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Introduction
It is a common and well-accepted hypothesis in cell biology that intermediate filaments (IF) proteins are present only in metazoan cells, whereas they are absent in unicellular eukaryotic organisms and plants. However, this consensus may need to be reconsidered, since our group very recently succeeded in the characterization of a novel nuclear protein, Dictyostelium NE81, as the first lamin-like protein in a lower eukaryote. Based on the current knowledge, we draw a model for nuclear envelope organization in Dictyostelium in this Extra View and we review the experimental data that justified this classification. Furthermore, we provide unpublished data underscoring the requirement of posttranslational CaaX-box processing for proper protein localization at the nuclear envelope. Sequence comparison of NE81 sequences from four Dictyostelia with bona fide lamins illustrates the evolutional relationship between these proteins. Under certain conditions these usually unicellular social amoebae congregate to form a multicellular body. We propose that the evolution of the lamin-like NE81 went along with the invention of multicellularity.

Lamins are the major components of the nuclear lamina and serve not only as a mechanical support, but are also involved in chromatin organization, epigenetic regulation, transcription and mitotic events. Despite these universal tasks, lamins have so far been found only in metazoans. Yet, recently we have identified Dictyostelium NE81 as the first lamin-like protein in a lower eukaryote. Based on the current knowledge, we draw a model for nuclear envelope organization in Dictyostelium in this Extra View and we review the experimental data that justified this classification. Furthermore, we provide unpublished data underscoring the requirement of posttranslational CaaX-box processing for proper protein localization at the nuclear envelope. Sequence comparison of NE81 sequences from four Dictyostelia with bona fide lamins illustrates the evolutional relationship between these proteins. Under certain conditions these usually unicellular social amoebae congregate to form a multicellular body. We propose that the evolution of the lamin-like NE81 went along with the invention of multicellularity.

A lamin in lower eukaryotes?

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phosphorylation consensus sequence directing transport to the rod domain and a CaaX-box at their C-terminus. The NLS is required for transport into the nucleus. As a prerequisite for nuclear envelope breakdown (NEBD), phosphorylation by CDK1 mediates disassembly of the lamina network at the onset of mitosis. Finally, the CaaX-box serves as a signal for post-translational lipidation and processing. Lamins are prenylated by farnesyl transferase at the cysteine residue of the CaaX-box ("C" stands for cysteine, "a" for aliphatic amino acid residues, "X" for the amino acid residue determining the type of prenyl moiety). Processing proceeds with cleavage of the three C-terminal amino acid residues (=aaX) either by Ras converting enzyme 1 (RCE1) or by ZMPSTE24 and carboxymethyltransferase of the farnesylated cysteine residue by isoprenylcytochrome methyl transferase (ICMT). While this is the last processing step in case of B-type lamins, prelamin A is cleaved once again by ZMPSTE24, removing the last 15 (in case of human lamin A) amino acid residues including the farnesyl anchor. Thus, B-type lamins stay associated with the nuclear envelope (NE) due to their lipid anchor, while A-type lamins require an interaction with inner NE proteins or with B-type lamins for their recruitment to the NE. The lamin-based nuclear lamina is intimately linked to the cytosolic cytoskeleton through LINC complexes (linker of the nucleoskeleton and cytoskeleton), which consist of Sun-family proteins in the inner nuclear membrane interacting with so-called KASH domain proteins in the outer nuclear membrane. The latter interact directly or indirectly with actin filaments, cytoplasmic intermediate filaments and microtubules. In vegetative cells, LINC complexes are also responsible for the tight linkage of the centrosome, i.e., the main microtubule-organizing center, to the nucleus. Through these interactions the nuclear lamina not only influences the mechanical properties of the nucleus, but also that of the whole cell and, thus, lamins are thought to protect cells against mechanical stress. Yet, with regard to the diverse interactions of the nuclear lamina with chromatin-associated proteins such as histones, transcriptional regulators and chromatin modifiers, it is obvious that they do not only serve as a mechanical support, but that lamins are also involved in chromatin organization, epigenetic regulation, transcription and mitotic events. These various important functions are also reflected by the symptoms of laminopathies. These devastating, inheritable diseases including muscular dystrophy, cardiomyopathy, partial lipodystrophy and progeria are caused by mutations of the lamin A gene, or of genes encoding lamin-binding or lamin-processing proteins. Bearing in mind these universal functions for all eukaryotic cells, it should be expected that non-metazoan cells need a functional equivalent of a nuclear lamina or even lamin-like proteins as well. Yet, none of the sequenced genomes of plants (e.g., Arabidopsis and Physcomitrella), fungi (e.g., Saccharomyces and Aspergillus) or protozoans (Plasmodium and Trypanosoma) disclosed a lamin or lamin-like protein; even though the first observations of a filamentous lamina were made half a century ago in two unicellular eukaryotes, Amoeba proteus and Gregarinia melenoplia. Indeed, the existence of lamins in Saccharomyces, Tetrahymena, Physarum and dinoflagellates has been postulated due to cross-reactivity of lamin-specific antibodies. However, in all of these cases the nuclear envelope-associated coiled-coil proteins characterized on the molecular level were evolutionary unrelated to lamins. This is why lamins or lamin-like proteins are generally considered to be absent in lower eukaryotes.

