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The role of sequences unique to nuclear intermediate filaments in the targeting and assembly of human lamin B: evidence for lack of interaction of lamin B with its putative receptor

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SUMMARY

The mechanism by which human nuclear lamin B is targeted and assembled has been studied by transfecting into mammalian cells lamin mutants deleted of three sequences unique to lamins. Nuclear lamins contain an extra 42 amino acids (aa) in their rod domains and NLS and CAAX motifs in their tail domains, which distinguishes them from cytoplasmic IF proteins. These three sequences act in concert to ensure correct temporal and spatial assembly of lamin B. Deletion of any one of these three sequences from lamin B did not significantly disrupt nuclear lamina targeting, but when two or more of these sequences were deleted, targeting was severely compromised. The CAAX motif is necessary for the efficient integration of lamin B into an already formed nuclear lamina, since lamin B CAAX$^-$ mutants had reduced targeting to the lamina when arrested in S phase of the cell cycle. CAAX-deficient mutant lamin B proteins were soluble and not associated with membranes at mitosis, proving that the CAAX motif is responsible for association of human lamin B with membranes. In addition, CAAX$^-$ mutant lamin B proteins fractionated independently of the lamin B-receptor (LBR), indicating that these two proteins do not bind directly to each other.

Key words: Intermediate filament, Lamin, Nucleus, CAAX motif

INTRODUCTION

Intermediate filament (IF) proteins are a heterogeneous group of proteins that assemble into 10 nm filaments either as long arrays in the cytoplasm, or as a cage-like cross-hatched meshwork beneath the nuclear envelope (Aebi et al., 1986; reviewed by Fuchs and Weber, 1994). Found in all cell types and the major cytoskeletal component in some cells, both cytoplasmic and nuclear IFs have been implicated in diverse cellular functions (reviewed by Hutchinson et al., 1994; McLean and Lane, 1995; Ho and Liem, 1996; Lee and Cleveland, 1996; Rao et al., 1996; Fuchs, 1997; Lenz-Bohme et al., 1997).

Structurally, IF proteins are characterized by a highly conserved central α-helical 'rod' domain flanked by less conserved 'head' and 'tail' domains (reviewed by Conway and Parry, 1988; Stewart, 1993; Shoeman and Traub, 1993; Heins and Aebi, 1994). The rod domain in vertebrate cytoplasmic IF proteins is generally 310 amino acids (aa) in length, although there are exceptions of some highly divergent IF proteins that possess slightly smaller or longer rods domains (Wallace et al., 1998; Hess et al., 1998). In contrast, nuclear lamins possess rod domains that are 352 residues long due to an insert of a 42 aa sequence consisting of six heptad repeats with α-helical properties (see Nigg, 1989; Weber et al., 1989a). Interestingly, invertebrate IF proteins also possess the 42 aa insert, leading to the hypothesis that they are the ancestral precursors of lamin proteins and that cytoplasmic IF proteins having evolved from lamins by loss of the 42 aa insert (Dodemont et al., 1990; Doring and Stick, 1990; Way et al., 1992). In addition, nuclear IF proteins differ from cytoplasmic IF proteins by containing a nuclear localization signal (NLS) in their tail domains (Loewinger and McKeon, 1988; Moir et al., 1995) and typically a COOH-terminal CAAX motif (where C is cysteine, A an aliphatic amino acid and X is variable; Holtz et al., 1989; Nigg et al., 1992; Moir et al., 1995). The NLS facilitates transport of proteins to the nucleus, whereas the CAAX motif is post-translationally modified in a complex series of steps that involve addition of isoprenyl and carboxyl methyl groups to the cysteine and eventual proteolytic cleavage of the C-terminal AAX residues. The CAAX modifications are thought to be involved in anchoring lamins to the nuclear envelope, and possibly also aid in lamina assembly, although there is conflict regarding these roles (see Lutz et al., 1992; Firmbach-Kraft and Stick, 1993).

There are three major lamins expressed in mammalian cells, termed lamins A, B and C, with A and C being alternatively spliced products of the same gene (see Moir et al., 1995). The

CAAX motif is present in lamins A and B but is missing from lamin C, suggesting that a collinear CAAX motif is not necessary for localization and assembly. Instead, nuclear envelope targeting of lamin C could occur by oligomerization with the CAAX-containing lamins, A and B, which are both initially translated with CAAX motifs but differ in their post-translational processing and maturation pathways. A protease removes the last 18 aa, including any CAAX modifications from lamin A, shortly after it is incorporated into the lamina (Weber et al., 1989b). In contrast, the CAAX-associated modifications remain permanently associated with lamin B.

The retention of CAAX modifications confers different biochemical properties onto lamins. This is especially noticeable at mitosis when nuclear and some cytoplasmic IFs are disassembled as a result of phosphorylation (Gerace and Blobel, 1980; Chou et al., 1990; Peter et al., 1991; Heald and McKeon, 1990; Ward and Kirschner, 1990; Luscher et al., 1991; Foisner, 1997). Lamins have been classified into two types, based on their solubility properties at mitosis. A-type lamins, typified by lamins A and C in humans, are found as soluble proteins after centrifugation, irrespective of Triton X-100 treatment (Gerace and Blobel, 1980). In contrast, B-type lamins, typified by lamin B in humans, are insoluble under similar centrifugation conditions but become soluble after Triton X-100 treatment. In the absence of detergent, lamin B is believed to associate with fragments of the disassembled nuclear envelope through its permanently attached CAAX-encoded modifications, whereas upon treatment this association is disrupted (Gerace and Blobel, 1980; Weber et al., 1989b).

In this report we have investigated the role of the three lamin-specific sequences, the 42 aa insert, the NLS and the CAAX motif, in lamin B targeting and assembly, by deleting them singly and in all possible combinations. Transfection of these mutants into four different cell lines has provided new insight into the roles these sequences play in lamina targeting.

MATERIALS AND METHODS

Construction of lamin constructs

Full-length and mutant lamin molecules were constructed from human lamin A, B, and C cDNAs obtained from the following sources: lamins A and C were in pUC19 (gift of Dr G. Blobel, Rockefeller University); lamin B was in pBluescript KS(−) (gift of Dr Charles Glass, Scripps Research Inst.; Pollard et al., 1990).

All lamin constructs were expressed using the modified CMV-NF-L expression plasmid (Lee et al., 1993; Janicki and Monteiro, 1997) and detected using the monoclonal antibody 9E10 specific for the myc epitope corresponding to 12 aa of h-myc sequence (Monteiro et al., 1994) that was used to tag each protein at its C-terminal end. For CAAX-containing constructs, the myc-tag preceded the CAAX motif (see Monteiro et al., 1994).

pLamB(–CAAX) or construct B–

The 5′ half of lamin B was first inserted into CMV-NF-L (Lee et al., 1993) by digesting the latter with *Xba*I (which cleaves just 3′ of the CMV promoter), followed sequentially by filling the overhang with T4 DNA polymerase and digestion with *Hin*dIII. The resulting approximately 3.3 kbp fragment containing the CMV promoter, devoid of NF-L sequences, was ligated with the 5′ portion of lamin B (corresponding to codons 1-117 of lamin B) obtained as a 741 bp *Eco*RV-*Hin*dIII double-digested fragment of lamin B in pBS.KS(–). Next the 3′ end of lamin B was added together with the myc-tag.

The myc-tag was obtained by cutting pNF (Monteiro et al., 1994) to completion with *Sal*I (which cleaves just 5′ of the myc-tag) followed by partial digestion with *Hin*dIII (there are two *Hin*dIII sites, one located 5′ of the MSV promoter in pNF and another 3′ of the NF-L untranslated sequences). The approximately 4.0 kbp fragment, containing vector sequences together with the myc-tag and 3′-NF-L untranslated sequences, was isolated and ligated with a 3′ fragment of lamin B generated by PCR amplification using primer 5′-GCTGCTCCTCAACTATG-3′ and primer 5′-ACGATGAGCTGCG-GCCGCTCGAGGCTTCTATTGGATGCTCT-3′. The two primers both contained 17 bases at their 3′ end that were complementary to lamin B sequences encoding aa 116-122 and 576-582, respectively. The primers were used to PCR amplify the region between codons 116-582 of lamin B, excluding the CAAX motif. The approximately 1.2 kbp PCR product was double-digested with *Hin*dIII and *Xho*I (note all end restriction sites in this and subsequent PCR fragments were added through the primers) and ligated with the pNF fragment. The resulting clone was cut with *Hin*dIII and the approximately 2.5 kbp fragment containing lamin B 3′ coding sequences linked in-frame with the myc-tag, was isolated and ligated into the *Hin*dIII cut CMV expression plasmid containing the 5′ portion of lamin B (described above). Recombinants containing the 3′ lamin B fragment inserted in the appropriate orientation corresponded to lamin B that was myc-tagged and deleted of the CAAX motif (Fig. 1; referred to as construct B–).

pLamB(+CAAX) or B

Lamin B containing both a myc-tag and a CAAX motif was constructed by PCR using primer 5′-GCTGCTCCTCAACTATG-3′ and primer 5′-TACCGTCGACTCACATGATGCTGCAGTTGGGG-CTCTGGGTATTCAAGTCCTCTTCAG-3′. The latter contained 32 bp at its 3′ end, 17 of which were complementary to the myc-tag in construct B–, preceded by 15 bases to add the CAAX* sequence (where *refers to a TGA stop codon). The primers were used to amplify the C-terminal region between codons 118 of lamin B and the myc-tag, thereby adding the CAAX sequence back. The approximately 1.2 kbp PCR product was then reamplified with the first primer described above and primer 5′-CGGAATTCAG-GCCTGATATCTGCGCATCGCGACTCACATGATGCTGCAGT-3′ (containing 17-bp at the 3′ end complementary to the CAAX motif, followed by a *Stu*I restriction site to facilitate ease of cloning). The resulting product was cut with *Hin*dIII and *Stu*I and substituted for the corresponding C-terminal portion in construct B–, generating lamin B that was myc-tagged followed by the CAAX motif and referred to as construct B.

