided by Georg Krohne (Biozentrum, Würzburg). The open reading frame was isolated by double digestion with *Eco*RI and *Eco*RV, the DNA fragment was blunt ended with T4 DNA polymerase and cloned into the *Bgl*II site of pSP64T. The B2-SaaX mutant was generated by site-directed mutagenesis with the use of the QuickChange mutagenesis Kit (Stratagene) with the following primer pair: 5′-GAACAACATCCAGAGGCTCCTCTGTCATGTAAAC-3′ and 5′-GTTTACATGACAGAGGAGCCTCTGGATGTTGTTC-3′. Flag-epitope-tagged *Xenopus laevis* prelamin A (accession number X06345) was generated by amplifying the open reading frame excluding the start methionine codon by PCR with a sense primer containing a *Eco*RI recognition site 5′-GGAATTCTGAGAC-CCAGGTCAGAAG-3′ and an antisense primer containing a *Xho*I recognition site 5′-CGGCTCGAGTGGCATCCGGTTAC-3′. Cycling parameters were as described (Hofemeister et al., 2000). The PCR product was double digested with *Eco*RI-*Xho*I and cloned into *Eco*RI-*Xho*I double-digested pCS2+ (Flag) vector (Hofemeister et al., 2000). Flag-epitope-tagged *Xenopus laevis* lamin B1 was generated accordingly. The sense primer contained an *Avr*II recognition site 5′-GCCCTAGGGGCCACTGCCACCCCTAGC-3′ and the antisense primer a *Sna*BI recognition site 5′-TCCTACGTATCACATGATG-GCGCAG-3′. The *Avr*II-*Sna*BI double-digested PCR product was cloned into an *Xba*I-*Sna*BI double-digested pCS2+Flag-*Hin*dIII vector. The *Hin*dIII recognition site in front of the Sp6 RNA polymerase promoter of pCS2+Flag had been destroyed by digestion with *Hin*dIII, blunt ending with T4 DNA polymerase and re-ligation. Myc-epitope-tagged *Xenopus* lamin LIII was generated by PCR with the sense primer that encoded a myc-epitope 5′-GATCCACCATG-GAGGAGAAGCTGATTTCTGAGGAGGATTTGGCCACATCTAC-CCCCAGC-3′ and the antisense primer 5′-GATCCATTACATGATG-GAACAGCTGG-3′. The PCR product was cloned into the *Eco*RI digested and blunt ended pCS2+myc vector.

RNA techniques, electrophoresis, immunoblotting and cell culture

These techniques were essentially as described (Hofemeister et al., 2000).

Nuclear envelope spread preparations

Oocyte nuclei were manually isolated in ‘5:1 buffer’ (83 mM KCl, 17 mM NaCl, 10 mM Tris-HCl, pH 7.2) containing 10 mM $MgCl_2$. Isolated nuclei were placed on a glass coverslip and attached to the glass by gentle pressing. The envelope was opened with fine forceps

and the nuclear content was washed away with a stream of buffer. In this way a large part of the nuclear envelope firmly attached to the glass surface and could be stained without further fixation. Spreads were blocked for 15 minutes with 0.3% bovine serum albumin (BSA) and incubated for 15 minutes with appropriate dilutions of primary antibody, washed for 15 minutes with several changes of 5:1 buffer, incubated for another 15 minutes with Cy3-conjugated goat anti-guinea pig IgG (Dianova, Hamburg, Germany). After washing for 15 minutes with 5:1 buffer, spreads were mounted with Fluoromount-G (Southern Biotechnology Association, Birmingham, AL).

Cryostat sectioning of oocytes

Oocytes were fixed in 20% dimethylsulfoxide in methanol overnight at –20°C (Dent et al., 1989) transferred to methanol and stored at –20°C until use. Methanol was replaced by PBS (140 mM NaCl, 2.7 mM KCl, 8.1 mM Na_2HPO_4, 1.5 mM KH_2PO_4, pH 7.3), specimens were gently agitated rolling end over end using a turning wheel at room temperature for 10 minutes, the buffer was replaced by infiltration solution (15% fish gelatin, 16% sucrose in water) and again agitated on a turning wheel for 24 hours. Oocytes were frozen in liquid nitrogen mounted on a cryostat holder. 10 μm sections were taken and transferred onto poly-L-lysine-coated slides. Sections were post-fixed in acetone at –20°C for 2 minutes, air dried for 20 minutes and transferred into a staining tray containing PBS. All the remaining steps were done at 37°C. Blocking was carried out for 30 minutes with 5% non-fat dry milk in PBS. Incubation with the appropriate dilutions of primary antibody in 1% non-fat dry milk in PBS was for 2 hours, followed by three washes of 10 minutes each with 1% non-fat dry milk in PBS. Incubation with Cy3-conjugated goat anti-mouse IgG (Dianova) was for 1 hour at room temperature, followed by three washes in PBS with 1% non-fat dry milk at room temperature. Sections were mounted in Fluoromount-G. Immunofluorescence microscopy was performed as described (Hofemeister et al., 2002).

Electron microscopy

Oocyte nuclei were isolated in 83 mM KCl, 17 mM NaCl, 10 mM HEPES-KOH, pH 7.2, transferred into 2.5% glutaraldehyde in the same buffer and fixed for 30 minutes on ice. They were then transferred into 2% osmium tetroxide in 50 mM sodium cacodylate (pH 7.2), incubated for 1 hour on ice, washed three times in water, and heavy metal stained for 12 hours in 0.5% aqueous uranyl acetate at 4°C. Dehydration, embedding, sectioning and electron microscopy was carried out as described (Stick and Krohne, 1982).