**NE81 Shares Many Structural and Functional Features of Lamins**

Already in the late seventies the existence of a nuclear lamina in Dicytostelium amoebae was proposed, since electron micrographs of Dicytostelium cells had revealed a thin, continuous, low electron-density layer between the inner nuclear membrane and the chromatin. Its appearance in ultrathin sections was very reminiscent of the previously characterized fibrous (nuclear) lamina of animal cells. However, as in other lower eukaryotes, the proteins involved in formation of a putative nuclear lamina in Dicytostelium were completely unknown. Recently, we succeeded in identification and characterization of a lamin-like protein in Dictyostelium d discoideum. It was found serendipitously in a proteomic screen for centrosomal and centrosome-associated proteins. We called it NE81 since it has a calculated molecular mass of 81 kDa and a corresponding GFP fusion protein is targeted to the nuclear envelope. The fact that it could be copurified with isolated centrosomes and protogena indicated a potential role in the centrosome/nucleus linkage, which will be discussed below. Sequence analysis of the 716 aa protein instantly revealed striking similarity to lamins with a central, α-helical coiled-coil rod domain flanked by a head and long tail domain. With a length of 370 aa the rod domain has a comparable length to that of lamins and it is also predicted to be interrupted by non-helical linkers. Moreover, the rod domain is directly preceded by a CDK1 phosphorylation consensus sequence and is followed by a basic nuclear localization sequence. The position of these signals with respect to the rod domain matches exactly those to lamins. Finally, the NE81 protein sequence ends with a CaaX-box (CLIM). The methionine at the X-position is characteristic for lamins, and indicates farnesylation instead of geranylgeranylation. Taken together, the sequence features already strongly suggested that NE81 could be a lamin-like protein, since the only known inner NE proteins with a CaaX-box are lamins and Drosophila kugelkern, another lamina protein, which is clearly distinguished from lamins by its much shorter α-helical coiled-coil region.

This idea was confirmed by all experimental data. NE81 was localized at the nuclear envelope during the entire cell cycle. Immuno-electron microscopy using anti-NE81 antibodies revealed an association of the protein with the inner nuclear envelope. As it holds true for lamins, proper localization of NE81 along the nuclear envelope requires the presence of a functional CaaX-box. A first indication for this requirement came from the analysis of a Dicytostelium str mutant knockout strain lacking the isoprenylcytochrome methytransferase IcmA. Although IcmA was still evenly distributed at the nuclear envelope in these cells, we also observed clusters of NE81 within the nucleus in about 50% of...
These cells (Fig. 1A). These clusters were never observed in control cells. This suggests that processing of NE81 including isoprenylcysteine methylation is required for proper localization of the protein at the nuclear envelope. If the CaaX-box is deleted completely, the respective GFP fusion proteins (GFP-NE81\(\Delta\)CLIM) are exclusively present in clusters within the nucleus that were reminiscent of those of endogenous NE81 in IcmA deficient cells. This behavior was also observed in a new strain where the CaaX-box cysteine was point mutated to serine (Fig. 1B). In EM images, these protein clusters appeared as sponge-like structures of low electron density. Both CaaX-box mutated GFP fusion proteins (GFP-NE81\(\Delta\)CLIM and GFP-NE81SLIM) showed the same cell cycle-dependent behavior, i.e., the GFP-fluorescent clusters in the nucleus disappeared at the G/M transition (Fig. 1B) and reappeared in telophase. This behavior and the low density of GFP-NE81\(\Delta\)CLIM clusters showed that GFP-NE81\(\Delta\)CLIM clusters were no aggregates of misfolded protein. Rather they were likely to represent three-dimensional assemblies that were formed and dissociated in a cell cycle-dependent fashion. Fluorescence recovery after photobleaching (FRAP) experiments disclosed a similar behavior of the full-length GFP-NE81 protein. It was almost immobile during interphase, which was in agreement with the idea that it forms a stable nuclear lamina composed of assembled NE81 chains. However, between early mitosis and cytokinesis, CDK1 behaves very similar to NE81 in FRAP experiments. Integrated into the nuclear lamina it shows little or no mobility.26,27 Yet at the G/M transition, as an early event in nuclear envelope breakdown (NEBD), the nuclear lamina disassembles, a process initiated by CDK1 phosphorylation of lamins.9 Like in many fungi and lower eukaryotes, there is no NEBD in Dictyostelium amoebae, however, the postulated nuclear lamina needs to become softer in order to allow constriction during cytokinesis. This could be achieved by partial disassembly of NE81 resulting in an increased mobility. The timing of disappearance and re-formation of GFP-NE81\(\Delta\)CLIM and GFP-NE81SLIM clusters as well as the increased mobility of full length GFP-NE81 fits exactly to the activity window of the master regulator of mitosis, CDK1. Indeed we could show that the serine residue within the putative CDK1 phosphorylation site at position 122 is required for this dynamic behavior. In the non-phosphorylatable S122A point mutant of GFP-NE81\(\Delta\)CLIM, the GFP fluorescent clusters in the nucleus persisted throughout mitosis. We concluded that, similarly to lamins, phosphorylation negatively regulates an inherent capacity of NE81 to form high order polymers. Endogenous NE81 becomes isoprenylated and anchored to the inner nuclear envelope favoring polymerization in two dimensions. However, CaaX-box mutations preclude prenylation and processing, and therefore prevent proper attachment to the inner nuclear membrane. This,
Moreover, we observed misshapen nuclei with irregularly condensed chromatin (32%), supernumerary centrosomes (7%) and missing centrosomes (5%). All of these are phenotypes virtually absent in control cells (Fig. 1E). Overexpression of GFP-NE81 also caused irregularly shaped nuclei, blebbing of the nuclear envelope and disorganized chromatin (Fig. 1D).

