BAF is required for emerin assembly into the reforming nuclear envelope

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SUMMARY

Mutations in emerin cause the X-linked recessive form of Emery-Dreifuss muscular dystrophy (EDMD). Emerin localizes at the inner membrane of the nuclear envelope (NE) during interphase, and diffuses into the ER when the NE disassembles during mitosis. We analyzed the recruitment of wildtype and mutant GFP-tagged emerin proteins during nuclear envelope assembly in living HeLa cells. During telophase, emerin accumulates briefly at the 'core' region of telophase chromosomes, and later distributes over the entire nuclear rim. Barrier-to-autointegration factor (BAF), a protein that binds nonspecifically to double-stranded DNA in vitro, co-localized with emerin at the 'core' region of chromosomes during telophase. An emerin mutant defective for binding to BAF in vitro failed to localize at the 'core' in vivo, and subsequently failed to localize at the reformed NE. In HeLa cells that expressed BAF mutant G25E, which did not show 'core' localization, the endogenous emerin proteins failed to localize at the 'core' region during telophase, and did not assemble into the NE during the subsequent interphase. BAF mutant G25E also dominantly dislocalized LAP2β and lamin A from the NE, but had no effect on the localization of lamin B. We conclude that BAF is required for the assembly of emerin and A-type lamins at the reforming NE during telophase, and may mediate their stability in the subsequent interphase.

Key words: Nuclear envelope, Lamin A, Lamin-associated polypeptide 2, MAN1, Emery-Dreifuss muscular dystrophy, Barrier-to-autointegration factor, Chromosome

INTRODUCTION

The nucleus provides a physical framework for chromosome organization, yet is highly dynamic. The nuclear envelope (NE) disassembles at the onset of mitosis, and is reconstructed around the chromosomes during telophase, re-establishing the architecture of the nucleus for the next interphase. Disassembly and reassembly of the NE are crucial for the progression of mitosis in higher eukaryotic cells. Membrane proteins that bind to chromosomes are proposed to play an important role in nuclear assembly (Gant and Wilson, 1997). The best studied of these proteins are lamin B receptor (LBR) (Worman et al., 1990) and lamina-associated polypeptide-2 (LAP2) (Foisner and Gerace, 1993; Furukawa et al., 1995). Both LBR and LAP2 bind to chromosomes in vitro and are recruited to chromosomes at the earliest stage of NE assembly in vivo (Furukawa et al., 1997; Ellenberg et al., 1997; Haraguchi et al., 2000). LBR interacts with HP1, a protein required for repressive chromatin structure in *Drosophila* (Ye and Worman, 1996). LAP2 has a different chromatin partner, named barrier-to-autointegration factor (BAF) (Furukawa, 1999). BAF, a DNA binding protein, is highly conserved among multicellular animals from nematodes to humans. BAF was discovered as a cellular factor that prevents retroviral DNA from self-integrating in vitro, and is proposed to bridge double-stranded DNA molecules in a non-sequence-specific manner (Lee and Craigie, 1994; Lee and Craigie, 1998; Zheng et al., 2000). However, the biological role of BAF in uninfected cells remains unknown.

LAP2β is related to another integral membrane protein of the nuclear envelope, named emerin (Manilal et al., 1996; Nagano et al., 1996; Yorifuji et al., 1997; Shumaker et al., 2001). The loss of emerin gene product is responsible for an X-linked recessive disease, Emery-Dreifuss muscular dystrophy (EDMD) (Bione et al., 1994). Most X-linked EDMD patients are null for emerin protein, although a few patients express mutated emerin that is mislocalized to the ER. Emerin binds both A- and B-type lamins (Clements et al., 2000; Fairley et al., 1999). Mutations in the gene encoding A-type lamins, *LMNA*, are responsible for an autosomal dominant form of EDMD and dominant lipodystrophy (Bonne et al., 1999; Raffaele Di Barletta et al., 2000; Cao and Hegele, 2000; Shackleton et al., 2000; Genschel and Schmidt, 2000; Wilson et al., 2001). In *LMNA*-null mouse cells, emerin is not specifically retained at the NE, but is also found in the ER, and the NE localization of emerin is restored by exogenous expression of *LMNA* (Sullivan et al., 1999). Thus, emerin localization depends at least in part on A-type lamins, and loss of emerin from the NE may contribute to the *LMNA*-null mouse phenotype.

Human emerin is a serine-rich 254-residue protein with an apparent mass of 34 kDa in SDS-PAGE (Bione et al., 1994), and is phosphorylated in a cell-cycle-dependent manner (Ellis et al., 1998). Emerin belongs to the LEM (LAP2, Emerin, MAN1) family of nuclear proteins, which have the ~43-residue 'LEM'-domain (Lin et al., 2000). The LEM domain of LAP2 mediates its binding to BAF, as shown by yeast two-hybrid analysis (Furukawa, 1999) and biochemical assays with purified BAF dimers and BAF-DNA nucleoprotein complexes (Shumaker et al., 2001). Emerin also binds directly to BAF in a LEM-domain-dependent manner, suggesting that LEM proteins constitute a family of BAF-binding proteins, as described in the accompanying paper (Lee et al., 2001).

To test the hypothesis that emerin and BAF interact in vivo, we analyzed the dynamic behavior of emerin and BAF during NE assembly in telophase human (HeLa) cells. We used computerized time-lapse fluorescence microscopy (Haraguchi et al., 1997; Haraguchi et al., 1999) to follow emerin and BAF tagged with green fluorescent protein (GFP-emerin and GFP-BAF) in living HeLa cells, in combination with indirect immunofluorescence in fixed cells. We also analyzed a series of emerin truncations, and emerin and BAF proteins with biochemically-characterized mutations. We found that emerin and BAF are co-enriched during telophase in the central region of the assembling nuclear rim (designated the 'core' region), near spindle-attachment sites. However this 'core' localization did not depend on microtubules or the cytoskeleton. Importantly, we found that emerin localization depends on its ability to bind BAF. Furthermore, transient expression of a BAF mutant that does not localize at the 'core' region selectively blocked the nuclear envelope localization of endogenous emerin, LAP2β and lamin A, but not lamin B.