For immunoelectron microscopy, nuclei were fixed in 4% formaldehyde, 0.2% glutaraldehyde in 83 mM KCl, 17 mM NaCl, 10 mM HEPES-KOH (pH 7.2) for 30 minutes, washed several times in buffer, blocked for 30 minutes in 1% BSA in buffer and incubated in anti-lamin B2-specific monoclonal antibody (mAb) L7-8C6 (ascites fluid diluted 1:200) for 1 hour. Nuclei were washed three times for 10 minutes each and then processed for immunogold staining (Langbein et al., 2002).

Results

Lamins synthesized in oocytes associate with the nuclear envelope

To study the architecture of nuclear laminae with an experimentally altered composition we chose *Xenopus* oocytes as an experimental system. The amount of ectopically synthesized protein can be controlled simply by varying the amount of injected RNA. Nuclear envelopes can easily be isolated manually for both biochemical and structural studies. In contrast to somatic cells where the tight association of peripheral chromatin prevents a face-on view of the lamina, the inner aspect of the oocyte nuclear envelope is freely accessible to light and electron microscopic analysis.

Synthetic RNAs encoding either wild-type or N-terminal epitope-tagged versions of *Xenopus*, lamins B1, B2, LIII and prelamin A were injected into the cytoplasm of *Xenopus* oocytes. After incubation for 16 hours, the nuclei were isolated and either used unfractionated or separated into nuclear envelope and nuclear content. Proteins of whole nuclei as well as of nuclear envelopes and nuclear contents were separated by SDS-PAGE and analyzed by immunoblotting to monitor the subnuclear distribution of the additional lamin proteins. As controls, nuclei of non-injected oocytes were processed in parallel. Blots were first probed with an antibody that specifically recognizes the experimentally introduced lamin. To distinguish the additional lamin LIII from the endogenous LIII, epitope-tagged versions of LIII (either Flag- or myc-tag) were introduced and detected with the respective tag-specific antibody. Similarly, prelamin A was produced as a Flag-A chimera, as no *Xenopus* lamin A-specific antibody is available.

Endogenous lamin LIII was exclusively located in the nuclear envelope (Fig. 1; compare lanes 2 and 3, 5 and 6). Even with the most sensitive detection methods and at very high protein loads, LIII was never detected in the nuclear interior under steady state conditions (Fig. 1D') (Stick and Krohne, 1982; Firmbach-Kraft and Stick, 1993). Lamins B1, B2, and Flag-LIII, when excessively synthesized upon RNA injection, efficiently accumulated in the nuclear envelope. Similarly, lamin A associated with the envelope. However, 16 hours after RNA injection, small amounts of lamin A were still detected in the nucleoplasmic fraction (Fig. 1D, lane 2), indicating either that the integration of lamin A into the nuclear envelope was slower or that the association of lamin A with the envelope was less stable. All blots were reprobed with an antibody specific for LIII, to monitor the distribution of the endogenous lamin. None of the reprobed blots showed lamin LIII signals in the nuclear content fractions (Fig. 1A'-E'), demonstrating that the intranuclear lamin A signal in Fig. 1D (lane 2), was not due to contamination with nuclear envelope-associated material.

The fractionation procedure was further controlled using a lamin B2-SaaX mutant in which the CaaX cysteine residue had been replaced with a serine residue, so that the resulting mutant protein could neither be isoprenylated nor processed further (reviewed by Glomset and Farnsworth, 1994). As expected from previous results, the non-modified lamin B2 protein accumulated within the nucleus but remained in the nucleoplasm (Fig. 1E, lanes 2 and 3). Re-examination of the proteins by immunoblotting showed a distribution typical of endogenous lamin LIII (Fig. 1E').

Nuclear lamin distribution in oocytes

Next we analyzed the subcellular distribution of experimentally introduced lamins on cryostat sections through oocytes that had been fixed at different times after RNA injection. Endogenous lamin LIII showed a continuous peripheral lamina staining as typically observed in sections through any other tissue. Because of the lobulated surface of the oocyte nuclei and to some contortion during the sectioning process, the lamina staining appeared fuzzy or frayed in some areas (Fig. 2A). Essentially the same continuous nuclear

envelope staining was observed when the gene encoding prelamin A was expressed in oocytes (Fig. 2B). In addition, prelamin A-synthesizing oocytes often showed a nucleoplasmic lamin A veil, a faint homogenous staining of the nuclear interior (Fig. 2B, and not shown). Such injected oocytes could be kept in culture for up to one week. Remarkably, the abundance and immunostaining intensity of the lamin A veil increased after prolonged incubation times whereas the distribution of the endogenous lamin remained unchanged (not shown).