Thus, all phenotypes observed upon NE81 overexpression or knockout indicate a role of NE81 in functions addressed to lamins in metazoan cells such as maintenance of nuclear architecture, chromatin integrity and crosstalk of nuclear structures with the cytoskeleton.

Is NE81 a Lamin or a Lamin-Like Protein?

The many similarities to lamins raise the question whether NE81 should be considered a lamin-like protein or a real lamin. Due to the functional similarities regarding its role in nuclear integrity, centrosome/nuclear attachment and mechanical stability of cells as well as its cell cycle-dependent regulation, it is now clear that NE81 functions like a lamin in turn, could cause cluster formation by polymerization in three dimensions within the nuclear interior.

Overexpression of GFP-NE81ΔCLIM revealed a further function shared by NE81 and lamins. GFP-NE81ΔCLIM cells were unable to grow in shaking culture indicating an increased sensitivity to mechanical stress and were hypersensitive against mechanical shear forces. Increased mechanosensitivity of GFP-NE81ΔCLIM cells supports the idea that an NE81-based nuclear lamina could serve as a mechanical abutment for cysnucleus croskeletal elements just as in cells of higher eukaryotes, where the nuclear lamina is connected to the cytoskeleton through LINC complexes. Although no LINC complexes have been characterized in Dictyostelium yet, several components of the interaction of the nucleus with the cytoskeleton are known. These are Sun1, Kif9, LIS1, CP148, interapin and centrin B (cenB). While interapin is an actin-binding outer nuclear envelope protein linking the nucleus to the actin system, all others are related to the microtubule system. Although not binding to Sun1, interapin functionally interacts with Sun1, the classical LINC complex component. Bona fide KASH domain proteins of the outer nuclear membrane have not been identified so far in Dictyostelium. Instead, Sun1 resides in both nuclear membranes and is thought to link the centrosome and its microtubule arrays on the cytoplasmic side of the nucleus with the clustered centromeres inside the nucleus (2). At the centrosomal side, the centrosomal component CP148 appears to be involved in this linkage, while the binding partners at the centromere cluster are unknown. Interference with dynein function by point mutation of its regulator LIS1, or knock-out of the membrane-associated orphan kinesin Kif9, also disrupted the linkage of the centrosome to the nucleus under-scoring an important role of the microtubule system for this process (Fig. 2).