pLamB(–42 aa/–CAAX) or G

To delete the 42 aa insert from pLamB(–CAAX), primer 5′-CCATGGCGACTGCGACC-3′ (complementary to aa 1-6 of lamin B) and primer 5′-TATCGGATTCCTCGAGGTTGAGGAGCAGCTG-GTC-3′ (containing 17-bp at the 3′ end that were complementary to sequences immediately upstream of the 42 aa insert beginning at codon 120 of lamin B) were first used to PCR amplify sequences upstream of the 42 aa insert. Next sequences 3′ of the 42 aa insert were PCR amplified using primer 5′-CTATCGATCGGCCGCTC-GAGGATCTGAAGGATCAGATT-3′ together with the 41 bp primer that was used previously to make pLamB(–CAAX). The two products were substituted for corresponding fragments in construct B–, linked together by their common *Xho*I sites, thereby precisely deleting the 42 aa from the rod domain (changing the sequence from DQLLLN-YAKKES...30 aa...KSLEGD-LEDLKD to DQLLLN-LEDLKD; Fig. 1, construct G).

pLamB(–NLS/–CAAX) or E

To delete the NLS from lamin B, primer 5′-TCACGAG-GATCCCGAGTGGAAGAATCAGAGGCG-3′ (containing 18 bp complementary to sequences immediately downstream of the NLS)

and primer 5′-CAGACAACACACCATTC-3′ (complementary to 3′ NF-L untranslated sequence) were used to PCR amplify the region between aa 421 and the end of the 3′ untranslated region of NF-L of construct B–. The PCR product was digested with *Ava*I and used to replace the corresponding *Ava*I fragment from construct B–. By this process 18 aa containing the presumptive lamin B NLS were deleted, changing the aa sequence in that region from VTVSR-ASSSRSVRTTRGKRKRVD-VEESE... to VTVSR-VEESE.

pLamB(–NLS) or D, pLamB(–42 aa) or F, pLamB(–42 aa/–NLS) or H, pLamB(–42 aa/–NLS/–CAAX) or I

These constructs were made by substituting appropriate fragments between the constructs described above using standard restriction digestion and cloning procedures.

pLamA(–CAAX) or A– and pLamC or C

Lamins A and C were myc-tagged using either primer 5′-CAGTGAATTCGTCGACTCGGGGGCTGGAGTTGCC-3′ or primer 5′-CAGTGAATTCGTCGACGCGGCGGCTACCACTCAC-3′ (each of which contained 17 bp complementary to either the six carboxyl-terminal coding residues of lamin C, or the sequence beginning 11 codons upstream from the stop codon of lamin A, respectively) in combination with primer 5′-GACCTGGAGGCTCTGCTG-3′ (complementary to the common coding sequence in both lamins A and C). The two products were each digested with *Apa*I and *Sal*I and the resulting lamin A and C tail fragments were substituted for the NF-L tail in construct pLamA(+NF/tail) (Monteiro et al., 1994). The resulting recombinants were lamin A missing 10 C-terminal residues, and full-length lamin C, both with C-terminal myc-tags.

pLamA(+CAAX) or A

pLamA(–CAAX) was cut completely and pLam(NF/head&tail+NLS+CAAX) partially, with *Eco*RI and *Sal*I, and the large fragment from the former and the 350 bp fragment from the latter, containing the CAAX motif preceded by the myc-tag, were ligated together. By this procedure, 9 of 10 C-terminal residues of lamin A, including its CAAX motif, were fused back and in-frame onto the 3′ end of the myc sequence of pLamA(+CAAX).

All of the PCR-generated changes in the constructs were sequenced and found to contain the desired changes without any additional change being observed.

Cell culture and DNA transfection

HeLa, NIH 3T3, mouse L cells and SW13(–) cells seeded onto coverslips were grown in DMEM containing 10% fetal bovine serum and transfected with 15 μg plasmid DNAs as calcium phosphate precipitates (Graham and van der Eb, 1973). Stably transfected HeLa cells were isolated essentially as described by Monteiro and Cleveland (1989).

Immunofluorescent staining

Cells on coverslips were stained at the times indicated in the text and their fluorescence images captured under a Zeiss Axiovert 135 microscope using a ×100 Plan-Apo objective lens as described previously (Janicki and Monteiro, 1997). Primary antibodies used were the mouse monoclonal anti-myc 9E10 antibody (Evan et al., 1985), rabbit anti-lamin antibody (generated in this laboratory using recombinant-purified, bacterially expressed, human lamin C), rabbit anti-calreticulin antibody (StressGen, Victoria, Canada) and a rabbit anti-coilin antibody (gift of Dr Joseph Gall). Confocal microscopy and image processing was performed using a Leica confocal and imaging system (Leica Inc.) using a ×100 objective, with the kind help of Dr Joseph Gall (Carnegie Institution, Baltimore, MD).

Synchronization, transfection and cell cycle arrest of HeLa cells

HeLa cells at mitosis, collected by shake-off (Monteiro and Mical, 1996) were seeded onto coverslips in multiple dishes. Half of the dishes were treated with 1 μg/ml aphidicolin to block cell cycle progression at S phase. 4 hours after plating both aphidicolin-treated and untreated cells were transfected with constructs A, A–, B, B–, C, D, E and H shown in Fig. 1. At 3, 15, 24, 48, 72 and 96 hours post-transfection, coverslips were removed and stained for expression of the lamin proteins using the anti-myc antibody. Searching field by field at 1000× magnification, a minimum of 100 transfected cells were observed for each time point and scored for the percentage of transfected cells having the transgenic protein localized to the nuclear envelope. The results shown are from individual experiments.

Protein preparation, SDS gel electrophoresis and immunoblotting

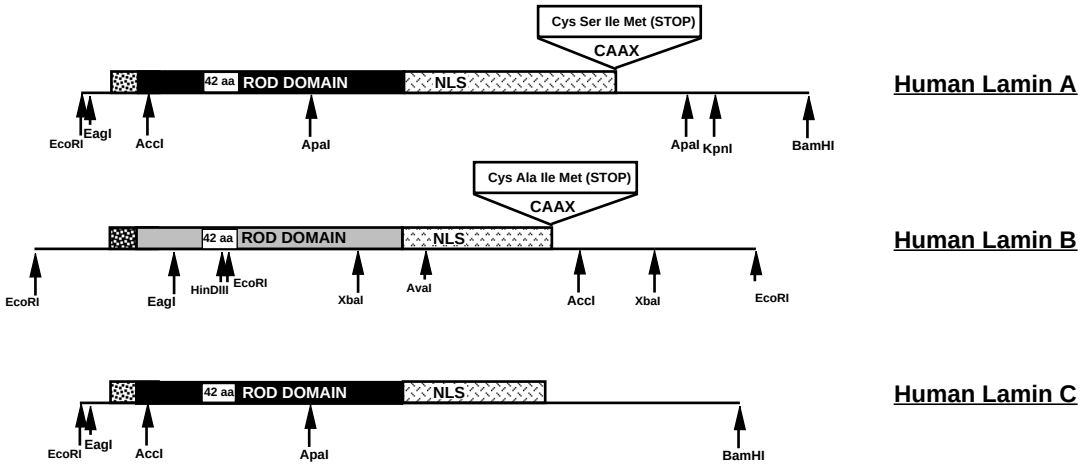
HeLa cells, 24 hours post-transfection, were washed three times with cold standard PBS buffer and lysed in ice-cold 1% Triton X-100, 10 mM triethanolamine-HCL, pH 7.4, 140 mM KCl, 1.5 mM $MgCl_2$, 5 mM iodoacetamide, 1 mM Pefabloc (Boehringer-Mannheim). After 10 minutes incubation on ice the lysates were centrifuged at 140,000 ***g*** for 15 minutes. The supernatant was removed and the pellet resuspended in lysis buffer (Monteiro and Mical, 1996) to a volume equal to that of the supernatant. Equivalent volumes of the supernatant and pellet fractions were separated by SDS-PAGE and immunoblotted as previously described (Monteiro et al., 1994; Janicki and Monteiro, 1997). The solubility properties of lamins in interphase and mitotic cells was analyzed by the method described in Monteiro et al. (1994). For this analysis too, equal portions of the supernatant and pellet fractions were immunoblotted, enabling a quantitative comparison to be made of the proportion of lamins that were either soluble or insoluble.

Antibodies used for immunoblotting were: a rabbit polyclonal antibody generated against purified recombinant human lamin C (which recognizes both human lamin A and C because of their sequence similarity), mouse monoclonal 9E10 anti-myc antibody, mouse monclonal anti-lamin B (Matritech, Inc., Cambridge, MA), or human autoantibodies that react with the lamin B receptor (characterized by Lin et al., 1996).