MATERIALS AND METHODS

Cells and reagents

HeLa cells were obtained from the Riken Cell Bank (Tsukuba Science City, Tsukuba, Japan). Hoechst 33342 was purchased from Calbiochem (La Jolla, CA). GFP fusion constructs of full-length human emerin (Nagano et al., 1996) and truncated emerin (Tsuchiya et al., 1999), and anti-emerin antibodies (Yorifuji et al., 1997) were generous gifts of K. Arahata and Y. Tsuchiya (National Institute of Neuroscience of Japan). Rabbit polyclonal serum 3273 against human BAF was prepared by injecting rabbits with a keyhole limpet hemocyanin (KLH)-conjugated synthetic peptide (NH3-CSQKHRDFVAEPMGEKPV-COOH) representing a region near the N-terminus of human BAF. BAF is almost identical between mammalian species, and this peptide was previously shown to be effective as an antigen (Furukawa, 1999). Serum 3273 detected one major band with an apparent molecular weight of 10 kDa (corresponding to BAF) on western blots of total HeLa cell extracts, and also recognized purified recombinant BAF protein on blots (data not shown). Serum 3273 antibodies were affinity purified using nitrocellulose membranes immobilized with purified recombinant human full-length BAF protein. The purified antibody was concentrated on Centricon-10 spun columns (Amicon, MA), replacing the eluent buffer with phosphate-buffered saline (Gibco BRL), and used for immunofluorescence studies. An independent rabbit polyclonal antibody against the synthetic peptide (residues 4-20 of mammalian BAF) was a generous gift of Kazuhiko Furukawa (Niigata University, Japan). Plasmids encoding human BAF and BAF mutant G25E were a generous gift of Robert Craigie (National Institutes of Health, USA). Mouse monoclonal antibody 101-B7 (IgG) against human lamin B was purchased from Matritech, Inc. (Cambridge, MA). Mouse monoclonal anti-LAP2β antibody 6G11 against residues 313-330 of human LAP2β were the generous gift of Crafford Harris (R. W. Johnson Pharmaceutical Research Institute, Raritan, NJ). Mouse monoclonal antibody (IgM) against lamin A/C was generated as follows: lamins A, B and C were purified from rat liver nuclei as described previously (Georgotos and Blobel, 1987), and 25 mg of a mixture of lamins A, B and C were injected intraperitoneally into an 8-week-old BDF1 mouse (Japan SLC, Inc., Japan). Two subsequent injections followed at 1-week intervals with the same dose. One month after the third injection, the mouse was given a booster injection of the same dose. Four days later, spleen cells from the mouse were fused with the mouse myeloma cell line P3U1 using standard methods. The hybridoma culture supernatants were screened by immunofluorescence microscopy of HeLa cells and normal rat kidney (NRK) cells, and by western blotting of rat liver nuclear lamina fraction and HeLa cell extract. The monoclonal antibody, TiM92, stained the nuclear rim in HeLa cells and in NRK cells, and specifically recognized lamins A/C on immunoblots (data not shown). TiM92 (the IgM subclass) was purified from ascites fluid by chromatography on a hydroxyapatite column.

Plasmid construction

Plasmid pET15b-BAF was described previously (Lee and Craigie, 1998). To construct GFP-BAF and GFP-BAF-G25E plasmids, the coding regions of wild-type or mutant BAF were PCR-amplified using the following primers: 5′-CGGTCGACGAGCAGCCATCATC-ATCATCAT-3′ and 5′-CCGGATCCCTACAAGAAGGCATCACA-CCAT-3′. PCR products were digested with *Sal*I and *Bam*HI, and inserted into the CMV promoter-driven pEGFP-C3 vector (Clontech Laboratories, Inc., Palo Alto, CA) at the 3′ end of GFP using the *Sal*I and *Bam*HI sites in the vector. GFP-fused emerin and GFP-fused truncated emerins were obtained from the Arahata laboratory. Plasmid pET11c-emerin-m24 encodes a mutant in which four amino acids are changed to alanines at residues 24-27, and lacks the C-terminal transmembrane domain (Lee et al., 2001). To make a GFP fusion to emerin-m24 that included the transmembrane domain, the coding region of pET11c-emerin-m24 was first PCR-amplified using primers 5′-CGTCCGGACTCAGATCCATGGACAACTAC-3′ and 5′-GCGG-ATCCCTGGCGATCCTGGCCCAG-3′. Second, the PCR product was digested with *Bsp*EI and *Bam*HI, and inserted in the pEGFP-C1 vector at the *Bsp*EI and *Bam*HI sites. Finally, this construct was digested with *Sac*I and *Bam*HI, and ligated with the *Sal*I/*Bam*HI fragment from full length GFP-emerin plasmids that include the transmembrane domain. The DNA sequence of all fusion plasmids were confirmed using a ABI377 DNA sequencer (Applied Biosystems, Norwalk, CT).

Microscope system setup

A DeltaVision microscope system (Applied Precision Inc. Seattle, WA) was used in this study. For temperature control during live observation, the microscope was placed in a temperature-controlled room, and kept at 37°C, as described previously (Haraguchi et al., 1997; Haraguchi et al., 1999).

Fluorescence microscopy in living cells

Cells were grown in a glass-bottom culture dish (MatTech, USA). GFP fusion plasmids (1 μg) were transfected into cells with LipofectaminePlus (Gibco BRL) according to manufacturer's methods except that the incubation time with DNA was reduced to 1.5 hour. Cells expressing GFP fusion proteins were stained with 100 ng/ml of Hoechst 33342 (a DNA-specific fluorescent dye) for 5-30 minutes, then cultured in phenol red-free DME medium supplemented with 10% fetal bovine serum in a CO_2 incubator for at least 30 minutes before microscopic observation. For microscopic observation, Hepes pH 7.3 (final concentration 25 mM) was used to avoid the need for CO_2 gas. Fluorescently stained living cells were imaged on a Peltier-cooled charge-coupled device (Photometrics) using an Olympus