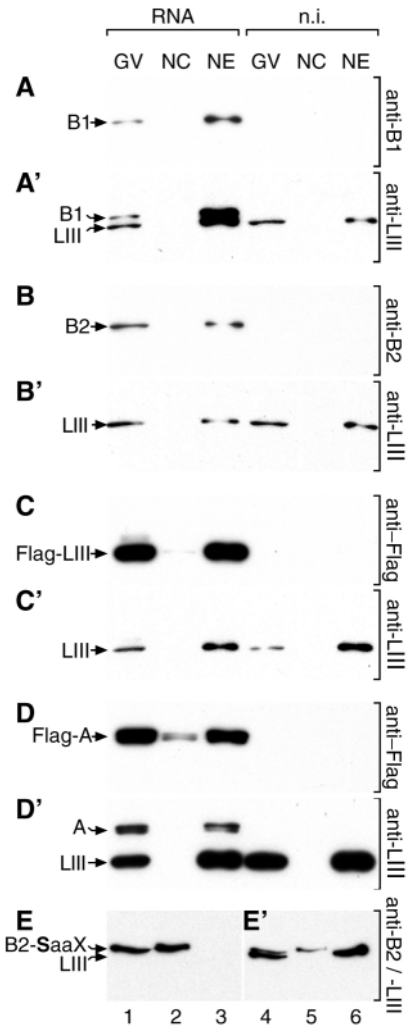

Fig. 1. Lamins synthesized in oocytes associate with the nuclear envelope. (A-E') Lamins were expressed in *Xenopus* oocytes by RNA injection. 16 hours after injection nuclei were manually isolated and either processed directly (GV, lane 1) or separated into nuclear content (NC, lane 2) and nuclear envelope (NE, lane 3). Fractions were separated by SDS-PAGE and lamins were detected by immunoblotting using chemiluminescence. Material from three nuclei was loaded in each lane. Lamin B1 was detected with mAb L7-4A2 (A), lamin B2 with mAb L7-8C6 (B,E), lamin Flag-LIII and Flag-A with mAb M2 (C and D, respectively). Control fractions of uninjected oocytes (n.i.) were processed in parallel (lanes 4-6). All blots were reprobed with lamin LIII-specific mAbs, mAb L6-5D5 (A'-D') or mAb NUC195 (E'). Note that blots were not stripped before reprobing, therefore, residual chemiluminescence signal from the first immunoreaction was still detectable, as seen in A'-E'.

Expression of genes encoding lamins B1 or B2 resulted in a different distribution. Both were associated with the nuclear envelope (Fig. 2C-E) (lamin B2 not shown). The distribution of these lamins, however, was discontinuous, often appearing as series of immunofluorescent dots of variable size at or very close to the nuclear envelope (Fig. 2D,E). Here, the nucleoplasm did not display any immunostaining, thus corroborating the nuclear fractionation results shown in Fig. 1.

To analyze lamin distribution in more detail, isolated nuclear envelopes were attached to coverslips with their nucleoplasmic side facing up and were examined by immunofluorescence microscopy (Fig. 2F-L). Nuclear envelope spread preparations of uninjected oocytes were uniformly stained with a lamin LIII-specific antibody, demonstrating that the endogenous lamin LIII was evenly distributed over the oocyte lamina (Fig. 2F). Additional synthesis of epitope-tagged lamin LIII showed essentially the same uniform distribution, with both myc- and Flag-tagged versions (Fig. 2G and not shown), indicating that the excess LIII protein was integrated into the existing lamin network and that the nature of the epitope tag did not influence the distribution of the proteins. At very high levels of gene expression that were achieved either by increasing the amount of RNA in a single injection or by two successive injections, LIII additionally accumulated in small dots (Fig. 2H). However, nuclear envelope spreads made upon overexpression of lamin B1 and B2 gene constructs revealed a strikingly different pattern dominated by the formation of granules and patches of variable size and shape up to sizes exceeding a micrometer (Fig. 2I,J), which were stained intensively with the lamin-specific antibodies used. However, the nuclear envelope regions between these structures remained practically unstained (compare Fig. 2I,J with the control preparation of an uninjected oocyte shown in 2L).

We then followed the time course of formation of such dots and patches by preparing envelope spreads at different times after RNA injection. Mostly, small dots were seen two to four hours after RNA injection. Thereafter the immunostained structures grew with time and eventually fused into large lamin-positive aggregates. Careful microscopic analysis indicated that these structures were located above the plane of the nuclear envelope, corresponding in situ to the nucleoplasmic side of the nuclear envelope. These structures also appeared to be stable over the entire period of oocyte in vitro culture, i.e. up to seven days. Lamins included in these structures were obviously resistant to extraction with buffers of high ionic strength as well as buffers containing high concentrations of non-ionic detergents. This suggested that the lamins here were assembled into supramolecular structures. Such patch formations occurred even at very low levels of expression of B1 or B2, as observed with wild-type (Fig. 2I,J) and epitope-tagged versions (Fig. 2D,E and results not shown). The distribution of the endogenous lamin, however, was unaffected in the presence of additional lamin B1 or B2. LIII did not accumulate in these B1- or B2-induced patches to a significant extent (not shown).

In line with the results obtained in sections, the excessive lamin A also showed uniform distribution throughout the nuclear envelope in spread preparations and was essentially indistinguishable from that of the endogenous lamin LIII (Fig. 2K). We never observed that the additional lamin A induced structures similar to those observed with lamins B1 and B2.