RESULTS

Model system used for studying lamin targeting and assembly

The mechanisms controlling intracellular targeting and assembly of nuclear IFs were investigated using human lamin A, B and C mutants (see Fig. 1). In particular, the roles of the CAAX motif, NLS and the 42 aa insert were studied by deleting them singly or in combination from lamin B (see Fig. 1). The properties of the CAAX motif in lamin B targeting were compared to its function in lamin A and C, since lamin C lacks the motif, whereas it is removed from lamin A shortly after targeting to the lamina (see Introduction). Constructs were transfected into mouse 3T3, mouse L cells, human HeLa and human SW13(–) cells, and expression was followed over time using the 9E10 antibody (Evan et al., 1985), which recognized the myc-tag incorporated at the C terminus of each construct. In human cells, endogenous myc protein, recognized by the 9E10 antibody, was not detectable at the antibody dilution used, and therefore did not obscure study of the myc-tagged IF proteins by immunofluorescence (Fig. 3, upper panel). Moreover, results were confirmed using the mouse cells in which endogenous myc is not recognized by the 9E10 antibody. All examples shown are of the targeting properties in HeLa cells, with identical results obtained with the other three



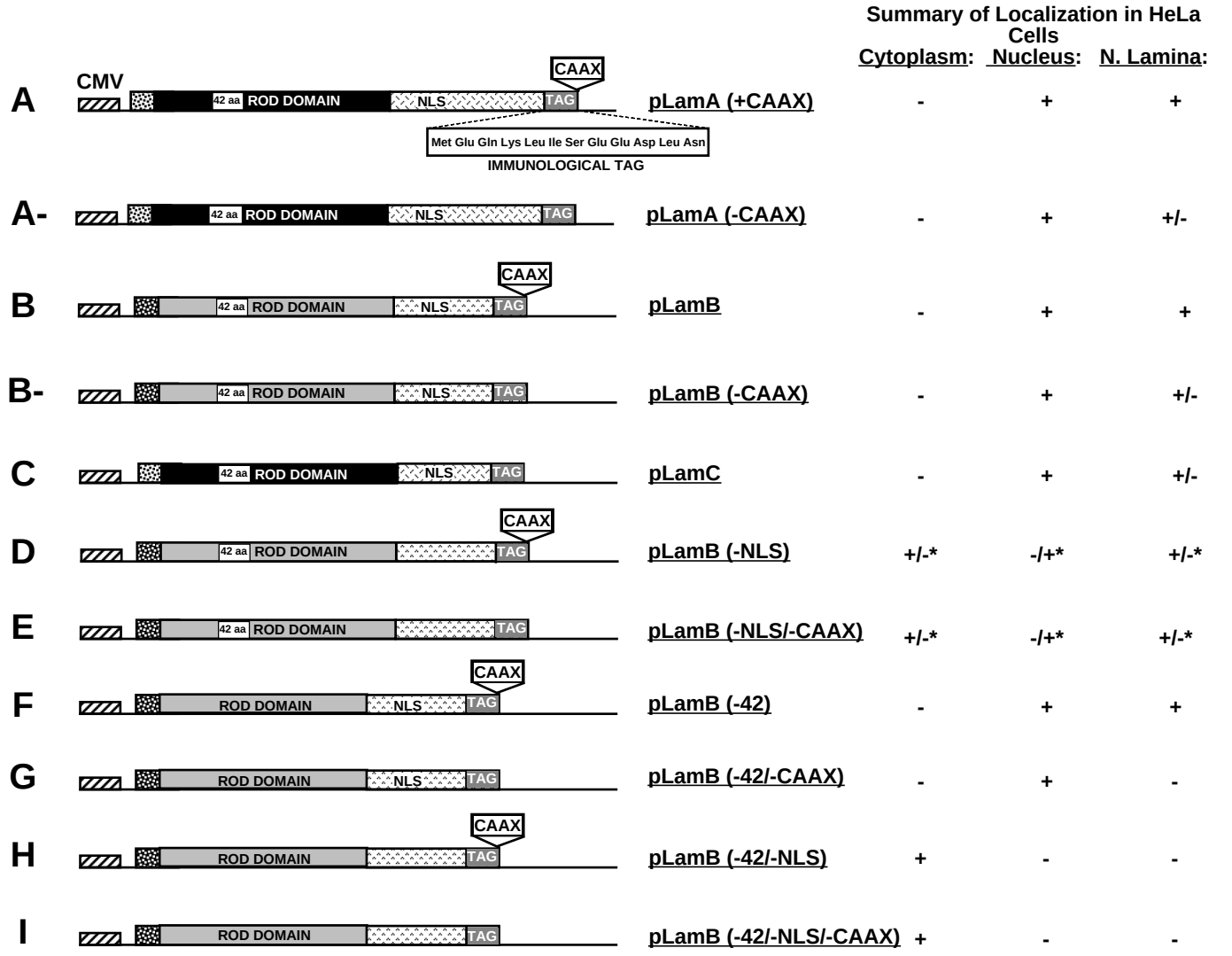


Fig. 1. Schematic drawings of lamin expression constructs. The three wild-type lamin cDNAs together with restriction sites used for cloning and generation of constructs A-I are depicted. Shading of head, rod and tail domains in each construct is maintained according to their parental source. The CMV promoter was used to drive expression of each construct. Each of the expressed proteins was tagged with 12 aa of h-myc sequence (TAG) recognized by the 9E10 monoclonal antibody. The presence or absence of the 42 aa insert, NLS and CAAX motif in each construct is indicated. A summary of the targeting properties of each construct is shown to the right of the constructs: +, presence; –, absence; *, location is dependent upon time post-transfection.

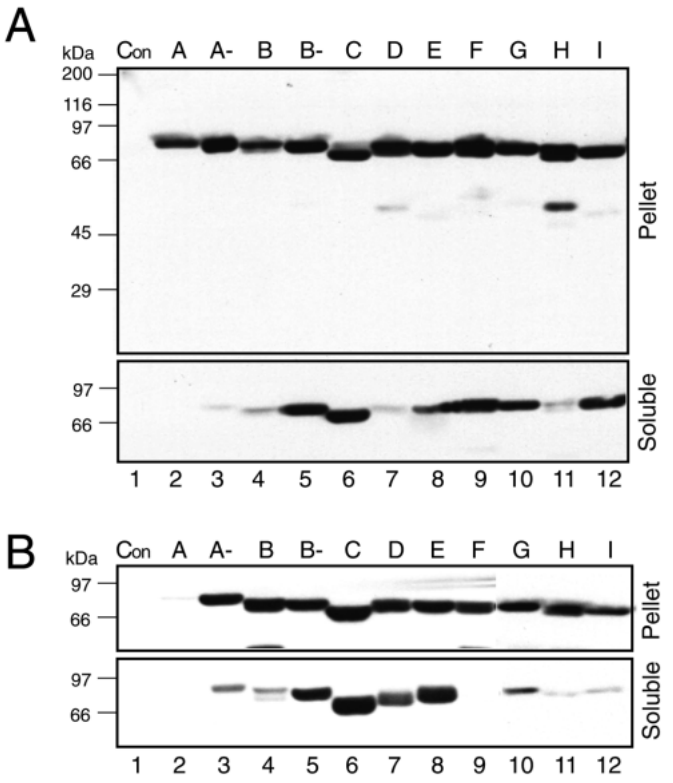


Fig. 2. Immunoblot analysis of lamin proteins expressed in HeLa cells. HeLa cells were either mock transfected (control) or transfected with plasmid DNAs corresponding to constructs A-I (lettered according to Fig. 1) and after 20 (A) and 44 (B) hours the expression and accumulation of IF proteins in Triton X-100 insoluble and soluble fractions was determined by immunoblotting with the anti-myc antibody. The positions of molecular mass markers (kDa) are indicated on the left.

cell lines. Immunoblots of extracts prepared from transfected cells contained immunoreactive polypeptides of the expected sizes for each construct (Fig. 2). Interestingly, these immunoblots demonstrated that cells transfected with constructs possessing a CAAX motif all have immunoreactive closely spaced doublet bands (see Fig. 2), which was especially noticeable on short exposures of the autoradiographs, suggesting that one or both bands correspond to postranslationally modified polypeptides encoded by the CAAX motif.

Two important points should be remembered when considering the results of these transfection studies. First, deletion or myc-tagging of lamin proteins, as with any other protein, could result in misfolding or mislocation of the proteins, although we believe this to be unlikely since consistent results were obtained with multiple constructs. Second, endogenous lamins may influence the phenotypes of the mutant transfected lamins and therefore novel phenotypes are likely to represent dominant effects due to the mutations. Studies in cell lines that lack lamins are not possible since none are known to exist.

The CAAX motif promotes rapid association of lamins with the nuclear envelope

The role of the CAAX motif in the assembly and localization of lamins A and B was studied using constructs containing or lacking the motif (Fig. 1A,A–,B,B–). When the motif was present, both lamins A and B displayed uniform nuclear staining with a few intranuclear speckles and a prominent rim at all times post-transfection (Fig. 3A,B). Confocal microscopy of 0.6 μm sections through these nuclei revealed rim staining which colocalized with the staining pattern of endogenous lamin A/C, consistent with targeting of lamin B to the nuclear lamina (Fig. 4a-c). In contrast, when the CAAX motif was deleted from lamins A and B, the proteins accumulated within nuclei as multiple aggregates at time intervals up to 24 hours post-transfection, similar to the behavior of transfected lamin C, which always lacks the motif (Fig. 3A–,B–,C). The aggregates for the large part did not colocalize with coilin by double immunofluorescence staining (Fig. 5a,b), indicating that they were not generally associated with coiled bodies or RNA processing centers. There were fewer but larger aggregates in lamin B- transfected cells, but for all three constructs, progressively fewer aggregates were seen 48 and 72 hours post-transfection. Over time, lamins A–, B– and C localized to the nuclear lamina, as seen by the nucleus becoming more uniformly stained with a brighter but less distinct rim compared to the CAAX-containing lamin A and B constructs.