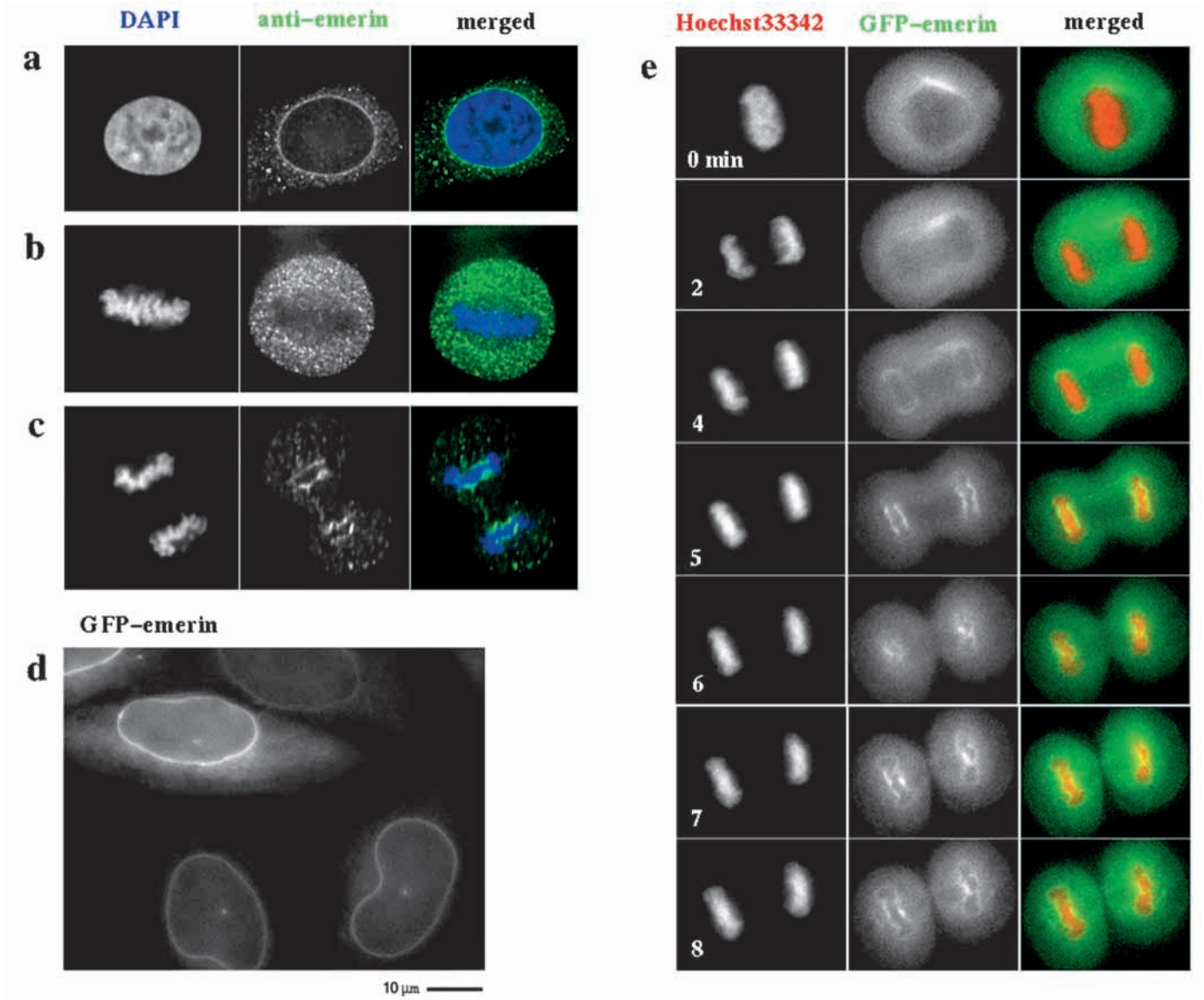

Fig. 1. Intracellular localization of emerin in HeLa cells. (a-c) Emerin stained with the antibody in fixed cells:(a) interphase; (b) metaphase; and (c) telophase stages of mitosis. (d-e) GFP-emerin in living cells: (d) interphase; (e) metaphase through telophase; time-lapse images in (e) were obtained at one-minute intervals in the same cell. Chromosomes were stained with DAPI in fixed cells, and with Hoechst 33342 in living cells. Bar, 10 μm.

oil immersion objective lens with a high numerical aperture (40×/NA=1.35) as described previously (Haraguchi et al., 1997; Haraguchi et al., 1999; Haraguchi et al., 2000).

Indirect immunofluorescence staining

Cells were fixed in a mixture of 3.7% formaldehyde and 0.2% glutaraldehyde (final concentrations) for 10 minutes at room temperature, and permeabilized with 0.1% Triton X-100 in PBS for 5 minutes at room temperature. After fixation, cells were treated twice with 0.1% sodium borohydride in PBS for 15 minutes at room temperature to block unreacted glutaraldehyde, and then incubated for 1 hour at room temperature with 1% BSA in PBS. Antibodies against the primary antigen (emerin, BAF or LAP2β) were then added to cells at dilutions of 1:100-500, and incubated for 18 hours at 4°C. Cells were washed four times with 2 ml PBS and stained with a fluorescent (Cy3-, Texas Red-, rhodamine- or Alexa-conjugated) secondary antibody at a dilution of 1:200-1000 for 3-4 hours at room temperature. Finally, the cells were washed three times with PBS, and then incubated sequentially with 20, 40, 60 and 80% glycerol containing PBS, 2.5% 1,4-diazabicyclo-2,2,2-octane (DABCO) and 0.5 μg/ml DAPI. The cells were mounted in 90% glycerol, containing 2.5% DABCO as an anti-fading reagent. Methanol fixation was used for immunofluorescence staining with anti-lamin A and anti-lamin B antibodies as described previously (Haraguchi et al., 2000).

Fluorescent microscopic images were obtained with an Olympus microscope IX70 using an oil immersion objective lens (UApo 60UV, NA=1.4) and high-selectivity filters. Serial optical section data (15-30 focal planes at 0.5 μm intervals) were collected on a Peltier-cooled charge-coupled device (Photometrics) and computationally processed by a 3D deconvolution method.

RESULTS

We first examined the subcellular localization of endogenous emerin in HeLa cells using a specific antibody (see Materials and Methods). As expected for interphase cells, endogenous

emerin immunolocalized predominantly at the NE (‘rim’ staining; Fig. 1a). During metaphase, emerin is dispersed into the ER network upon NE breakdown (Fig. 1b) and reassembles around chromosomes in early telophase (Fig. 1c). Emerin is first localized at the central region of telophase chromosomes, near spindle attachment sites (the ‘core’ region; Fig. 1c). This telophase specific ‘core’ localization was previously seen in another human cell type, HEp-2 (Dabauvalle et al., 1999), and is also seen in normal human fibroblast WI38 cells (T. Haraguchi, unpublished), suggesting that ‘core’ localization is a general property of emerin in dividing cells.

To examine the dynamic behavior of emerin in living cells, GFP-emerin was transiently expressed in living HeLa cells and monitored by time-lapse fluorescence microscopy. Cells expressing GFP-emerin were double-labeled for chromosomes using the DNA-specific fluorescent dye, Hoechst 33342. GFP-emerin was localized at the NE during interphase (Fig. 1d) and the majority was dispersed into the ER upon NE disassembly (Fig. 1e, 0 minutes), as expected. The GFP-emerin reappeared on the assembling NE during early telophase (4 minutes after the metaphase-anaphase transition in Fig. 1e) and then specifically localized to the ‘core’ region of the telophase chromosome mass (Fig. 1e, 5 minutes) for 3-4 minutes, before becoming uniformly distributed in the NE (Fig. 1e, 8 minutes). This dynamic behavior of GFP-emerin was consistent with that of endogenous emerin, demonstrating that the fusion to GFP did not disrupt emerin dynamics.

Since the ‘core’ region is close to the spindle attaching sites (see Fig. 7 in Haraguchi et al.) (Haraguchi et al., 2000), we tested whether microtubules mediated the ‘core’ localization. Surprisingly, treatment with nocodazole or vinblastin, under conditions that depolymerize microtubules and mitotic spindles in living HeLa cells (Haraguchi et al., 1997), did not disrupt the ‘core’ localization of emerin (Fig. 2). This result suggested that ‘core’ localization is independent of microtubules and microtubule-mediated events, and therefore might reflect a chromosome-based structure. Treatment with cytochalasin D, an actin-depolymerizing agent, also had no effect on ‘core’ localization (not shown), suggesting that the ‘core’ structure was also independent of actin.

The LEM-domain is necessary and sufficient for emerin localization at the ‘core’

To identify regions in emerin responsible for its ‘core’ localization, we expressed a series of truncated emerins as GFP-fusion proteins in HeLa cells. Each truncation removed residues from the N-terminus (Δ1-64, Δ1-106, Δ1-133, Δ1-155, Δ1-175 and Δ1-222) or C-terminus (Δ226-254, Δ175-254, Δ109-254, Δ66-254 and Δ38-254) (Tsuchiya et al., 1999), as shown in Fig. 3a.

First, we determined the region needed for ‘core’ localization. The GFP-emerinΔ66-254 construct, which has only the N-terminal 65 residues (including the LEM domain), localized correctly at the ‘core’ region of telophase chromosomes (Fig. 3c-e), but failed to localize at the interphase NE (Fig. 3b). All other constructs that included residues 1-65 also showed the ‘core’ localization, whereas the shortest construct, containing N-terminal residues 1-37 (Δ38-254) did not (Fig. 3a). In striking accordance, the GFP-emerinΔ1-64 construct, which lacks residues 1-64, did not localize at the ‘core’ region (Fig. 3g) although it did localize at the NE (Fig. 3f). A point mutation of the leucine residue at position 64 to phenylalanine also weakened the ‘core’ localization of emerin (data not shown), consistent with evidence that residues outside the LEM domain can influence binding to BAF (Shumaker et al., 2001). These results showed that the N-terminal 65 residues, which include the LEM-domain, were both necessary and sufficient for the ‘core’ localization of emerin. We concluded that ‘core’ localization constitutes an in vivo assay for emerin binding to BAF, since BAF is also present in the ‘core’ (see below), and because emerin residues 1-65 include the LEM domain, which directly mediates binding to BAF (residues 1-43) (Shumaker et al., 2001; Cai et al., 2001; Wolff et al., 2001; Lee et al., 2001).