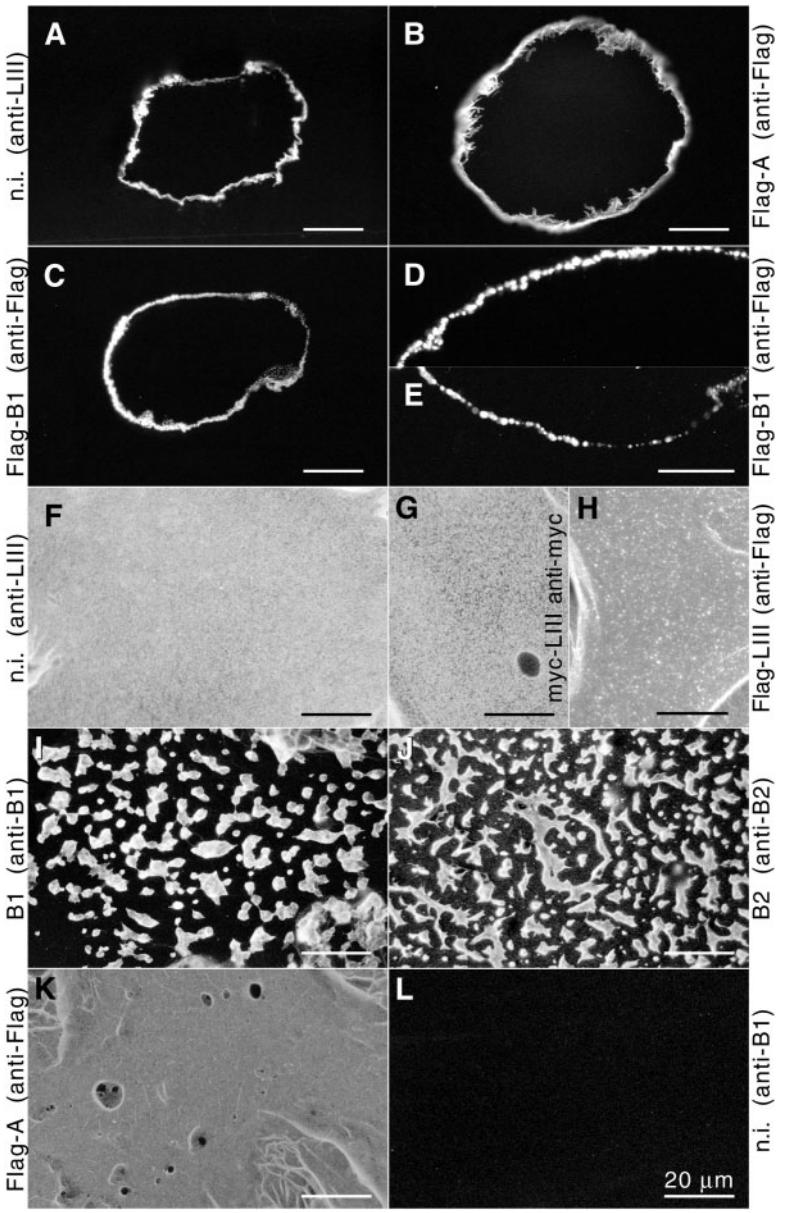

Fig. 2. Indirect immunofluorescence analysis of oocytes synthesizing nuclear lamins. Cryostat sections (A-E) and nuclear envelope spread preparations (F-L) are shown. The type of RNA injected is indicated at the margins of each panel. n.i., non-injected control oocytes. Oocyte lamin LIII was detected with mAb NUC195 (A) or mAb L6-5D5 (F), Flag-A, Flag-B1 and Flag-LIII with mAb M2 (B-E,H,K), lamin B1 and non-injected control envelopes were reacted with mAb L7-4A2 (I,L); myc-LIII with mAb 9E10 (G); lamin B2 with mAb L7-8C6 (J). Cy3-conjugated goat anti-mouse IgG was used as a secondary antibody. Brighter staining along folds of the nuclear envelope in F-H, K is due to folding of the nuclear envelope during the spreading procedure. Bars, 20 μm.

Electron microscopy of lamin-induced intranuclear membrane structures

Next, lamin-induced structures were analyzed by transmission electron microscopy of isolated oocyte nuclei. We concentrated our analysis on lamin B2-induced structures as this lamin could be detected with a monoclonal antibody suitable for immunoelectron microscopy. Here the localization of the additional lamin B2 coincided with the appearance of extensive arrays of intranuclear membrane-like cisternae similar in appearance to the nuclear envelope cisternae but lacking structures resembling pore complexes. These cisternae were often stacked and were always found in the vicinity of the inner nuclear membrane (Fig. 3A). The similar spatial distributions indicated that the granules and patches observed with the light microscope (Fig. 2) and the intranuclear arrays of stacked membrane-like elements represented different views of the same structures. Each of the membrane-like arrays was in contact with the inner nuclear membrane usually at one end, and the contact area was usually rather small (Fig. 3B,C). In several places the inner nuclear membrane and the intranuclear cisternae-like structures were in parallel (Fig. 3A-C). In serial sections, however, we never observed continuity between the intranuclear cisternae and the nuclear envelope. The intranuclear cisternae showed a tendency to form stacks in which the adjacent cisternae were separated by a characteristic narrow nucleoplasmic space. In addition, intranuclear vesicles of variable size were also frequently found near the cisternal stacks (Fig. 3B). Clearly, nuclear pore complex-like structures were not detected in these cisternal structures, thus excluding any relationship to intranuclear annulate lamellae (for reviews see Ghadially, 1988; Kessel, 1992).

Immunoelectron microscopic analysis of isolated oocyte nuclei revealed that the excess lamin B2 was located at or within the intranuclear membrane arrays (Fig. 3D). Immunogold labeling was strongest at the outermost membrane sheets and weak in or absent from the inner cisternae of the arrays probably because of limited accessibility of the inner regions of the multi-layered stacks to immunogold particles. In these experiments, the inner nuclear membrane was only very sparsely decorated with B2-specific antibodies, in line with the immunofluorescence data presented (Fig. 2J).