Lamins A and B possessing a CAAX motif also had different biochemical properties compared to the CAAX-deficient constructs (A–,B–,C) when analyzed by Triton X-100 extraction. Relatively little, if any, of the two lamins with a CAAX motif were soluble in Triton X-100 either after 20 or 44 hours post-transfection, whereas approximately 5-10% of lamin A–, 50% of lamin B– and 50% of lamin C were soluble at these times (Fig. 2A). The reason why a relatively smaller proportion of the lamin A– protein was soluble compared to the other two CAAX-deficient lamins, lamins B– and C, is not known, but it suggests that the pathway of lamin A assembly may be different from the latter. Since all three of the CAAX-deficient lamins formed nuclear aggregates after 20 hours post-transfection it is possible that the soluble protein extracted upon detergent treatment represents lamin proteins that are unaggregated, or that the lamin A– aggregates are more Triton X-100-insoluble than those of lamin B– or C. If the latter is true than the lamin A– aggregates seen in this study would appear to be different to those formed by the lamin AΔ10 isoform (Machiels et al., 1995, 1996), which contains a 30 aa deletion in the lamin A tail region, and which is soluble in Triton X-100. Apart from a few exceptions (constructs D and F), the inclusion of a CAAX motif onto all of the lamin A and B constructs caused a decrease in the solubility of the lamins, suggesting that this motif facilitates more rapid integration of lamins into either the lamina and/or into insoluble assembly precursors, since unassembled, membrane-associated proteins should have been solubilized by Triton X-100 treatment.

We next investigated whether the time-dependent lamina targeting seen in the $CAAX^-$ constructs (A–,B–,C) was linked to the cell cycle and events such as nuclear envelope breakdown or whether targeting was strictly a function of time. To address this question, we monitored lamina targeting in mitotically synchronized HeLa cells that were either allowed to progress through the cell cycle or arrested in S phase by aphidicolin treatment. The percentage of cells targeting the lamin constructs to the nuclear envelope was determined by

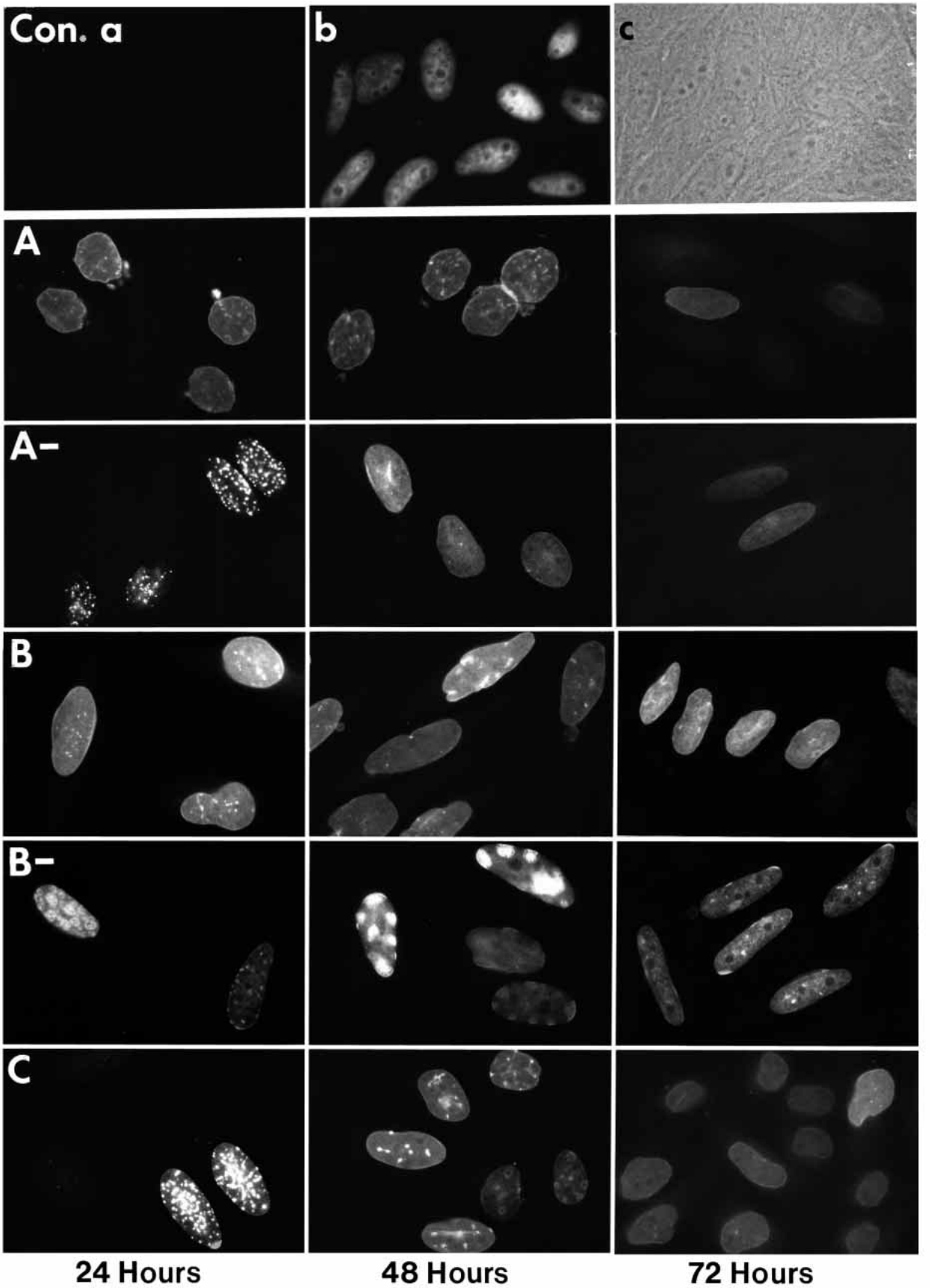
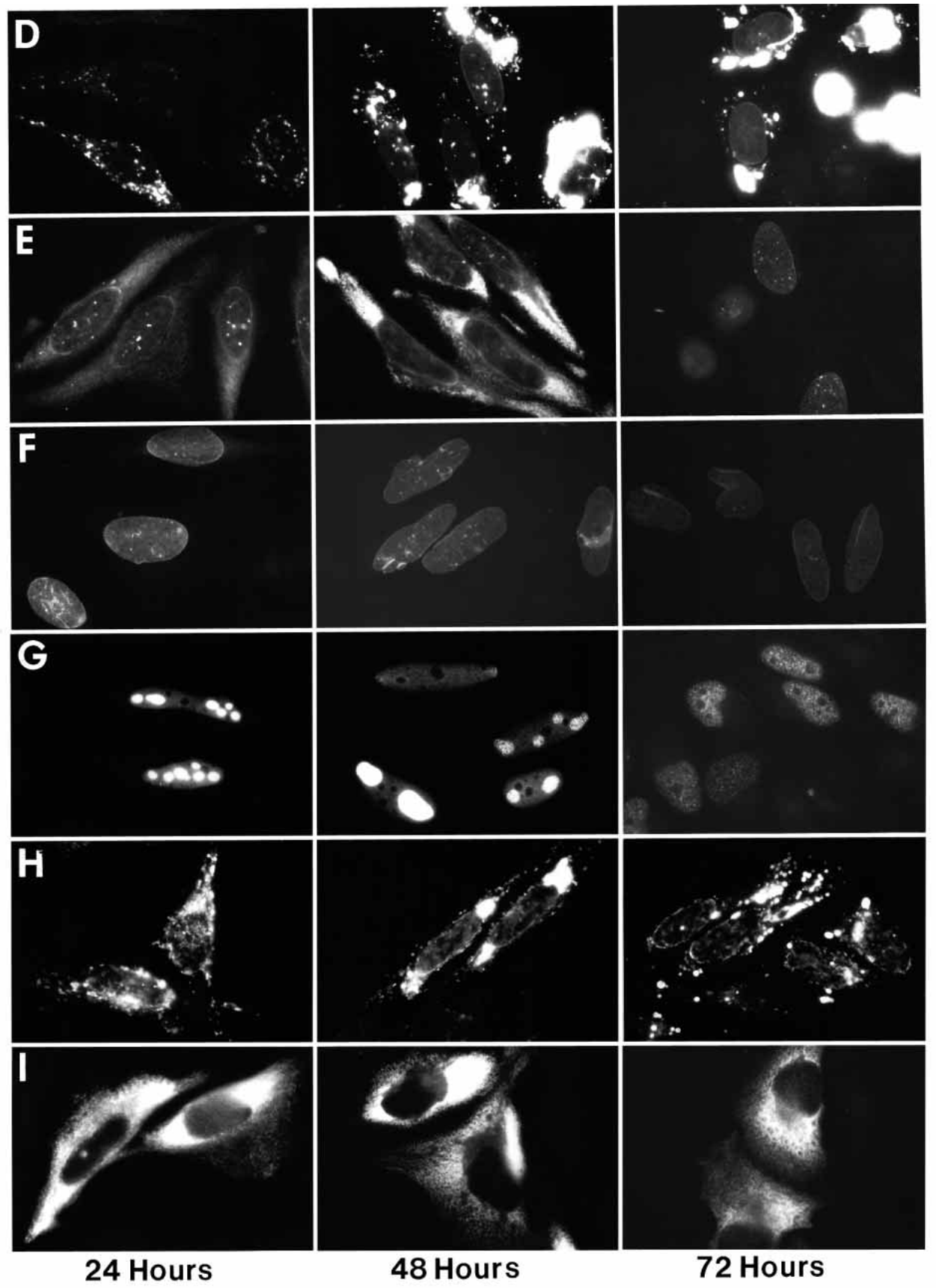


Fig. 3. Immunofluorescence localization of IF proteins expressed in HeLa cells over time. HeLa cells were transiently transfected with plasmid DNAs of constructs A-I and stained after 24, 48 and 72 hours for the expressed lamin proteins using the anti-myc antibody. (Con a,b,c) Control



mock-transfected HeLa cells stained with the anti-myc antibody (a), the corresponding DAPI staining (b), and a phase-contrast image (c) of the same cells.