Östlund et al. previously showed that the nucleoplasmic domain of emerin is necessary and sufficient for its nuclear envelope localization (Östlund et al., 1999). To dissect the nuclear envelope targeting domains in relation to the ‘core’ binding domain, we expressed and localized each GFP-emerin

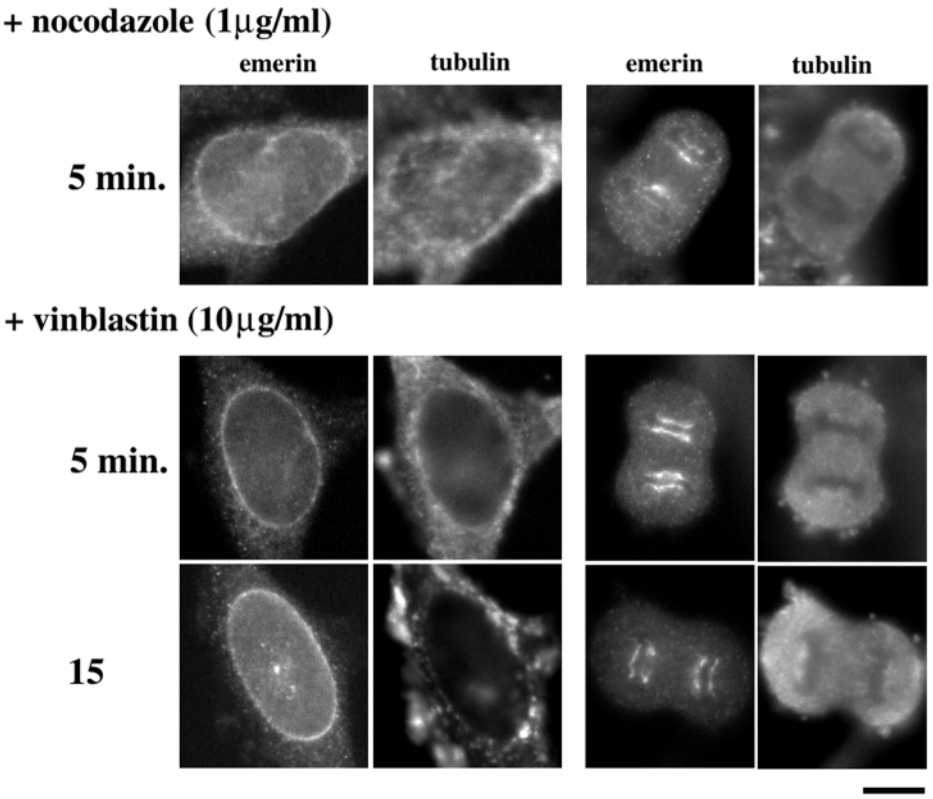

Fig. 2. Emerin ‘core’ localization does not depend on microtubules. Cells treated with nocodazole or vinblastine were fixed and stained with antibodies against endogenous emerin. Bar, 10 μm.

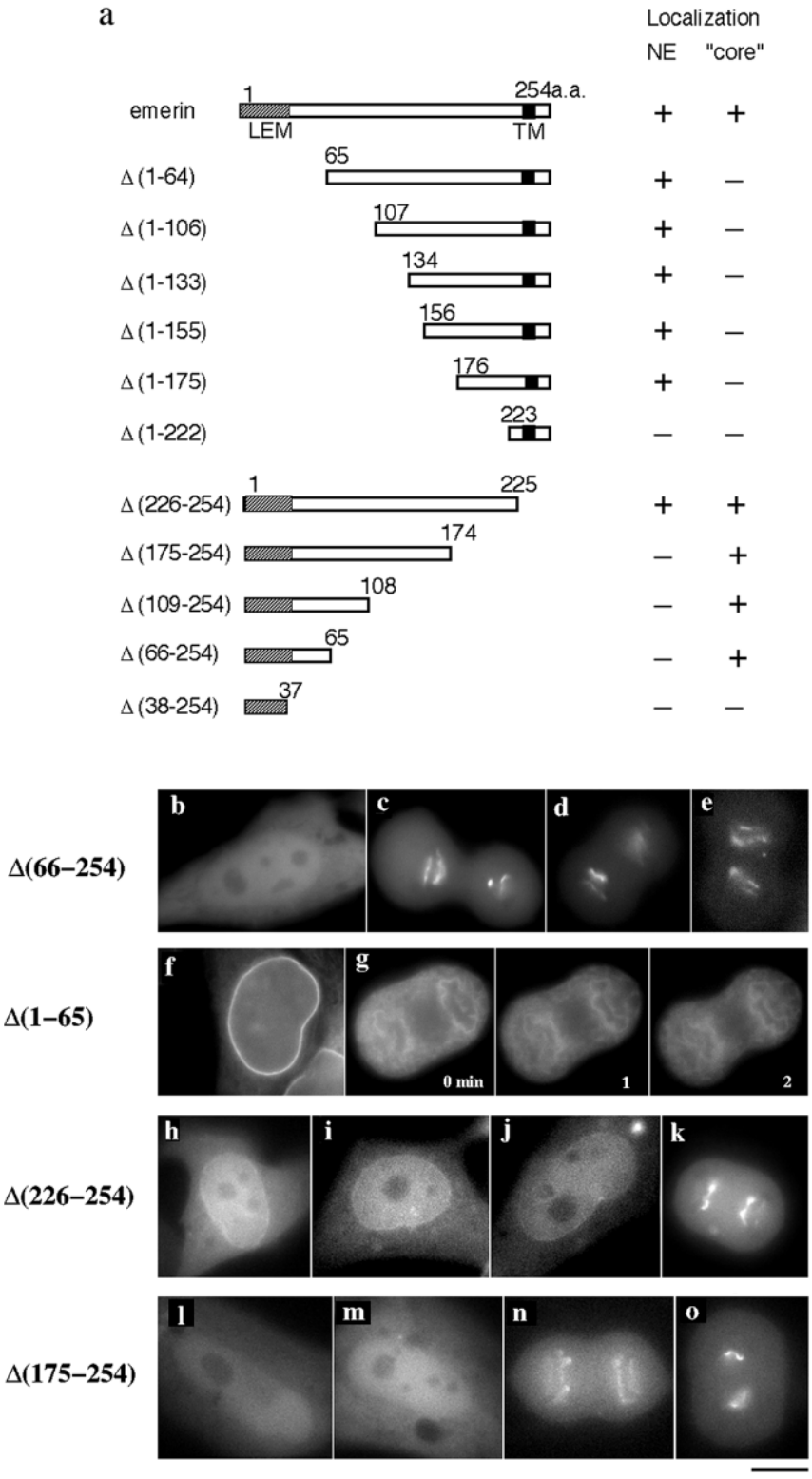

Fig. 3. Molecular domains of emerin necessary for its localization. (a) Molecular structure of emerin and its truncations. GFP was fused to their C-terminus. Localization of each truncation at the NE and the 'core' is summarized: N-terminal 65 residues are necessary and sufficient for the 'core' localization of emerin; C-terminal domain is required for nuclear membrane localization. Examples are shown for Δ66-254 (b-e), for Δ1-64 (f,g), for Δ226-254 (h-k), and for Δ175-254 (l-o). In (g) time-lapse images at 1 minute intervals are shown. Bar, 10 μm.

construct during interphase. As shown in Fig. 3b, GFP-emerinΔ66-254, which contains a putative bipartite nuclear localization signal (NLS) at residues 31-47, did not localize at the NE. By contrast, the GFP-emerinΔ1-64 construct, which lacks the putative NLS, did localize at the NE (Fig. 3f). Thus, this putative NLS is not necessary for nuclear membrane localization when emerin is expressed during interphase. By contrast, GFP-emerinΔ226-254, which lacks the transmembrane domain and the C-terminal tail, localized mostly inside the nucleus, as reported previously (residues 3-228) (Östlund et al., 1999). However, this protein also unexpectedly localized at the NE during interphase, albeit less prominently (Fig. 3h-j), and still localized at the 'core' region during telophase (Fig. 3k), consistent with having an intact LEM-domain. The weak NE localization of GFP-emerinΔ226-254 was totally lost in GFP-emerinΔ174-254 (Fig. 3l-m), confirming that residues 174-225 can mediate NE localization to some extent in the absence of the transmembrane domain (Östlund et al., 1999). Altogether, our results suggest that emerin may have affinities for multiple partners at the inner nuclear membrane. Emerin residues 174-225 are not required to bind lamins in vitro, and are instead proposed to interact with an unidentified partner at the nuclear envelope (Lee et al., 2001).