Interaction of CaaX-containing proteins with membranes can be prevented when the CaaX cysteine residue is replaced by any other amino acid residue. To determine which lamin parts are involved in the induction of intranuclear membrane formation and whether this depends on CaaX-mediated modifications, the lamin B2-SaaX gene mutant was expressed in oocytes. As shown by gel electrophoretic analysis, B2-SaaX protein accumulated in the nucleoplasm but did not associate with the nuclear envelope (Fig. 1E). The formation of intranuclear cisternal arrays was never observed in oocyte nuclei expressing the B2-SaaX gene construct (Fig. 3E), suggesting that interaction with membranes is a prerequisite for formation of intranuclear membrane stacks as described above.

The membrane-targeting motifs of N-Ras induce formation of intranuclear vesicular structures

We have previously shown that a NLS in conjunction with a

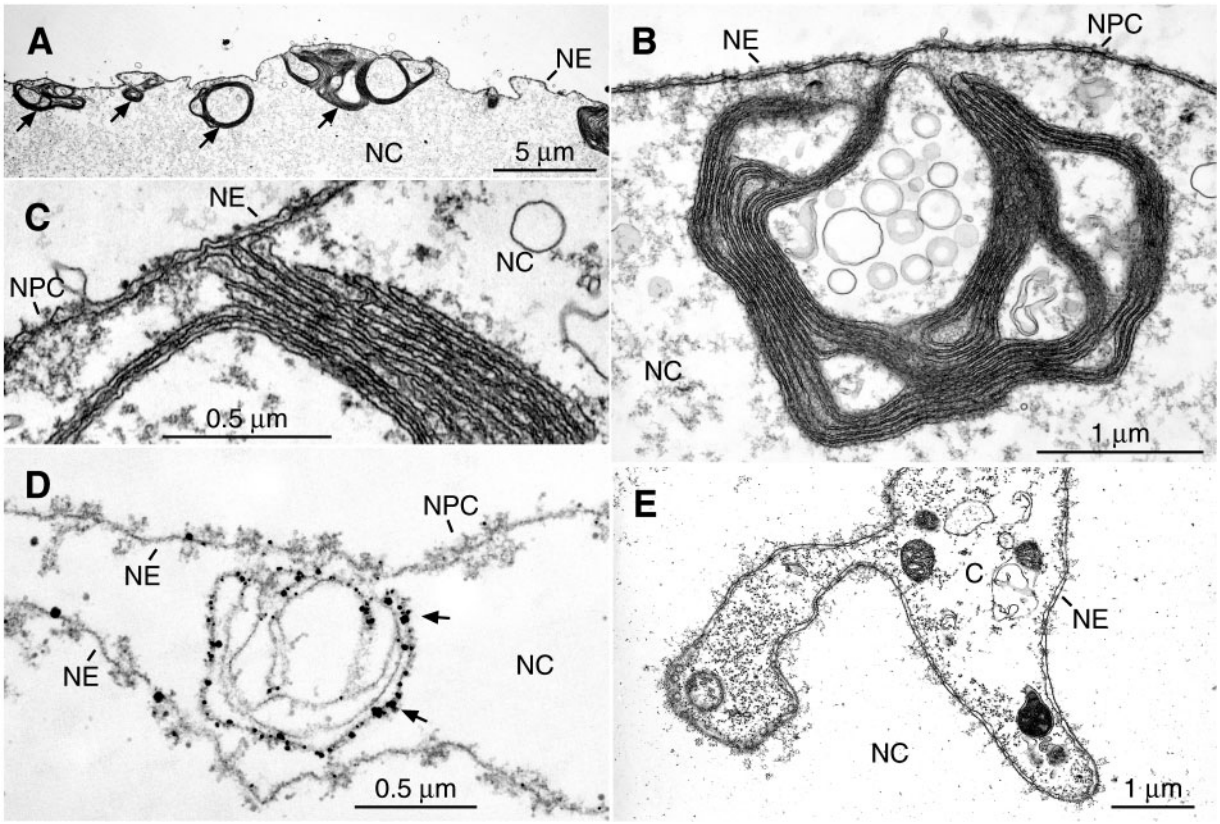

Fig. 3. Electron microscopy of lamin-induced intranuclear membrane structures. (A-E) EM sections of isolated oocyte nuclei are shown. Oocytes were injected with RNA encoding lamin B2 (A-D) or lamin B2-SaaX (E). Nuclei were isolated and processed for electron microscopy. Sections were stained with uranyl acetate and lead citrate (A-C,E) or processed for pre-embedding immunoelectron microscopy (D). Lamin B2 was detected with mAb L7-8C6 and nanogold-coated secondary antibody. Arrows in A indicate membrane arrays attached to the nuclear envelope. Arrows in D indicate gold particles decorating the outer membrane cisternae of a lamin B2-induced membrane array. C, cytoplasm; NC, nucleoplasm; NE, nuclear envelope; NPC, nuclear pore complex. Bar, 5 μm (A); 1 μm (B,E); and 0.5 μm (C,D).