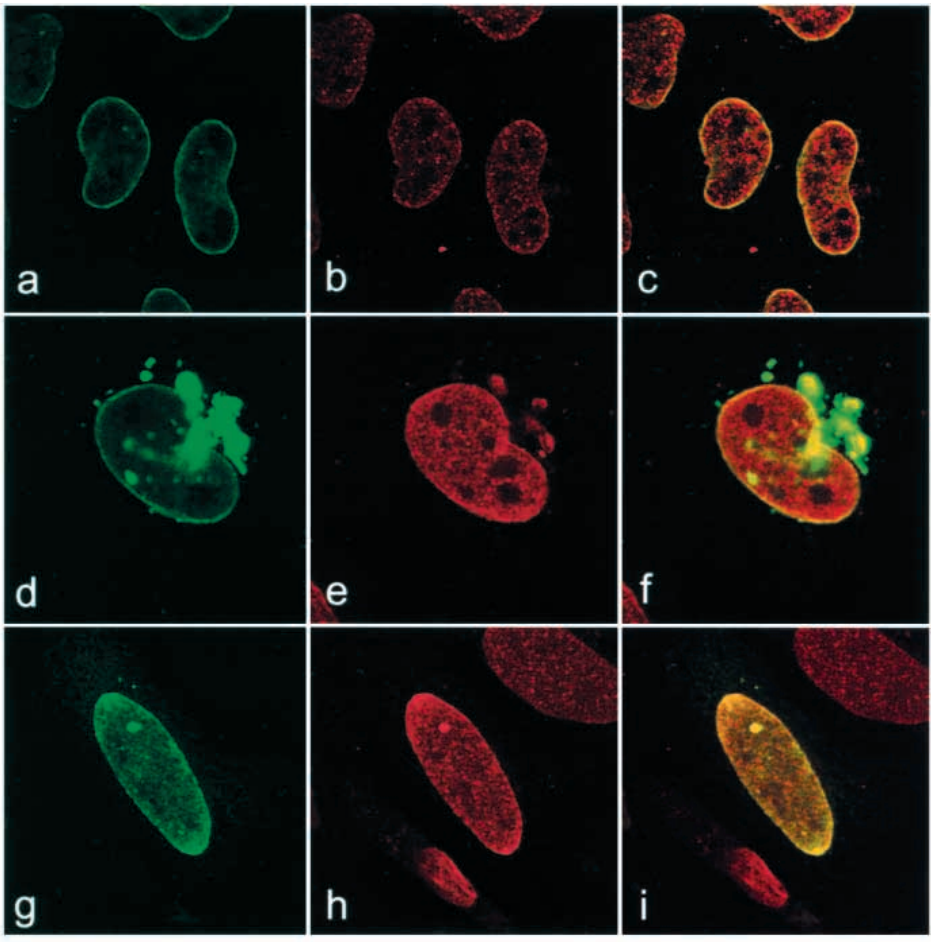


Fig. 4. Confocal microscopy showing targeting of lamin mutants to the nuclear lamina. HeLa cells were transiently transfected with plasmid DNA of constructs B (a,b,c), D (d,e,f), or E (g,h,i), and after 48 hours were double stained for expression and localization of transgenic lamin myc-tagged polypeptides using the anti-myc antibody followed by a fluorescein-conjugated donkey anti-mouse antibody or, for endogenous lamins, with a rabbit polyclonal anti-lamin A/C antibody followed by a rhodamine-conjugated donkey anti-rabbit antibody. Left panels show the fluorescein staining, the center panels the rhodamine staining, and the right panels the result of merging the fluorescein and rhodamine staining of the same groups of cells. Two important points should be noted. First, the mutant lamins were localized to the nucleus with rim fluorescence (left) suggestive of lamina localization. Second, apart from some cytoplasmic staining for constructs D and E, the merged images could be precisely overlapped, especially at the nuclear rim (indicated by the yellow colour produced upon merging the green and red colour images). There was no evidence for a corona of green fluorescence at the nuclear rim, which would support evidence for the proteins being localized outside the nucleus. Rather these data are consistent with intra-nuclear and lamina localization of the mutant lamins.

immunofluorescence microscopy at time intervals spanning at least three cell cycles for both dividing and S phase-arrested cells (the cell cycle is approx. 20-23.5 hours long in HeLa cells; Monteiro and Mical, 1996). This analysis confirmed the importance of the CAAX motif in rapid lamina targeting, as the lamin A and B constructs possessing the motif were quickly targeted to the lamina, with 75% having appropriate targeting after 3 hours and almost 100% by 24 hours (Fig. 6, constructs A and B). Blocking the cell cycle did not inhibit targeting of the CAAX-containing lamins as similar results were obtained in aphidicolin treated cells (Fig. 6, constructs A and B).

In contrast, deletion of the CAAX motif from lamins A and B reduced targeting by approximately 30-50% at time points prior to the first mitosis (Fig. 6, constructs A– and B–; mitosis occurred in these transfected cells approximately 20-23 hours after plating), a rate similar to that of lamin C which normally lacks the CAAX motif (Fig. 6, construct C). Despite being

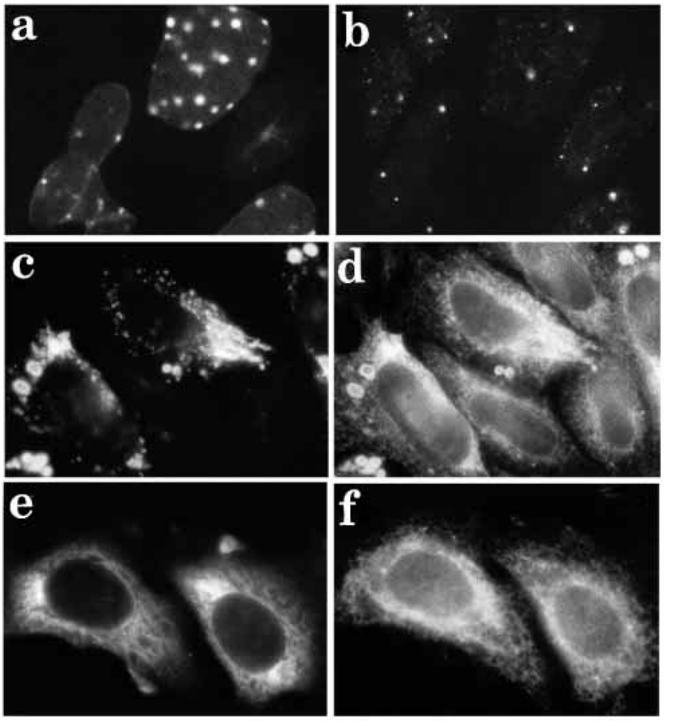


Fig. 5. Demonstration that lamin proteins with CAAX motifs are associated with membrane structures by double immunofluorescence microscopy. HeLa cells were transiently transfected with constructs C (a,b), H (c,d) and E (e,f), and after 48 hours were double stained with anti-myc and either anti-calreticulin or anti-coilin antibodies. Left panels show the anti-myc staining and on the right are the same cells double stained for coilin (b) or calreticulin (d,f).

delayed, all three CAAX$^-$ constructs eventually achieved 90% targeting 48 hours post-transfection in the dividing cells. In aphidicolin-arrested cells, however, targeting of the CAAX$^-$ lamins differed, with the lamin B CAAX$^-$ mutant being especially compromised in that only 50% of cells achieved proper lamina targeting, whereas lamin C targeting was slightly delayed, and lamin A CAAX$^-$ targeting slightly enhanced (see Fig. 6). Collectively, these data demonstrate that the CAAX-motif targets lamins to the nuclear envelope in a cell cycle-independent fashion and that mature lamins A and C, which lack the motif, do not require nuclear envelope breakdown for lamina incorporation as appropriate targeting of these CAAX$^-$ constructs still occurred in S phase-arrested cells. In contrast, lamin B deleted of its CAAX motif required cell cycle progression for efficient lamina integration. We suspect that nuclear envelope breakdown at mitosis may be the cell cycle event that may be crucial for efficient integration of the lamin B CAAX$^-$ mutant.