BAF accumulates at the telophase 'core'

Emerin interacts directly with BAF through the LEM domain (Lee et al., 2001) and the above results showed that the LEM-domain of emerin is required for its 'core' localization. We therefore tested the hypothesis that BAF itself localizes at the 'core' region, by using indirect immunofluorescence to localize endogenous BAF during mitosis. Our hypothesis was supported by two independent antisera against BAF, both of which stained the 'core' region in early telophase chromosomes (Fig. 4a,b).

To further understand the dynamic behavior of BAF, we transiently expressed a GFP-BAF fusion protein in living HeLa cells, and localized BAF during the cell cycle. As shown in Fig. 4d, GFP-BAF was diffusely non-localized during metaphase and early anaphase, but then dramatically localized at the 'core' region for a limited time during telophase – specifically, from 5 minutes to 9 minutes after the metaphase-anaphase transition. By 9 minutes after the metaphase-anaphase transition, some GFP-BAF staining had begun to disperse into the cytoplasm. Simultaneous detection of GFP-BAF and emerin (with anti-emerin antibodies) in fixed

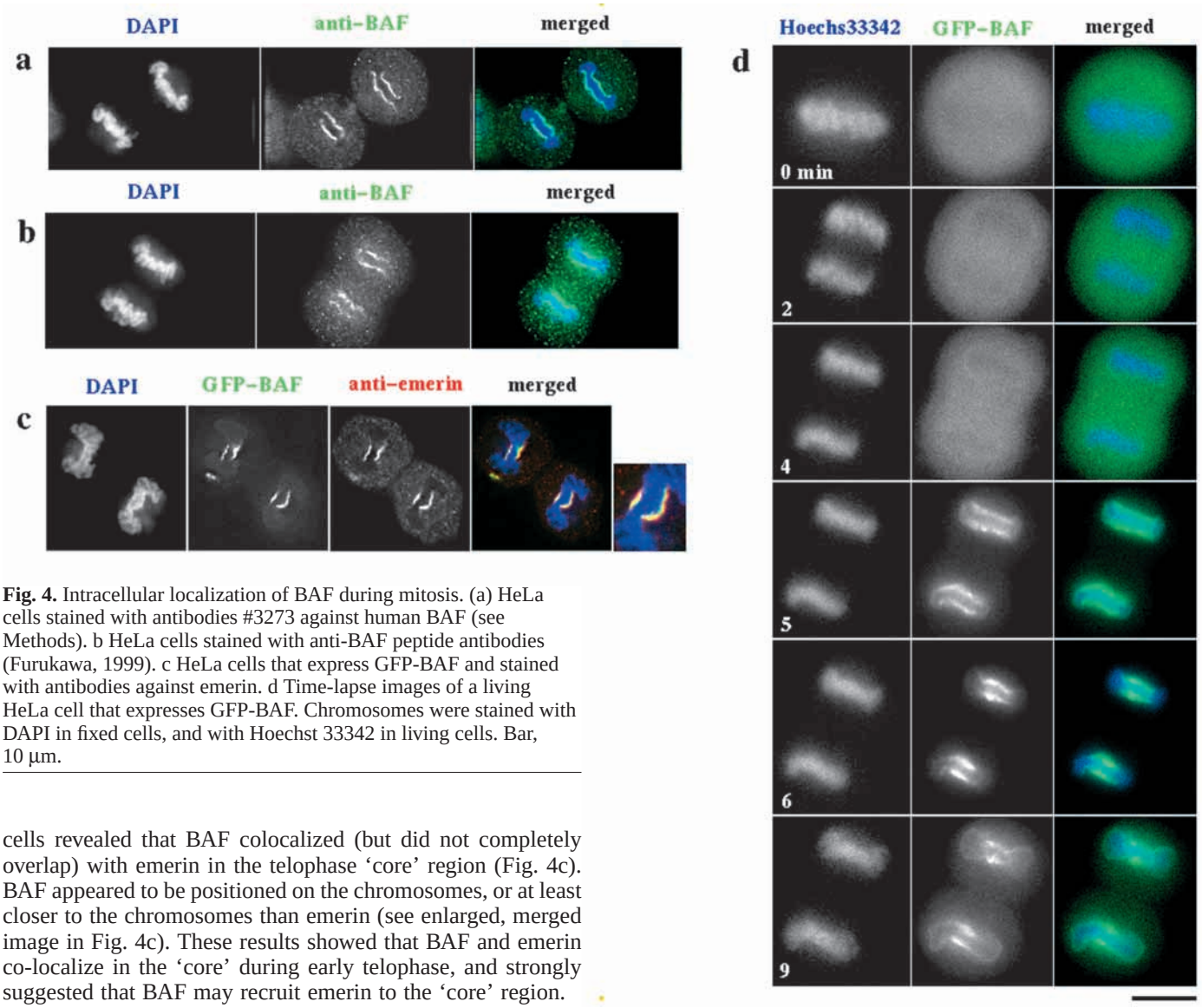

Fig. 4. Intracellular localization of BAF during mitosis. (a) HeLa cells stained with antibodies #3273 against human BAF (see Methods). b HeLa cells stained with anti-BAF peptide antibodies (Furukawa, 1999). c HeLa cells that express GFP-BAF and stained with antibodies against emerin. d Time-lapse images of a living HeLa cell that expresses GFP-BAF. Chromosomes were stained with DAPI in fixed cells, and with Hoechst 33342 in living cells. Bar, 10 μm.

cells revealed that BAF colocalized (but did not completely overlap) with emerin in the telophase 'core' region (Fig. 4c). BAF appeared to be positioned on the chromosomes, or at least closer to the chromosomes than emerin (see enlarged, merged image in Fig. 4c). These results showed that BAF and emerin co-localize in the 'core' during early telophase, and strongly suggested that BAF may recruit emerin to the 'core' region.

