

ABSTRACT Structural protein 4.1, which has crucial interactions within the spectin-actin lattice of the human red cell membrane skeleton, also is widely distributed at diverse intracellular sites in nucleated cells. We previously showed that 4.1 is essential for assembly of functional nuclei *in vitro* and that the capacity of 4.1 to bind actin is required. Here we report that 4.1 and actin colocalize in mammalian cell nuclei using fluorescence microscopy and, by higher resolution cell whole mount electron microscopy, are associated on nuclear filaments. We also devised a cell-free assay using *Xenopus* egg extract containing fluorescent actin to follow actin during nuclear assembly. By directly imaging actin under non-perturbing conditions, the total nuclear actin population is retained and is visualized *in situ* relative to intact chromatin. We detected actin initially when chromatin and nuclear pores began assembling. As the nuclear lamina assembled, but preceding DNA synthesis, a discrete actin network formed throughout the nucleus. Protein 4.1 epitopes also were detected when actin began to accumulate in nuclei, producing a diffuse coincident pattern. As nuclei matured, actin was detected both coincident with and also independent of 4.1 epitopes. To test whether acquisition of nuclear actin is required for nuclear assembly, the actin inhibitor latrunculin A was added to *Xenopus* egg extracts during nuclear assembly. Latrunculin A strongly perturbed nuclear assembly and produced distorted nuclear structures containing neither actin nor protein 4.1. Our results suggest that actin as well as 4.1 is necessary for nuclear assembly and that 4.1-actin interactions may be critical.

We are investigating the functions of the protein 4.1 family in nuclei, centrosomes and mitotic spindles in eukaryotic cells (1-3). Protein 4.1 is a superfamily of multifunctional structural proteins widely expressed in nucleated cells (4) whose prototypical member has well-defined interactions with spectrin, actin and integral membrane proteins in the human red cell membrane skeleton (reviewed by (5, 6)). We recently showed that protein 4.1 is essential for proper assembly of functional nuclei *in vitro* in *Xenopus* egg extracts and identified the spectrin-actin binding domain (SABD) of protein 4.1 as one of the 4.1 domains critical for this process. Furthermore, using a mutant 4.1 SABD unable to bind actin, we demonstrated 4.1-actin binding capacity is necessary for nuclear reconstitution (3). These observations prompted us to further investigate the roles of actin in nuclear assembly.

Actin is widely recognized as a major cytoskeletal component involved in dynamic processes such as cell motility and shape changes, cytoplasmic vesicle transport, and muscle contraction. Actin also has been reported for several decades to be intranuclear, in particular to be associated with the nuclear matrix (7-9), although many of these reports initially met with skepticism. Objections included possible cytoplasmic contamination, extraction and fixation artefacts, and the inability to detect nuclear actin using fluorescently labeled phalloidin. However, recent experimental data confirm and extend the early reports to convincingly provide a body of evidence documenting actin in the nucleus. These newer observations include characterization of actin and ARPS in chromatin remodeling and histone acetyl transferase complexes (reviewed by (10)), and identification of two functional leucine-rich nuclear export signals in actin (11). In addition to protein 4.1, other actin-binding proteins in nuclei have been identified (reviewed by (12) including a nuclear-specific myosin (13), a nuclear-specific spectrin (14) as well as cofilin (15).

We report here that 4.1 and actin colocalize in nuclei of cultured mammalian cells by fluorescence microscopy of fixed and live cells. Higher resolution electron microscopy of cell whole mounts showed actin and 4.1 epitopes closely associated on nuclear filaments. However, we also imaged nuclear actin directly during nuclear assembly *in vitro* using *Xenopus* egg extracts and fluorescently labeled actin. Under non-perturbing conditions, we initially detected actin when chromatin and nuclear pores began to assemble. A discrete nuclear actin network formed as nuclear lamina assembled, prior to DNA synthesis. We also report that actin acquisition is necessary for proper nuclear assembly and inhibition of either actin or 4.1 excludes both actin and protein 4.1 from being incorporated into nuclei, blocking nuclear assembly.

METHODS

Materials WI38 cells (CCL 75) were obtained from the American Type Culture Collection (Rockville, MD). BrdUrd was from Sigma, EYFP and ECFP vectors were from Clontech and Lipofectamine was from Invitrogen. Rhodamine-labeled actin was the kind gift of Dr. M. Welch (University of California, Berkeley, CA). Latrunculin A was generously provided by Dr. D. Drubin (University of California, Berkeley). IgGs against 4.1R SABD and 4.1R CTD have been described (1). Antibodies against BrdUrd were from BD Biosciences, MAb 414 against nuclear pore complex proteins from Babco, Mab C4 against actin was from ICN, secondary fluorescent antibodies from Molecular Probes and gold-bead-conjugated secondary antibodies were from Amersham.

***Xenopus* extracts and nuclear assembly reactions.** 10,000xg cytoplasmic *Xenopus* egg extracts were prepared as described (16) and made interphasic with 400mM Ca²⁺. For nuclear assembly, demembranated *Xenopus* sperm were added to 20μl egg extract on ice with rhodamine labeled actin and incubated at 20°C. Assembly reactions were observed directly after placing an aliquot of a reaction and Fix (16) on a slide and squashing with a coverslip. For immunostaining, reactions diluted with BRB80 (80mM PIPES, 2mM MgCl₂, 1mM EGTA, pH 6.8) containing 15% glycerol and 1% Triton X-100 were spun through 25% glycerol-BRB80 cushions onto coverslips, fixed in -20°C methanol, and probed with antibodies as described previously (3). Images were captured using a Nikon Eclipse E600 microscope equipped with a CCD camera and processed using Adobe Photoshop.