Isolation of stable cell lines expressing lamin B with and without the CAAX motif

To further investigate the role of the CAAX motif, seven HeLa

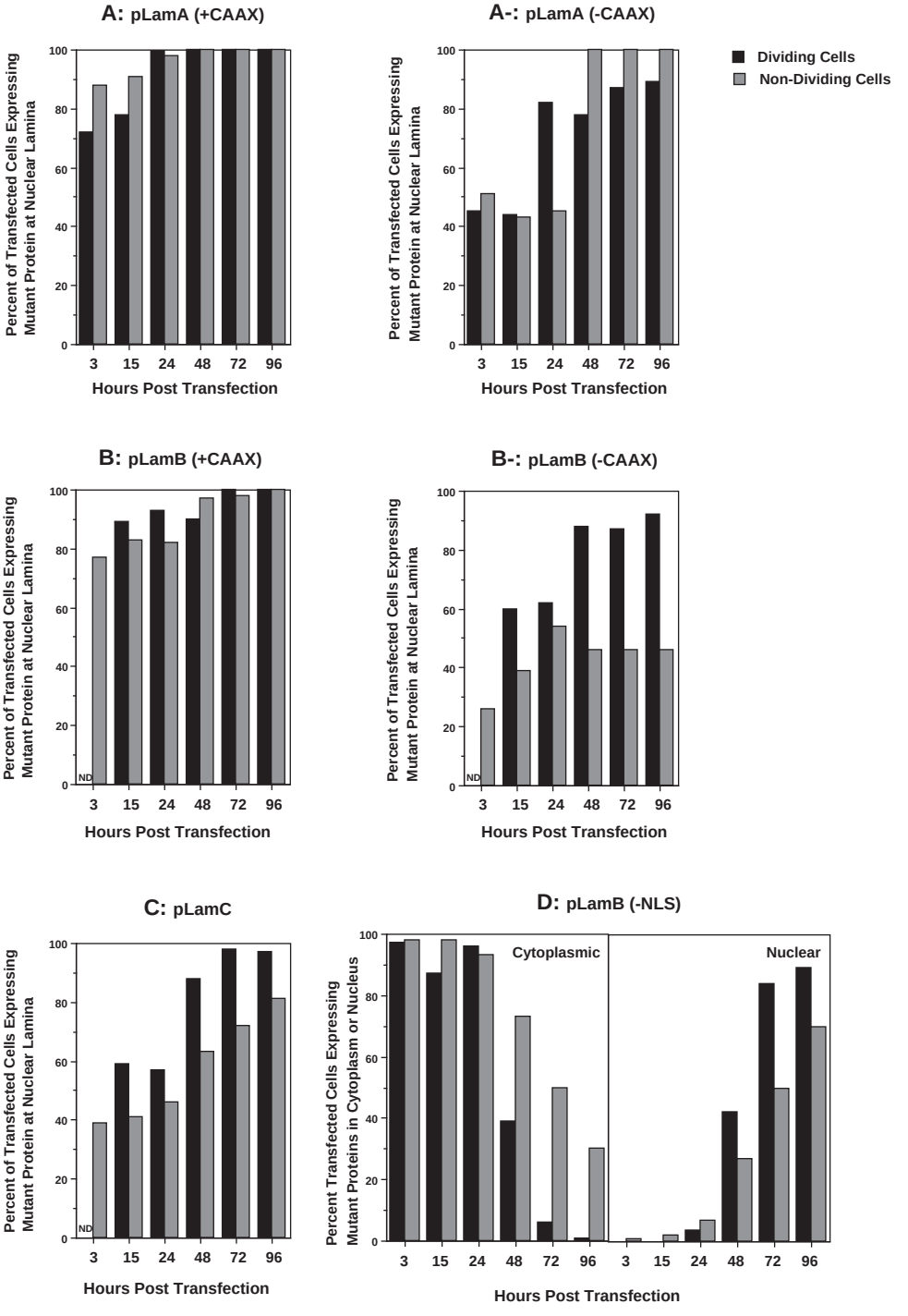
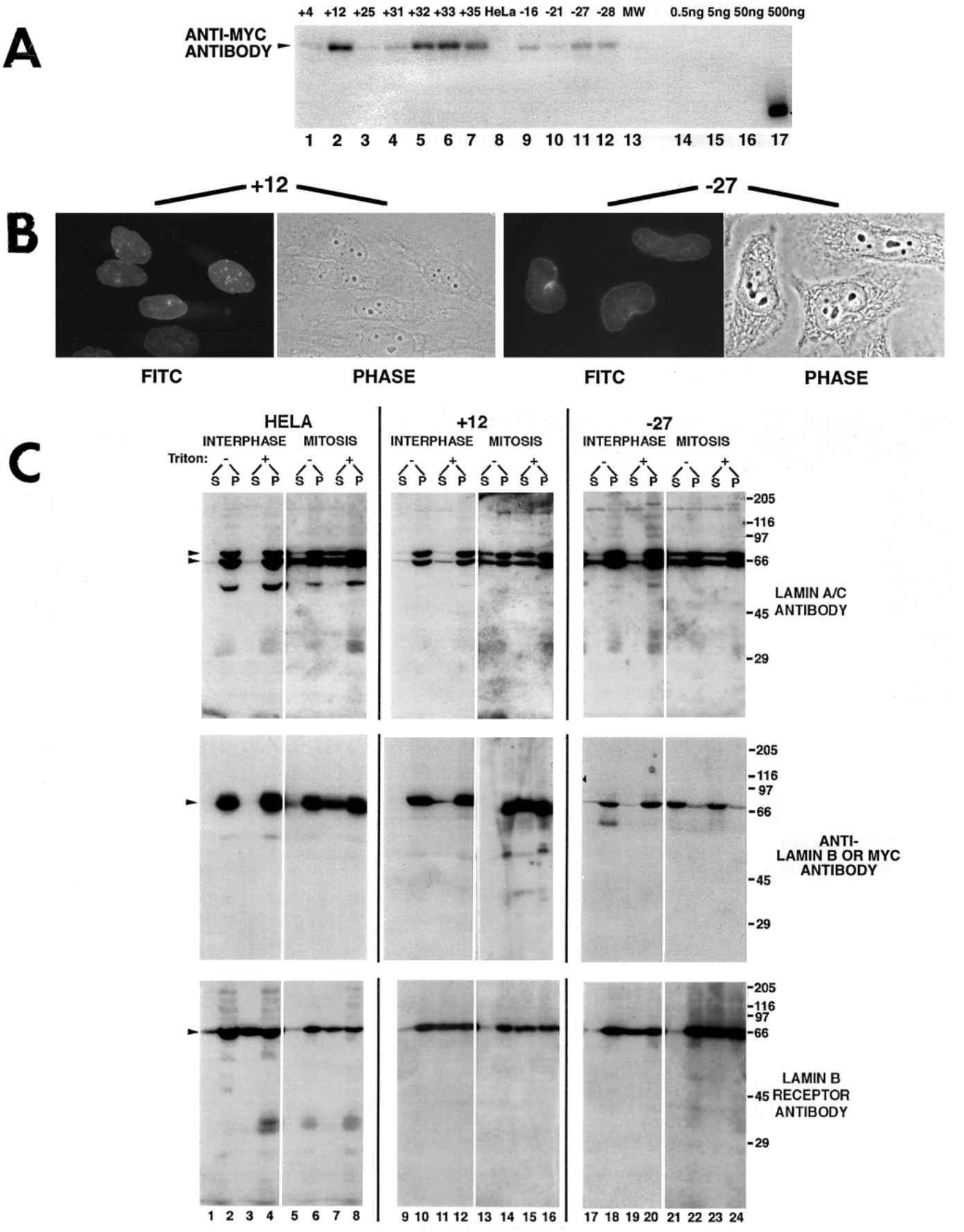


Fig. 6. Comparison of nuclear targeting of lamins containing or lacking a CAAX motif in synchronized HeLa cells. HeLa cells synchronized by mitotic shake-off were transfected with constructs A, A–, B, B–, C or D, in the presence (gray shading represents incorporation non-dividing cells) or absence (black shading represents incorporation in dividing cells) of aphidicolin. The percentages of transfected cells after 3, 15, 24, 48, 72 and 96 hours post-transfection that had nuclear lamina or cytoplasmic localization are shown.


A
+4 +12 +25 +31 +32 +33 +35 HeLa -16 -21 -27 -28 MW 0.5ng 5ng 50ng 500ng
ANTI-MYC ANTIBODY
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17
B
+12
-27
FITC
PHASE
FITC
PHASE
C
HELA
+12
-27
INTERPHASE MITOSIS
Triton: - + - +
S P S P S P S P
205
116
97
66
45
29
LAMIN A/C ANTIBODY
ANTI-LAMIN B OR MYC ANTIBODY
LAMIN B RECEPTOR ANTIBODY
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24

cell lines stably expressing lamin B with a CAAX sequence and four cell lines expressing lamin B deleted of the motif were isolated (Fig. 7). Clones +12 and –27, expressed the highest amounts of lamin B $CAAX^+$ and $CAAX^-$ protein, respectively, and were studied further. Comparison of protein lysates from the cell lines to known amounts of purified myc-tagged bacterially expressed trpE protein indicated that lamin B $CAAX^+$ and $CAAX^-$ transgene expression was approximately 0.5% and 0.2% of total cell protein, respectively (Fig. 7A). In both cell lines, the transfected lamin was predominantly localized to the nucleus, although in the $CAAX^-$ line nuclear rim staining was slightly more diffuse compared to the $CAAX^+$ line (Fig. 7B).