CaaX motif can act as a minimal targeting motif for the association of proteins with the inner nuclear membrane (Hofemeister et al., 2000). We therefore asked whether a protein lacking any lamin-specific sequences might also induce intranuclear membrane formation, using GFP chimeras containing nucleus-targeting sequences that were not derived from lamins. One of these chimeras contained at its N-terminus the NLS of the large T antigen, followed by six myc epitopes as tags, and at its C-terminus the last 11 amino acid residues of human N-Ras (NLS-MT-GFP-N-Ras). The construct was expressed in HeLa cells and its distribution was followed by fluorescence microscopy. The chimeric protein was enriched at the nuclear envelope but was also located in the nucleoplasm and the cytoplasm (Fig. 4A,B). In cells producing high levels of chimeric protein, vesicular and tubular structures were present that were decorated with chimeric GFP. These structures were either associated with the nuclear envelope or were present in the interior of the nucleus. Brightly fluorescent dots generally smaller than the nuclear structures were often present in the cytoplasm (Fig. 4A-C). As a control, a chimera lacking the N-Ras-specific CaaX sequences was expressed. The resulting protein was efficiently accumulated in the nucleoplasm, but did not show association with the nuclear envelope. Nuclei of transfected cells did not show any signs of additional intranuclear structures (Fig. 4D).

When, however, cells expressing NLS-MT-GFP-N-Ras chimeric gene constructs were analyzed by transmission electron microscopy, the nuclei of the transfected cells contained numerous intranuclear stacked membrane-like arrays (Fig. 5), resembling those found in oocytes synthesizing both lamin B1 and B2, and again the cisternae were free of pore complex structures. In contrast to the lamin-induced intranuclear membrane-like arrays in oocytes (Fig. 3), where attachment to the nuclear envelope was displayed only rarely, in the NLS-GFP-N-Ras gene construct-expressing cells, multilayered membrane cisternal stacks were frequently close to the inner membrane over relatively large areas. However, pore complexes were absent from the nuclear envelope in these areas. Such intranuclear membrane-like arrays were also frequently found in the nuclear interior (Fig. 5A-D) and these arrays often contained vesicles of variable diameters that were either associated with, or entirely surrounded by, stacked cisternae. We never observed such membrane structure stacks in the cytoplasm of these cells, although they should have been detected by the methods used (e.g. Cordes et al., 1996).

Our observation that intranuclear membrane proliferation can be induced by expression of a chimeric nuclear protein containing a CaaX motif bears the intriguing possibility that this might be a general property of CaaX-containing proteins rather than a peculiarity of particular lamin proteins.

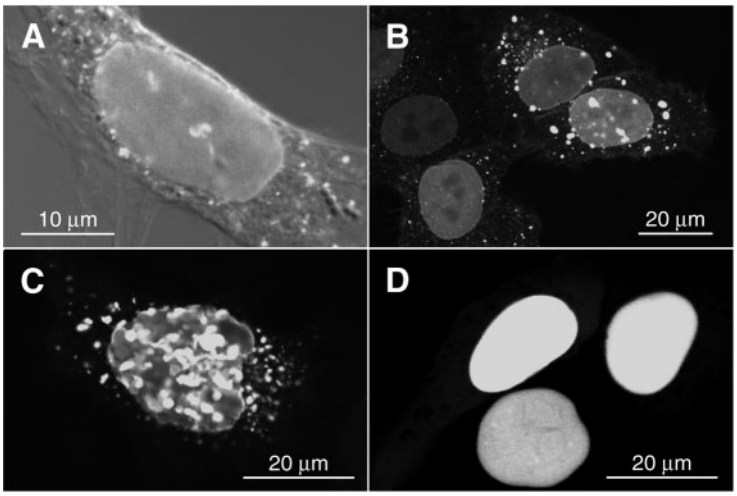

Fig. 4. Nuclear GFP containing the membrane targeting motifs of N-Ras induces intranuclear vesicular structures. HeLa cells transiently transfected with NLS-MT-GFP-N-Ras (A-C) or NLS-MT-GFP (D) chimeras were fixed 24 hours after transfection and examined by fluorescence confocal laser scanning microscopy. In A an overlay of the GFP fluorescence and differential interference contrast picture is shown. Note the presence of brightly fluorescing vesicular structures inside nuclei of cells expressing high levels of the chimeric GFP in C. Bar, 10 μm (A); 20 μm (B-D).

Discussion

By overexpression of gene constructs encoding nuclear lamin proteins in *Xenopus* oocytes we have shown that lamins B1 and B2, rather than being integrated into the endogenous lamina and forming thickened 'fibrous laminae' as repeatedly described in the literature [Fawcett and others (Fawcett, 1966; Höger et al., 1991), and references therein], induce the formation of intranuclear membrane-like structures that harbor the excess protein. By contrast, when genes encoding lamins A and LIII are overexpressed, the resulting proteins are targeted to the nuclear envelope and associate with the lamina in a manner indistinguishable from that of endogenous lamin LIII. These findings of differences between lamins are remarkable, as all four lamins are coexpressed in various combinations and relative ratios in different cell types during development and in adult tissues of *Xenopus laevis*.

Lamin LIII is the major constituent of the oocyte lamina (Stick and Krohne, 1982; Stick and Hausen, 1985; Stick, 1988), whereas lamins B1 and B2 are only minor components in oocytes and early embryonic cells, and lamin A is completely absent from these cells (Wolin et al., 1987; Lourim et al., 1996). From the gastrula stage onwards, lamins B1, B2 and LIII are integrated into a common nuclear lamina structure of embryonic nuclei (Benavente et al., 1985; Stick and Hausen, 1985). As development proceeds, the amount of lamin LIII per nucleus decreases, and this lamin is gradually replaced by lamins B1 and B2. Lamin A, which is expressed late in embryonic development and occurs in most somatic cells, always coexist with at least one of the B-type lamins (Wolin et al., 1987; Röber et al., 1989) (reviewed by Stick, 1987).