BAF recruits emerin to the 'core' region

To test the biological implications of emerin localization at the 'core', we localized an emerin mutant (mutant m24) with four amino acid substitutions in the LEM-domain that disrupt its binding to BAF in vitro (Lee et al., 2001). To create this mutant, residues 24-27 (GPVV) were changed to alanines (AAAA) in the context of full-length emerin fused to GFP (designated GFP-emerin-m24; Fig. 5a). This mutation abolishes binding to BAF in vitro, with possible enhancement of binding to lamin A (Lee et al., 2001). When expressed in interphase cells, GFP-emerin-m24 was localized normally at the NE (Fig. 5b), consistent with its ability to bind lamin A in vitro (Lee et al., 2001). These results were also consistent with our finding (Fig. 3) that the LEM-domain was not needed for emerin to localize at the nuclear envelope, if emerin protein was expressed in interphase cells, which have a functional nucleus. However, in cells that passed through mitosis and had to re-assemble the nucleus, GFP-emerin-m24 failed to properly reassemble in the NE. During telophase, GFP-emerin-m24 was not seen at the 'core' region, but instead localized uniformly on telophase chromosome surfaces; in these same cells, endogenous BAF was localized normally at the 'core' (Fig. 5c,d). This result was important because it demonstrated that the 'core' localization of emerin during telophase depends on its ability to bind BAF, whereas BAF localization at the 'core' does not require emerin. To our surprise, after these cells had exited mitosis and re-entered interphase (early G1), GFP-emerin-m24 remained dispersed in the cytoplasm, presumably in ER membranes (Fig. 5e). We conclude that the transient interaction of emerin with BAF at the 'core' region may be essential for emerin localization at the nuclear envelope during the subsequent interphase (see Discussion).

Mutated BAF interferes with assembly of emerin, LAP2β and lamin A into the NE

To test our hypothesis that BAF is required for emerin localization in the 'core' region, we expressed various missense point mutants of BAF fused with GFP in living HeLa cells. One such mutant, BAF-G25E (glycine at position 25 mutated to glutamic acid) affected emerin localization during telophase. This BAF mutant is inactive for binding to DNA (Umland et al., 2000), and inactive for binding to emerin and

LAP2 in vitro (M.S.-T. and K.L.W., unpublished), but might retain the ability to interact with BAF-DNA complexes. When GFP-BAF-G25E was transiently expressed in living cells, the 'core' localization of endogenous emerin at telophase was abolished (Fig. 6a; compare with Fig. 1c) even though GFP-BAF-G25E itself did not localize at the 'core' region (Fig. 6a). In addition, expression of GFP-BAF-G25E prevented emerin from localizing at the NE at a later stage of mitosis (Fig. 6b) and during the ensuing interphase (indicated by an arrow in Fig. 6c), whereas normal NE localization was seen in non-expressing cells (Fig. 6d; arrowheads in Fig. 6c). Controls showed that emerin localized normally in cells that expressed GFP fused to wildtype BAF (Fig. 6e).

To test the possibility that mutant BAF G25E might also disrupt other nuclear proteins, we localized lamin A, lamin B and LAP2β in cells that went through mitosis in the presence of GFP-BAF-G25E (Fig. 7). We first compared the timing of lamin A assembly in HeLa cells that were either untransfected (Fig. 7b), or transfected with the BAF mutant (Fig. 7a). Untransfected controls were important because most lamin A is imported into nuclei after the nascent nuclear envelope has assembled; thus, the lamin A signal at early times during nuclear envelope assembly can be weak or variable (Gant and Wilson, 1997; Broers et al., 1999; Moir et al., 2000). We found that the accumulation of lamin A at reforming nuclear envelopes was significantly retarded in HeLa cells that expressed GFP-BAF-G25E, with most lamin A remaining in the cytoplasm (Fig. 7a), compared to untransfected cells (Fig. 7b). Importantly, GFP-BAF-G25E had no detectable effect on the localization of lamin B (Fig. 7c). However, the other LEM-domain protein tested, LAP2β, also failed to localize at the reforming nuclear envelope in cells that had passed through mitosis in the presence of GFP-BAF-G25E, and often formed small punctate aggregates in the cytoplasm (Fig. 7d). Emerin also tended to form cytoplasmic aggregates in the presence of GFP-BAF-G25E (Fig. 6b), and colocalized with the LAP2β aggregates (data not shown). Such aggregates may represent the ER because it has been reported that emerin accumulates as aggregates within the ER when lamin A/C is eliminated from the NE (Vaughan et al., 2001). Consistent with previous reports (Foisner and Gerace, 1993), our untransfected controls showed rapid accumulation of LAP2β at reforming nuclear envelopes during telophase (Fig. 7e; also note the two non-expressing cells in Fig. 7d). These results suggested that BAF may specifically recruit and mediate the nuclear envelope assembly of at least two LEM-domain proteins, emerin and LAP2β, and the assembly of lamin A, but not lamin B, in vivo. In addition, we found that nuclear transport activity was not affected in nuclei reformed in the presence of GFP-BAF-G25E as detected by an NLS-conjugated fluorescent protein (data not shown), suggesting that nuclear transport machinery, such as nuclear pore complex, is assembled normally into the NE in those cells.

DISCUSSION

Emerin and BAF are conserved among multicellular eukaryotes, from *C. elegans* to *H. sapiens* (Cai et al., 1998; Cohen et al., 2001), suggesting that these proteins have fundamental biological roles. Both proteins (and lamins) are absent from single-cell eukaryotes such as yeast (Cohen et al., 2001), suggesting that emerin and BAF contribute uniquely to nuclear architecture or function in 'higher' eukaryotes. One such function can be NE reassembly during mitosis, because disassembly and reassembly of the NE are major events unique to 'higher' eukaryotes. Emerin accumulates transiently in a