The unusual, nuclear envelope-associated centrin B may be involved in anchoring of the nucleus/centromeric link to chromatin and/or an NE81-based nuclear lamina. The requirement of NE81 for the centrosome/nucleus linkage became clear in NE81 knockout cells, in which the centrosome was detached from the nucleus in more than 50% of all cases (Fig. 1C). Moreover, we observed misshapen nuclei with irregularly condensed chromatin (32%), supernumerary centrosomes (7%) and missing centrosomes (5%). All of these are phenotypes virtually absent in control cells (Fig. 1E). Overexpression of GFP-NE81 also caused irregularly shaped nuclei, blebbing of the nuclear envelope and disorganized chromatin (Fig. 1D).

Figure 2. Hypothetical model of nuclear envelope organization in the pericentrosomal region in Dictyostelium. In both nuclear membranes (black line) Sun1 is involved in linkage of the centrosome to an NE81-based nuclear lamina. On the centrosomal side, the centrosomal corona protein CP148 is directly or indirectly associated with Sun1, on the nuclear side Sun1 is directly or indirectly bound to NE81. Centromere proteins are also associated with Sun1 and mediate clustering of centromeres in the pericentrosomal region. The tight linkage between the cytosolic centrosome and the centromere cluster and nuclear lamina could be mediated by a self-interaction between SUN-domains or by an unknown protein within the perinuclear space. At the outer nuclear membrane both the kinesin Kif9 and Sun1-associated dynactin/dynamin link to microtubules and help to hold the centrosome close to the nucleus.
The many similarities shared between NE81 and lamins raise the question why NE81 was not detected earlier in searches for lamins in the sequenced genomes of social amoebae (Dictyostelium). One reason is that sequence similarities between NE81 and lamins are low and, thus, BLAST searches with lamins as a query yield numerous non-related coiled coil proteins. It appears that formation of a nuclear lamina enforces mainly a structural conservation of its main constituent, while sequence conservation is restricted to a few consensus sequences such as the CaaX-box, the NLS and the CDK1 site.

While sequence conservation is restricted to a few consensus sequences such as the CaaX-box, the NLS and the CDK1 site, structural conservation of its main constituent, nuclear lamina enforces mainly a structural compensation of knockdown of one of the lamins by RNAi. Whether it may be allowed to call NE81 a lamin will depend on the outcome of further analyses of its structure and supramolecular assembly.

NE81 in Other Lower Eukaryotes

The many similarities shared between NE81 and lamins raise the question why NE81 was not detected earlier in searches for lamins in the sequenced genomes of social amoebae (Dictyostelium). One reason is that sequence similarities between NE81 and lamins are low and, thus, BLAST searches with lamins as a query yield numerous non-related coiled coil proteins. It appears that formation of a nuclear lamina enforces mainly a structural conservation of its main constituent, while sequence conservation is restricted to a few consensus sequences such as the CaaX-box, the NLS and the CDK1 site. Even among Dictyostelium NE81 sequences vary strongly. The most closely related orthologue in Dictyostelium purpureum exhibits an amino acid identity of only 58% (74% similarity) to D. discoideum NE81. Compared with Dictyostelium fasciculatum and Polyphemus polyphemus NE81 sequence conservation is even lower with amino acid identities of 37% (56% similarity) and 34% (54% similarity), respectively (Fig. 3). These values reflect the phylogenetic distance between the two latter species, which belong to two different groups within the Dictyostelia than D. discoideum and D. purpureum.

We found no NE81-like protein in sequenced prototaxa including Entamoeba, Plasmodium, Trypanosoma and Paramecium. From an evolutionary point of view Dictyostelia are positioned between protists lacking lamins, and metazoa with a lamin-based nuclear lamina. We believe that the evolution of the lamin-like NE81 went along with a typical attribute of all Dictyostelia, i.e., the capability to form multimeric bodies from single cells under certain conditions. Future research may disclose whether the invention of a nuclear lamina based on a lamin-like protein may even have been a prerequisite to reach the multicellular state.

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Figure 3. For figure legend, see page 243.
Figure 3 (See previous page). Protein sequence alignment of two bona fide lamins with NE81 orthologs from four Dictyostelidae. Coding sequences from the social amoeba D. discoideum (starting with as position 99), D. purpureum (starting with as position 84), D. fasciculatum (starting with as position 110) and Polyphondylum pallidum (starting with as position 119) and Amphimedon queenslandica were derived from the respective genome projects (dictybase.org, saug.hi-bioba.de, www.metazome.net/amphimedon). In case of D. pallidum the predicted second intron had to be neglected since otherwise the deduced amino acid sequence was devoid of a Caax-box. Sequences were aligned using Multalin software. High consensus of amino acid similarity is colored in red, low consensus in blue. Blue dotted lines refer to the human lamin B1 sequences. Sequences were aligned using Multalin software; red consensus line is colored in red, blue consensus line in blue.