In oocytes, induction of formation of intranuclear cisternal stacks is noted even at low levels of lamins B1 and B2. The additional lamin B1 and B2 is clearly confined to the induced stacked arrays, in striking contrast to results obtained in transfection studies with cultured cells (Izumi et al., 2000) and with *Xenopus* embryonic cells. When synthesized in embryos,

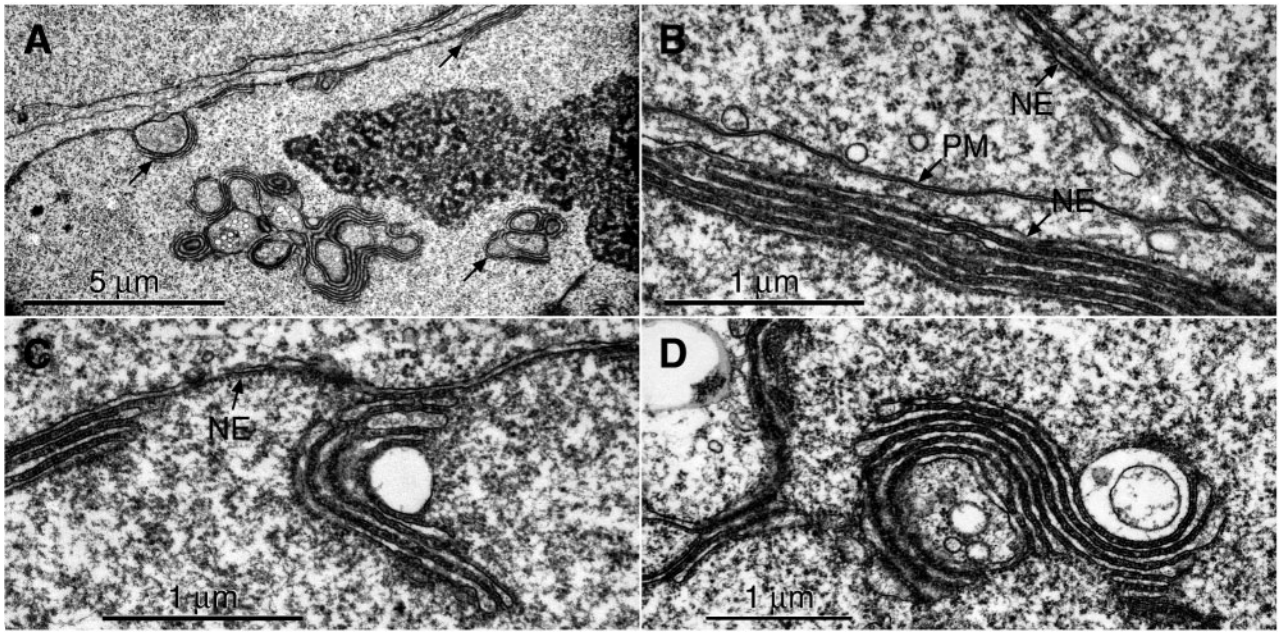

Fig. 5. NLS-MT-GFP-N-Ras chimeras induce formation of intranuclear membrane arrays in HeLa cells. (A-D) HeLa cells transiently transfected with NLS-MT-GFP-N-Ras were processed for EM 24 hours after transfection. Sections were stained with uranyl acetate and lead citrate. Note the formation of stacked membrane cisternae aligned with the nuclear envelope as well as the formation of membrane arrays within the nucleoplasm. NE, nuclear envelope; PM, plasma membranes. Bar, 5 μm (A); 1 μm (B-D).

lamins B1 and B2 are found at the nuclear envelope, giving rise to 'nuclear rim staining' (our unpublished results) as characteristic for proteins located in the nuclear lamina (see also Gerace et al., 1978; Krohne et al., 1978). One possible explanation might be that the oocyte nuclear envelope lacks factor(s) needed for the integration of lamins B1 and B2 into the existing lamina. The nature of these factors is not known. However, certain inner nuclear membrane proteins like those of the LAP2-family of proteins, which are differentially expressed in oocytes and early embryos, or the *Xenopus* LBR protein, which is redistributed from the ER to the inner nuclear membrane during early stages of development are potential candidates (Gajewski and Krohne, 1999; Schoft et al., 2003) and may play a role in these topogenic processes. On the other hand, lamin LIII is integrated into the existing lamina over a wide range of protein concentrations as shown by the uniform distribution of the excess protein. Induction of small intranuclear membrane arrays was only seen in oocytes expressing very high levels of the lamin LIII gene construct, and here the 'novel' lamin-containing spots appeared on the basis of an uniformly stained nuclear envelope.

Expression of genes encoding prelamin A did not induce formation of intranuclear membranes, and this difference in behaviour cannot yet be explained. However, the fate of lamin A after integration into the nuclear envelope might provide a clue: all four lamins, B1, B2, LIII and A, carry a CaaX motif at their C-termini and undergo CaaX-dependent isoprenylation and carboxyl methylation but A lamins lose their isoprene moiety soon after incorporation into the lamina by a lamin A-specific endoproteolytic processing (Weber et al., 1989; Beck et al., 1990; Hennekes and Nigg, 1994). Therefore, lipid-mediated interaction of A lamins with the inner nuclear membrane may be abolished whereas B-type lamins are permanently isoprenylated (Nigg et al., 1992; Firmbach-Kraft and Stick, 1993).