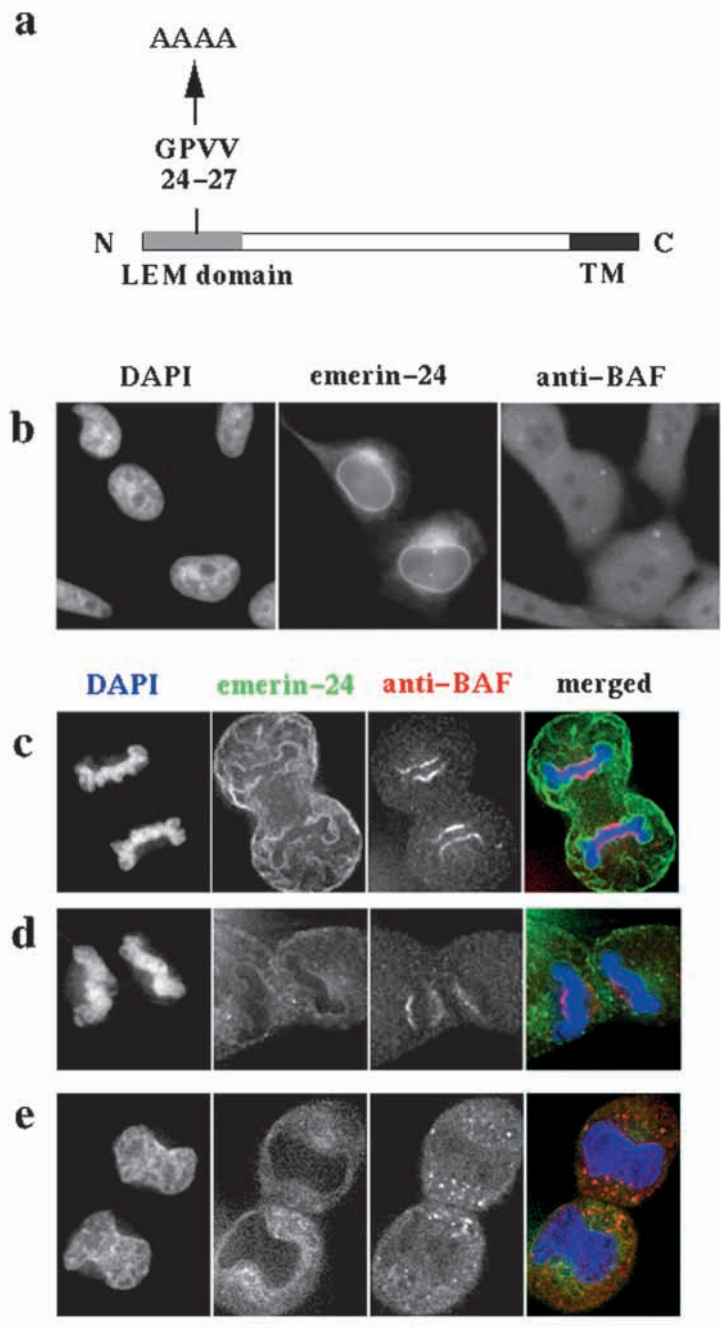

Fig. 5. Localization of GFP-fused mutant emerin-m24. (a) Molecular structure of GFP-fusion to mutant emerin-m24. (b) Localization of GFP-emerin-m24 in an interphase HeLa cell. (c-e) Localization of GFP-emerin-m24 and endogenous BAF in HeLa cells expressing the mutant emerin. GFP-tagged emerin-m24, and anti-BAF staining are shown in c, telophase in d and the next interphase in e. Chromosomes were stained with DAPI.

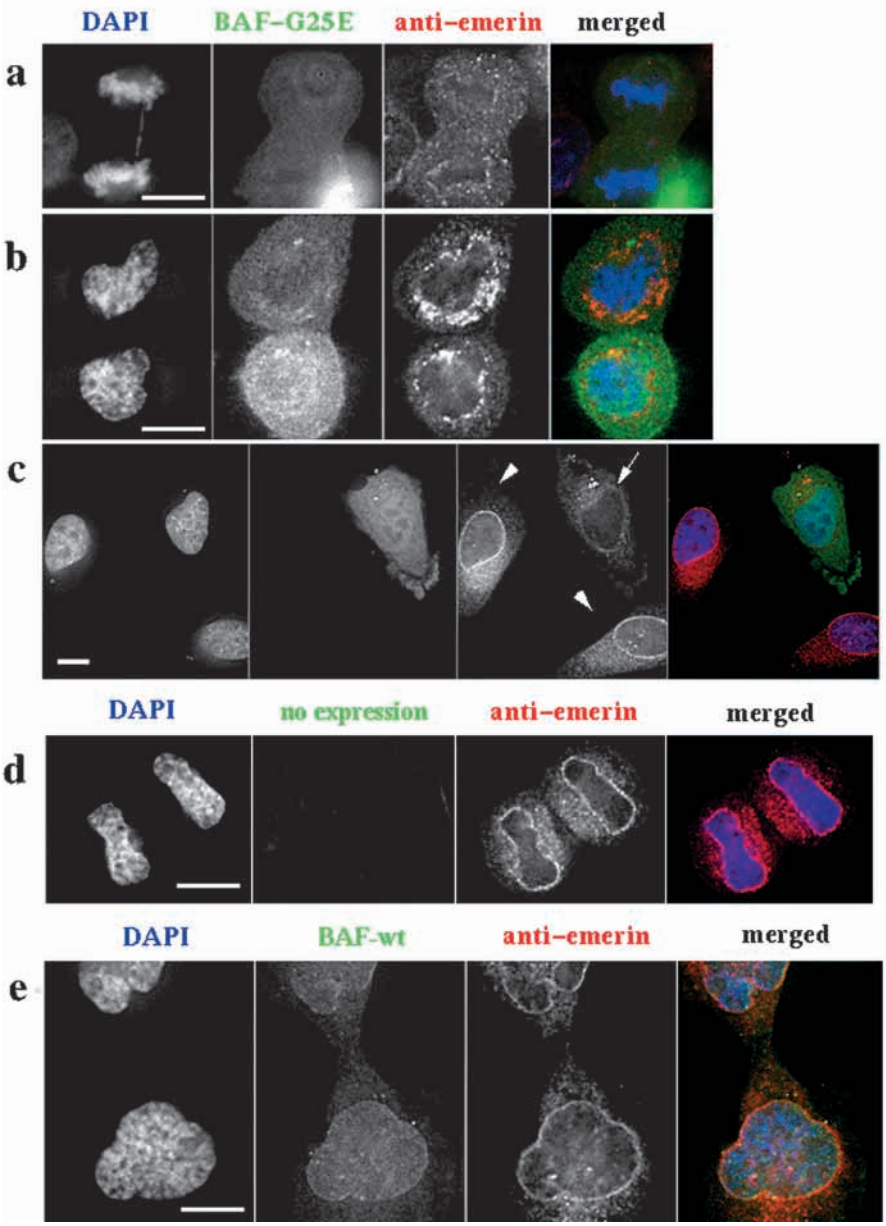

Fig. 6. Emerin does not accumulate at nuclear envelopes reassembled in the presence of mutant BAF-G25E. HeLa cells were stained with antibodies against endogenous emerin. (a) A telophase cell expressing GFP-BAF-G25E. (b) Cells expressing GFP-BAF immediately after cytokinesis. (c) An interphase cell that went through mitosis in the presence of GFP-BAF-G25E is indicated by the arrow; cells with no expression of GFP-BAF-G25E are indicated by the arrowhead. (d) A late telophase cell with no expression of GFP-BAF-G25E observed in the same culture dish as for b. (e) HeLa cells expressing GFP-BAF (wildtype) shortly after cytokinesis. Chromosomes were stained with DAPI. Bars, 10 μm.

specific region, designated the 'core' region, of the telophase chromosome rim in HeLa cells (Haraguchi et al., 2000) as well as in human HEp-2 cells (Dabauvalle et al., 1999). We now find that emerin localization at the 'core' requires its BAF-binding region, and depends on BAF's localization at the 'core'. Mutant BAF-G25E, which does not localize at the 'core', dominantly disrupts the NE assembly of emerin, LAP2β and lamin A, but not lamin B, in the subsequent interphase. Thus, the BAF-dependent 'core' structure during early telophase is important for re-establishment of nuclear lamina structure. These findings have important implications. We propose that BAF, lamin A, emerin and other LEM domain proteins may constitute a discrete structural element of the nuclear lamina, since lamin B was sheltered from the dominant effect of the BAF mutant. We also propose that BAF may be a structural element of the NE-chromatin interface, since BAF can theoretically anchor all LEM proteins and their attached lamin filaments to chromatin. Finally, our results suggest that telophase interactions between emerin and BAF at the 'core' may define a novel early phase in the assembly of A-type lamins.

The 'core' region: a BAF-dependent chromatin structure

The 'core' region is close to the spindle attaching sites (Haraguchi et al., 2000). Despite this proximity to kinetochores, our evidence strongly suggests that the 'core' region is independent of the cytoskeleton, because it was insensitive to depolymerization of microtubules and actin filaments. We propose that the 'core' region represents a previously unrecognized BAF-dependent chromatin structure that is distinct from centromeres and telomeres, and which mediates the reassembly of specific elements of the reforming NE. These elements include lamin A, emerin and probably other LEM proteins, and exclude lamin B and possibly LBR (see below). This idea is based on the dominant effects of mutant BAF-G25E, which interfered with the assembly or retention of endogenous emerin, lamin A and LAP2β (but not lamin B) at the NE in cells that went through mitosis. We think that chromosome surfaces outside the 'core' might mediate attachments between B-type lamins and their other binding partner(s), since LBR (which binds B-type lamins) is found outside the 'core' (Haraguchi et al., 2000). Other results also support this model. For example, A- and B-type lamins have distinct pathways of assembly during interphase (Moir et al., 2000; Steen and Collas, 2001) which might mirror distinct pathways of assembly during nuclear reformation.