The solubility properties of the transgenic lamin B proteins in the cell lines were compared to endogenous lamins at interphase and mitosis (Fig. 7). The solubility of endogenous lamins A, B and C in the cell lines was unaltered when compared to untransfected HeLa cells (Fig. 7C, top panels and left middle panel). At interphase, all three proteins were insoluble and non-extractable by Triton X-100 treatment, as would be expected for lamins that are assembled (Fig. 7C, top panels, lanes 1-4, 9-12 and 17-20, and middle panel, lanes 1-4). At mitosis, when the lamina is disassembled, endogenous lamin B found in the pellet fraction, probably due to its attachment to nuclear envelope membrane remnants through its permanently associated CAAX modifications, was released into the supernatant by Triton X-100 treatment (Fig. 7C, middle panel, lanes 5-8). Mature lamins A and C, which lack CAAX modifications, were more soluble at mitosis independent of detergent treatment (Fig. 7C, top panels, lanes 5-8, 13-16 and 21-24).

The transgenic lamin B protein in interphase cells of the +12 and –27 cell lines were insoluble, indicating that both had assembled (Fig. 7C, middle panels, lanes 9-12). However, they behaved differently at mitosis. In the +12 cell line the lamin B-$CAAX^+$ was solubilized in a Triton X-100-dependent manner (Fig. 7C, middle panel, lanes 13-16), similar to the behavior of endogenous lamin B (Fig. 7C, middle panel, lanes 5-8) whereas the lamin B $CAAX^-$ protein was soluble independent of detergent treatment (Fig. 7C, middle panels, lanes 21-24). These results indicate that membrane attachment of lamin B at mitosis is mediated by its CAAX motif, since deletion of the motif destroys membrane association.

Evidence for independent segregation of lamin B and its putative receptor

The lamin B $CAAX^+$ and $CAAX^-$ expressing cell lines offered an unusual opportunity to examine the association of lamin B with the putative lamin B receptor (LBR). If binding was mediated through sequences apart from the CAAX-associated modifications, then the two proteins should have cofractionated with one another in both cell lines. Instead the LBR fractionated independently of the lamin B proteins (Fig. 7C, bottom panels). Support for this conclusion is evident as both endogenous and transgenic lamin B proteins were almost completely insoluble in interphase extracts treated with Triton X-100, which differs to the approximately 50% solubility of the receptor. More significantly, the lamin B $CAAX^-$ protein was almost completely soluble in Triton X-100 untreated mitotic lysates of the -27 cell line (Fig. 7C, middle panel, lanes 21-22), whereas only a very small proportion of the receptor was soluble (Fig. 7C, bottom panel, lanes 21-22). Clearly the independent segregation of the two proteins indicates that lamin B does not bind directly to the LBR.

The roles of the NLS and 42 aa insert in lamin targeting

We next examined the roles of the putative NLS, the 42 aa insert and the CAAX motif in targeting and assembly, by deleting them individually and in all possible combinations from lamin B (Fig. 1, constructs D-I). Deletion of the NLS alone (Fig. 3D), or in combination with the 42 aa insert (Fig. 3H), changed the localization of lamin B from the nucleus to the cytoplasm, indicating that the deleted sequence is indeed the only NLS present in lamin B. The mutant proteins, which accumulated in the cytoplasm, appeared membrane-associated as they had a vesicular-like staining pattern, probably as a consequence of CAAX-associated modifications. Double immunofluorescence staining using an antibody against calreticulin, a calcium binding protein that is localized to the ER, and the anti-myc monoclonal antibody to detect the mutant lamin B proteins, indicated the vesicular staining pattern of the lamin B mutants overlapped the staining pattern of calreticulin (Fig. 5c,d; construct H), suggesting that the lamin B constructs were associated with cytoplasmic membranes. Interestingly, with increased time after transfection, additional anti-myc staining was observed within the nucleus (Fig. 3D,H, 48 and 72 hours; Fig. 4d,f; construct D). Confocal microscopy of sections taken through nuclei of cells transfected with construct D revealed rim fluorescence staining, which colocalized with the endogenous lamin A/C staining, consistent with localization of the mutant lamins to the nuclear lamina (Fig. 4d-f). An analysis of transfected synchronized cells indicated that this nuclear lamina targeting was principally cell cycle-dependent, although targeting still occurred in non-dividing cells but at much lower rates (Fig. 6, construct D; and

Fig. 7. Immunofluorescence and biochemical properties of stable cell lines expressing lamin B with and without a CAAX motif.
(A) Quantitation of lamin B proteins stably expressed in HeLa cells. Cell lysates (50 μg) from G418 resistant cell lines cotransfected with pSV2-neo (Monteiro and Cleveland, 1989) and construct B (lanes 1-7), or pSV2-neo and construct B– (lanes 9-12), together with HeLa cell lysate (lane 8) and known amounts of bacterially expressed trpE-myc protein (lanes 14-17) were immunoblotted with anti-myc antibody to quantify expression of myc-tagged lamins.
(B) Fluorescence and phase contrast images of lamin B $CAAX^+$ (+12) and lamin B $CAAX^-$ (–27) stable cell lines after staining with anti-myc antibody, showing nuclear lamina localization of the transfected products. (C) Biochemical fractionation of lamins and the lamin B-receptor in control HeLa cells, +12 and –27 stable cell lines. Cell lysates were prepared in the presence (+) or absence (–) of Triton X-100, and equal portions of the soluble (S) and pellet (P) fractions of the cell lysates were separated by SDS-PAGE on a 8.5% polyacrylamide gel, and immunoblotted with an anti-lamin A/C specific antibody (top panels), an anti-lamin B specific antibody (middle, left panel), a myc antibody (middle, center and right panels), or with human auto-antiserum against the lamin B receptor (bottom three panels). The top three panels indicate that the biochemical properties of endogenous lamins A and C are not changed in the +12 and –27 stable cell lines. The middle three panels demonstrate that the CAAX motif of lamin B is responsible for binding membranes at mitosis. The bottom three panels shows that the lamin B receptor fractionates independently from lamin B. Arrowheads on the left indicate the positions of full-length lamin A/C polypeptides in the top panels, lamin B in the middle panels, and the LBR in the bottom panels. The other smaller immunoreactive bands in each of the blots are presumed to be proteolytic products.

data not shown). We speculate that endogenous lamins may aid in the rescue of the mutant 'cytoplasmic' lamins (probably by dimerization or oligomerization) and that nuclear envelope breakdown at mitosis, which results in disassembly of lamins, could facilitate this process.

Deletion of the NLS from lamin B CAAX$^-$ changed the targeting properties of this construct in two noticeable ways. First, the double mutant (construct E) accumulated predominantly in the cytoplasm as a meshwork 24 hours post-transfection (Fig. 3E), with a staining pattern distinct from that of calreticulin (Fig. 5e,f) or vimentin (data not shown). However, at later times post-transfection, and especially noticeable after 72 hours, the staining of the lamin B NLS$^-$ CAAX$^-$ mutant was predominantly to the nucleus whereas in the CAAX$^+$ NLS$^-$ construct, additional vesicular-staining in the cytoplasm was routinely seen (compare Figs 3D,E and 4d,g, respectively). We should indicate that in cells in which the lamin B NLS$^-$ CAAX$^-$ mutant was highly expressed (based on the immunofluorescence staining intensity) there was prominent cytoplasmic staining in addition to nuclear staining, suggesting that nuclear lamina targeting of this mutant is somewhat inefficient.

Similar to the analysis of construct D, we used confocal microscopy to determine if the nuclear staining of the lamin B NLS$^-$ CAAX$^-$ mutant was consistent with properties expected for localization to the nuclear lamina. Confocal microscopy of 0.6 μm sections taken through nuclei of cells transfected with construct E revealed that the anti-myc fluorescence staining of the lamin B NLS$^-$ CAAX$^-$ mutant had a prominent rim, which is expected if localization is to the lamina. More significantly, the anti-myc fluorescence staining (shown in green colour; Fig. 4g) colocalized perfectly with the staining pattern of endogenous lamin A/C (shown in red colour; Fig. 4h) as evident by the yellow colour that was produced upon merging the transgenic and endogenous, green and red fluorescent colour images, respectively (Fig. 4g-i). There was no evidence for a corona of green fluorescence at the nuclear rim upon merging the green and red colour images, which would have supported evidence for the mutant lamins being localized outside the nucleus. Instead these data are consistent and highly supportive of localization of the mutant lamins to the nuclear lamina.

The 42 aa insert is not required for nuclear lamina targeting

Previous work suggested that the additional 42 aa in the lamin rod domain prevents lamins from associating with cytoplasmic IF proteins (Monteiro et al., 1994). However, when the 42 aa insert was deleted from lamin B so that the length of the rod domain equaled that of cytoplasmic IF proteins, the localization of the mutant protein appeared indistinguishable from wild-type lamin B (Fig. 3F). However, when these transfected cells were analyzed by immunoblotting for the presence of the mutant protein in pellet and soluble fractions there was a noticeable increase in the amount of soluble protein after 20 hours compared to wild-type lamin B (compare Fig. 2A, lanes 9 and 4, respectively). This solubility difference was less noticeable in lysates analyzed after 44 hours (Fig. 2B, lanes 9 and 4, respectively). These results suggest that deletion of the 42 aa insert in lamin B partially compromises the ability of the protein to assemble, especially at early times after transfection. To further define the role of the 42 aa insert, we created additional mutants lacking the NLS and/or the CAAX motif.

Deletion of both the 42 aa insert and CAAX motif (construct G) caused lamin B to accumulate into large aggregates in the nucleus when examined 24 and 48 hours after transfection, but by 72 hours, the protein was dispersed into smaller aggregates within the nucleus (Fig. 3G). Nuclear lamina targeting, defined by rim fluorescence, was not apparent for this construct. Similarly, lamina targeting was defective, or severely compromised, when the 42 aa insert and the NLS were both deleted (construct H; Fig. 3H). Deletion of all three sequences (the 42 aa insert, NLS and CAAX motif) from lamin B resulted in a mutant (construct I) that was unable to localize to the nucleus and which instead accumulated diffusely in the cytoplasm (Fig. 3I).