In vitro studies show that that mammalian lamins can form complexes in all possible combinations (Schirmer and Gerace, 2004) and in vitro cotranslation studies with *Xenopus* lamins support this, although in the latter case it is not known whether these complexes form at the dimer or at a higher oligomeric level (our unpublished results).

Endoplasmic reticulum (ER) membrane proliferation has been intensely studied (e.g. Wright et al., 1988; Schunck et al., 1991; Vergeres et al., 1993; Nishikawa et al., 1994; Wanker et al., 1995; Naik and Jones, 1998), including changes in ER structure occurring during cell differentiation and in response to external cues (e.g. Orrenius and Ericsson, 1996). Particularly striking examples are the formation of stacked cisternae on the outer nuclear envelope, called karmellae (Smith and Blobel, 1994; Parrish et al., 1995; Koning et al., 1996). Moreover, changes in the ER structure might also be caused by particular proteins, such as those containing one or more transmembrane ER-targeting domains. For example, a single transmembrane domain of a resident ER protein fused to a cytoplasmic GFP is sufficient to induce overproduction of ER membranes (Snapp et al., 2003). Apparently, lamins lack transmembrane domains and interact with membranes via their lipidated hydrophobic C-termini. We have show here that a chimeric GFP protein containing two targeting sequences, a NLS and a C-terminal CaaX motif, is sufficient to induce intranuclear membrane formations in HeLa cells. Remarkably, this chimera does not show any sequence similarity with lamins, except that it carries the same targeting motifs, which in this case are derived from the large T antigen and human N-Ras, respectively. This shows that the potential to induce the formation of intranuclear membrane structures might not be a feature of particular lamins but a more general phenomenon observed when proteins interact with membranes ectopically or at unusually high levels.

The morphology of the intranuclear membrane-like structures described in this study resembles that of stacked cisternae of the ER in several aspects. The induced proteins form flat cisternae that closely associate producing large stacks in which adjacent cisternae are separated by a narrow nucleoplasmic space of constant width. Further experiments are necessary to elucidate how the lamin molecules dimerize and associate into higher order assemblies (Stuurman et al., 1998). Studies with chimeric GFP proteins have shown that even low affinity dimerization of GFP might be sufficient to induce extensive stacking of ER cisternae (Snapp et al., 2003). We propose that the stacked membrane arrays might form by high or low affinity homotypic interaction of proteins that can associate with membranes via lipid anchors or are integrated into membranes by transmembrane domains.

Intranuclear membrane formations have previously also been observed in cells overexpressing nucleoporin Nup153 (Bastos et al., 1996) or nucleoporin Nup53p (Marelli et al., 2001) which also induce the appearance of bundles and arrays of membranous tubule formations that are either associated with the nuclear envelope or occur free in the nucleoplasm. However, as Nup153 does not contain transmembrane domains or other hydrophobic regions, it remains unclear how this protein might lead to intranuclear membrane proliferation. In contrast, in the case of Nup53p a potential amphipathic α-helix at its C-terminus seems to be responsible for the induction of intranuclear membrane formation (Marelli et al., 2001). Recently a deletion mutant of fibroblast growth factor receptor was described that lacks most of its extracellular region and its kinase domain but retains its transmembrane region (Sørensen et al., 2004). This protein localized to the nucleus and induced intranuclear membranes similar to those described here.

Electron microscopic studies of ER karmellae and other stacked ER arrays have established that these structures are often connected to the rest of the ER system, and photobleaching experiments have demonstrated a continuous flow of membrane proteins in and out of the stacked arrays (Snapp et al., 2003). In contrast to ER-derived membranes, continuity between inner nuclear membrane and intranuclear cisternae are never observed. At sites where both membranes are in close contact, they align tightly and form a narrow space identical to the spaces found between adjacent cisternae. The contact areas are large in cells expressing NLS-GFP-N-Ras, whereas they are restricted to small patches in lamin-induced membrane arrays. This might be explained by the distribution of the overexpressed proteins within the inner nuclear membrane. NLS-GFP-N-Ras shows a typical, although weak, nuclear rim staining. Alignment and zippering together of intranuclear cisternae with the inner nuclear membrane might therefore occur by interaction with the GFP chimeric protein located at the inner nuclear membrane. Overexpressed lamins B1 and B2 are not, or only to a very limited extent, incorporated into the existing lamina at the inner membrane.

Attachment points might therefore be limited to those areas in which small amounts of endogenous lamin B1 or B2, respectively, might be concentrated. Our findings suggest that homotypic interactions of lamin LIII are strongly favored over heterotypic interaction of LIII with B1 or B2.

As no continuity was found between intranuclear cisternae and the inner nuclear membrane, membrane delivery into the nuclear interior could occur by vesicle budding. However, bud formation has not yet been observed by electron microscopy.

In summary, our results show that CaaX-containing proteins when targeted into the nucleus can induce intranuclear membrane formation. These membranes form extensive arrays of stacked cisternae. Stacking is brought about by high or low affinity protein interaction. Intranuclear membrane stacks show similarity to organized smooth ER that forms when resident ER proteins are expressed at high levels, but the intranuclear membrane arrays are not continuous with the inner nuclear membrane. The capacity of particular lamins to induce membrane proliferation might be modulated by other cell type-specific expressed proteins. Our observations may serve as a cautionary note for studies using overexpression of lamins and other CaaX-containing proteins.

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