NE-chromatin interactions during nuclear reformation

Chromatin binding by nuclear membrane proteins is an essential first step in NE assembly. Our results suggest a model in which BAF may selectively recruit membrane proteins to a defined surface or structure on telophase chromatin. Other chromatin proteins that interact with NE proteins may also have roles in membrane recruitment or attachment. Heterochromatin protein (HP) 1 interacts with LBR in yeast

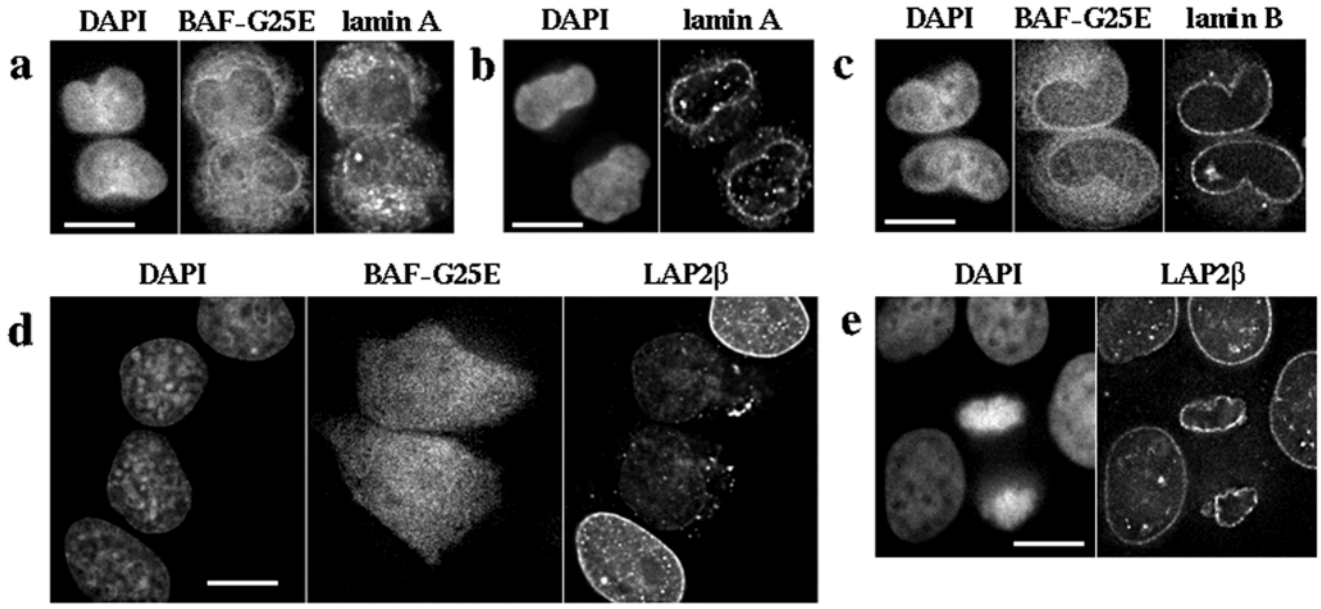

Fig. 7. Reassembly of lamins and LAP2β after cells go through mitosis in the presence of mutant BAF-G25E. HeLa cells were stained with antibodies against endogenous lamin A (a,b), lamin B (c), and LAP2β (d,e). (a) Telophase cells expressing GFP-BAF-G25E and stained with antibodies against lamin A. (b) Untransfected telophase cell stained with antibodies against lamin A. (c) Telophase cell expressing GFP-BAF-G25E and stained with antibodies against lamin B. (d) Cells expressing (middle two cells), and not expressing (outer two cells), GFP-BAF-G25E and stained with antibodies against LAP2β. (e) Untransfected cells stained with antibodies against LAP2β. Chromosomes were stained with DAPI. Bars, 10 μm.

two-hybrid assays (Ye and Worman, 1996). Mouse HP1 isoforms associate with the NE during interphase, and the N-terminal chromodomain of M31 (a mouse homolog of human HP1β) reportedly mediates the reassembly of LAP2β and B-type lamins at the chromosome rim (Kourmouli et al., 2000). However, we speculate that the LAP2β-BAF interaction might be predominant over the proposed LAP2β-HP1β interaction at early stages of nuclear reformation, since LAP2β failed to assemble into the NE in cells that expressed mutant BAF-G25E. The affinities of LAP2β (and other LAP2 isoforms) may be differentially regulated, since they are selectively phosphorylated by multiple kinases during both interphase and mitosis (Foisner and Gerace, 1993; Dreger et al., 1999; Otto et al., 2001). Emerin is also differentially phosphorylated (Ellis et al., 1998). Each pair of interactions (emerin-BAF, LBR-HP1, LAP2β-HP1, LAP2β-BAF, and others), may contribute uniquely to NE assembly during telophase, or to nuclear lamina structure during interphase. These ideas are testable for other LEM proteins including MAN1 (Lin et al., 2000), LAP2α (Dechat et al., 2000) and lem-3 (Lee et al., 2000), as well as other nuclear membrane proteins (Wilson et al., 2001).

BAF may mediate the assembly or stability of the A-type nuclear lamina

To explain the dominant effects of BAF mutant G25E on lamin A, we offer two possibilities. First, it has not been ruled out that BAF might interact directly with lamin A, and mediate its localization during interphase. However we favor a second possibility, in which LEM proteins that depend on BAF for their localization contribute to the assembly or stability of lamin A filaments; in this case, mutant-BAF-induced mislocalization of LEM proteins would cause downstream effects on the localization or stability of lamin A. Further studies are needed to understand the interactions of BAF with LEM-proteins and lamins.

Why is the LEM-domain not required to localize emerin expressed during interphase?

Our results suggest that the LEM-domain, and emerin-BAF interactions, are both essential and sufficient for emerin to localize at the 'core' during telophase. However, one result was paradoxical. When we expressed the non-BAF-binding emerin (mutant m24) during interphase, this protein localized to the nuclear envelope. This localization is readily explained by the diffusion-retention model for localization at the inner nuclear membrane (Östlund et al., 1999), in which newly-synthesized membrane proteins diffuse along the ER membrane, move along the 'pore membrane' domain, and are then retained at the inner membrane by binding to lamins or chromatin. Emerin mutant m24 can bind to lamin A but not BAF in vitro (Lee et al., 2001). In cells that underwent mitosis, this protein failed to localize at the 'core', which can be explained by its inability to bind BAF. However, we cannot explain why, during the subsequent G1-phase (interphase), the 'core-deficient' emerin protein did not become localized by a diffusion-retention mechanism. One possibility is that 'mature' nuclear lamina structure is not re-established in cells until many hours later, during G1 (Broers et al., 1999; Moir et al., 2000). Alternatively, emerin might need to interact with the 'core' to remove a mitotic post-translational modification that inhibits stable localization at the nuclear envelope.

In vivo evidence for a third functional domain within emerin

By localizing truncated versions of emerin, we found that residues 176-223 are sufficient to localize the C-terminal

region of emerin at the nuclear envelope. Mutations in this region do not interfere with emerin binding to either lamin A or BAF in vitro (Lee et al., 2001). Because inner membrane localization is thought to depend on binding to a stable component within the nucleus, such as lamins (Östlund et al., 1999; Fairley et al., 1999; Vaughan et al., 2001), we propose that residues 176-223 interact with a novel binding partner. This possibility will be tested in future work.

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