DISCUSSION

The data presented here demonstrate that three sequences present in vertebrate nuclear but not in cytoplasmic IF proteins, namely the 42 aa insert, the NLS and the CAAX motif, play distinct roles in nuclear lamina targeting and that any one of the three sequences can be deleted from lamin B without fully compromising targeting. However, when two or more sequences are deleted targeting is severely compromised, indicating that the signals act in concert to ensure correct temporal and spatial assembly of lamin B.

In contrast to earlier studies indicating that the NLS in lamins is essential for lamina targeting (Loewinger and McKeon, 1988; Holtz et al., 1989; Lourim and Lin, 1992; Schmidt and Krohne, 1995), our results indicate that it is possible to delete the NLS from human lamin B and still maintain lamina targeting, albeit with some deficiencies as mutant protein also accumulates in the cytoplasm. The discrepancies concerning the dispensability of the NLS may be due to differences in the methods used to construct NLS-deficient lamins. Most of the previous NLS-deficient mutants lacked large regions of the lamin tail domain, making it difficult to assess whether mutant phenotypes resulted from an absence of sequences necessary for lamin assembly or from the removal of the NLS specifically. In our studies only the small region encompassing the NLS was deleted, and lamina targeting was essentially preserved. The mechanisms however, by which the NLS-deficient lamin B mutants are transported into the nucleus are unclear, but there are at least two possibilities. They may either enter the nucleus by 'piggy back' association with other proteins, possibly endogenous lamins, or they partition into the nucleus after nuclear envelope breakdown at mitosis. Both mechanisms seem likely since studies of transfected cells that were either allowed to progress through the cell cycle or blocked in S phase indicate that nuclear lamina targeting was only reduced 20% in S phase-blocked cells (Fig. 6).

Deletion of the CAAX motif from lamin A and B resulted in the loss of membrane association, consistent with a large body of data indicating that CAAX modifications are involved in membrane targeting (Holtz et al., 1989; Krohne et al., 1989; Kitten and Nigg, 1991; Firmbach-Kraft and Stick, 1993; Hennekes and Nigg, 1994; Schmidt and Krohne, 1995). When present, the CAAX motif functioned to target newly synthesized lamins more rapidly to the nuclear lamina (as evident by nuclear rim fluorescence as well as by the insolubility of the protein in biochemical assays), whereas in

its absence, targeting was delayed. Transfection and monitoring of synchronized cells, that were either allowed to progress through the cell cycle or arrested at S phase by aphidicolin, indicated that lamins A, B and C integrate into the lamina by different mechanisms. Lamin A (CAAX$^-$) and lamin C were accelerated or delayed, respectively, but not blocked from assembly when cells were arrested in S phase, whereas lamin B (CAAX$^-$) targeting was reduced by approximately 50%. The results suggest that integration of lamin B into the nuclear lamina in non-dividing cells is more heavily dependent on the presence of its CAAX motif than are lamins A and C. This difference may have evolved because mature lamins A and C are both devoid of the CAAX motif whereas lamin B always contains the motif. These data provide support for accumulating evidence indicating that the individual lamins are not equivalent in assembly characteristics (Gerace et al., 1984; Horton et al., 1992; Pugh et al., 1997). For example, Gerace et al. (1984) demonstrated lamin A is incorporated into the lamina at a faster rate than lamin C, whereas Horton et al. (1992) demonstrated cell cycle-dependent integration of lamin C, but not lamin A, upon forcible expression in cells lacking the two lamins. Recent evidence suggests that lamin A may in fact assist in the assembly of lamin C (Pugh et al., 1997).

The reason why dissolution of the nuclear envelope is necessary for lamin B CAAX$^-$ integration is not known, but it is suggested that critical sites or factors at the nuclear lamina may have to be exposed by disassembly in order for incorporation of new lamin B. These putative sites or factors may be lamins themselves, the lamin B receptor (LBR) (Worman et al., 1988; Simos and Georgatos, 1992; Meier and Georgatos, 1994) or other lamin-associated proteins (LAPs; Foisner and Gerace, 1993). The generation of lamin B CAAX$^-$ expressing cell lines enabled a direct test of whether one of these factors is the putative lamin B receptor (LBR). The two proteins are thought to interact in vivo because lamin B binds with high affinity to LBR in vitro (Worman et al., 1988; Bailer et al., 1991) and the two cofractionate during subcellular fractionation (Meier and Georgatos, 1994). However, Triton X-100 extraction of interphase and mitotic cells stably expressing lamin B mutants indicated that lamin B CAAX$^-$ protein fractionates independently of the LBR, providing strong evidence that the two proteins do not bind directly to each other. Although unlikely, we cannot rule out the remote possibility that binding is facilitated by the CAAX motif of lamin B. Instead, we hypothesize that the association of lamin B with the membrane through the CAAX motif brings it into proximity with the integral membrane LBR protein. This would be consistent with results showing the hydrophobic modifications associated with the CAAX motif target proteins to membranes (Kitten and Nigg, 1991; Nigg et al., 1992; Monteiro et al., 1994).

A common feature of lamins A, B and C without CAAX motifs is their accumulation into intranuclear aggregates during early periods after transfection. Intranuclear lamin foci are also present during the normal cell cycle (Goldman et al., 1992; Sasseville and Raymond, 1995) and are also seen upon microinjection of lamins into cells (Goldman et al., 1992; Bridger et al., 1993; Pugh et al., 1997). The composition of these foci, including any proteins that lamins might be associated with, is not known although lamin B foci appear to correlate with sites of DNA replication (Moir et al., 1994). We believe that the high expression of lamins from the strong CMV promoter in our experiments may result in deposition of lamins into uncharacterized sites within the nucleus, which over time become less apparent as the proteins are slowly incorporated into the lamina. Clearly, deposition of lamins into foci suggests that incorporation of lamins without a CAAX motif is slow, since foci persist for up to 48 hours post-transfection. Either lamins in these foci are structurally different and require time for proper folding, or CAAX$^-$ lamin assembly into the nuclear lamina is slow, or new lamins are sequestered until utilized. Another possibility is that only a fixed amount of lamin can assemble into the lamina, with the excess accumulating into foci. This is unlikely, however, because foci in cells overexpressing lamins with a CAAX motif are much less apparent.

Previously, we suggested that the 42 aa lamin rod insert functions to prevent lamins from being trapped in the cytoplasm due to coassembly with cytoplasmic IF proteins (Monteiro et al., 1994). In those experiments, the rod domain of NF-L (310 residues) was lengthened by inserting the additional 42 aa and the ability of NF-L to coassemble with vimentin was decreased (Monteiro et al., 1994). In this study, the 42 aa insert was deleted from the lamin rod, making it equivalent in length to the rod domain of the cytoplasmic IF proteins. Interestingly, lamin B mutants lacking the 42 aa insert were not retained in the cytoplasm by coassembly with cytoplasmic IF but appeared to assemble at the lamina with a staining pattern indistinguishable from that of wild-type lamin B. Biochemical fractionation, however, indicated that the lamin B mutant with the 42 aa insert deleted was more soluble than its undeleted counterpart, suggesting the 42 aa segment is indeed required for efficient lamin B assembly. Although these data indicate that the 42 aa insert is dispensable for lamina targeting it is not possible to tell by light microscopy whether the shortened lamin B is correctly assembled. It also suggests that the length of the rod domain is not the only determining factor controlling segregation of cytoplasmic and nuclear intermediate filaments. Clearly, other sequence differences between lamin B and cytoplasmic IF proteins prevent coassembly.

In fact sequence comparison of veterbrate cytoplasmic and nuclear IF proteins reveals significant differences in the distribution of ionic charges between the two sets of molecules (see Parry et al., 1986; Meng et al., 1994; Letai and Fuchs, 1995). Lamins contain the 42 aa insert in a sub-region of the α-helical rod domain, termed helix 1B, which is rich in acidic and basic residues but of neutral charge, whereas the corresponding region in cytoplasmic IF is significantly more acidic but contains fewer charged residues (Parry et al., 1986). Since the initial steps in IF assembly occur by coiled-coil interactions of a pair of polypeptides arranged in parallel and in-register (Parry et al., 1985), the number of charged interchain stabilizing residues in lamins is believed to be considerably higher than that of cytoplasmic IF molecules, partly, but not only entirely, due to sequence differences in coil 1B (Parry et al., 1986; Letai and Fuchs, 1995). Deletion of the 42 aa insert would cause a significant change in these ionic interactions, but clearly this by itself is not sufficient to cause lamins to copolymerize with cytoplasmic IF proteins. Whether the end domains or the subsequent assembly of IF dimers into higher ordered structures (e.g. tetramer formation; Meng et al.,

1994) is critical in regulating coassembly of different IF proteins remains to be discovered.

In summary, we have shown that it is possible to delete any one of three signals (the 42 aa insert, the NLS, or CAAX motif) from lamin B and still retain targeting of the mutant lamin B molecules to the nuclear lamina. However, when two or more signals are deleted targeting is more severely compromised. Therefore, the three signals appear to act in concert with one another, ensuring timely and appropriate targeting of lamin B. Furthermore, our studies indicate LBR does not bind lamin B directly, suggesting that it may have other functions such as those proposed in chromatin binding (Ye and Worman 1994).

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