Western blotting of nuclei and BrdUrd labeling of nuclei assembled *in vitro* were done as described previously (3).

Immunofluorescent microscopy of human diploid fibroblasts. Growth of WI38 cells, preparation of nuclear matrix, immunofluorescent staining and transient transfections were performed as previously described (1). Controls using equivalent amounts of nonimmune IgG, no primary antibody, or vector alone were included in each experiment as appropriate.

Whole mount embedment-free immunoelectron microscopy. High resolution whole mount embedment-free EM of immunostained WI38 diploid human fibroblasts was performed as described (17). Briefly, cells grown on parlodion-carbon-coated sterile nickel grids were extracted, fixed with paraformaldehyde/glutaraldehyde, blocked with goat serum, incubated with primary antibody and immunostained with gold-bead-conjugated goat anti-rabbit or anti-mouse secondary antibodies. Samples were fixed again with glutaraldehyde, washed in 0.1M cacodylate, (pH 7.4), dehydrated in ethanol, and critical point dried. Sample grids thinly coated with carbon were viewed using a JEOL 1200EX electron microscope with a total tilt angle of 10° for stereo.

RESULTS

Localization of Protein 4.1 and Actin in Nuclei. Actin can be detected in nuclei of mammalian cells by immunofluorescence microscopy after cells are first extracted with detergent and high salt, and DNA removed by enzymatic digestion to reveal the nuclear matrix or scaffold. Previously actin and protein 4.1 isoforms have independently been detected in nuclear matrix by immunofluorescence microscopy and Western blot analysis in a variety of cell types (1, 4, 8, 9, 18, 19). Therefore to localize 4.1 relative to nuclear actin, we extracted cultured mammalian cells and probed nuclear matrix by double label immunofluorescence (Fig 1A). Anti-actin produced a punctate pattern along with a more diffuse staining in the nucleus. The punctate immunofluorescent labeling by protein 4.1 antibody was largely coincident with focal anti-actin immunofluorescent signals at this level of resolution.

In a second independent approach, actin and 4.1 proteins with fluorescent tags were expressed in live cells. Vectors with CFP fused to actin or YFP fused to an 80kD isoform of protein 4.1R containing an SABD were transfected into WI38 diploid human fibroblasts and live cells imaged at various times post-transfection. Whereas actin was detected on cytoplasmic filaments as well as in the nucleus at 24 hours, protein 4.1 initially was expressed only in the nucleus, colocalizing with the tagged nuclear actin (Figure 1B). At later times, nuclear 4.1 remained coincident with actin but was also detected in the cytoplasm. Transfected cells remained alive but ceased to divide even after 72 hours in culture.

In order to obtain more detailed information about an association between 4.1 and actin epitopes at higher resolution, we used double label immunoelectron microscopy. To analyze nuclear distribution of actin relative to 4.1 epitopes, we used unembedded cell whole mount electron microscopy to visualize continuous fibrous structures not appreciated in sectioned specimens. In stereo pair images of whole mount fibroblast nuclear matrix immunolabeled with different sized gold beads to detect antibodies against 4.1 SABD and actin, clusters of beads predominately decorate thick fibers in areas near dense structures (Fig 2). The 10-nm beads (actin epitopes) and the 5-nm beads (4.1 SABD epitopes) sometimes appear to be in subdomains of ~100nm or less apart on continuous fibers (Fig 2A,B) or sometimes intermingled (Fig 2C, arrow, is one example). Thus the higher resolution of EM affords detailed localization of nuclear actin and 4.1R SABD epitopes in closely associated microdomains within nuclear matrix.

Direct Imaging of Nuclear Actin. To circumvent potential issues of fixation or overexpression artefacts, an assay was devised to image nuclear actin without using perturbing conditions. For this assay, actin covalently labeled with rhodamine was added to *Xenopus* egg extracts and nuclear assembly was initiated by addition of demembranated *Xenopus* sperm. Nuclear actin and DNA were imaged directly in squashes of reaction aliquots by fluorescence microscopy. During the first 30 minutes, when sperm DNA remained highly condensed, actin was not detected (Fig 3). However, as DNA became decondensed and chromatin assembled, actin was detected in pronuclei (Fig 3, 30 minutes). Although initially diffuse, as nuclear assembly progressed, actin accumulated and formed an actin network throughout mature nuclei (Fig 3; 60, 70 minutes). In conclusion, using a novel non-perturbing assay, acquisition of actin was seen to occur in concert with chromatin formation during nuclear assembly and appeared as a lattice array within mature nuclei.

Acquisition of Nuclear Actin Relative to Nuclear Pores, Lamin, and DNA Synthesis. To determine in more detail when actin is incorporated into nuclei relative to other normal assembly markers, at various time points we sedimented nuclei from reactions containing rhodamine-labeled actin onto coverslips and immunostained them in parallel for nuclear pores, the underlying lamin network, or incorporation of BrdUrd into DNA. The internal rhodamine-labeled actin pattern was preserved during fixation so that secondary detection of actin was not required. Initially when DNA was relatively highly condensed there was no labeling of actin, pores, lamin or of incorporated BrdUrd (data not shown). At 30 minutes, diffuse actin immunofluorescence was detected (as previously, Fig 3) and irregularly distributed nuclear pore antigens were detected in association with chromatin (Fig 4A). Actin continued to accumulate as the pores became more regularly arrayed around chromatin. Lamin epitopes were detected at a slightly later stage than actin and pores, and formed a regular rim surrounding the chromatin as a distinct intranuclear actin pattern became visible (Fig. 4B). Finally, after pores and a lamina were assembled, BrdUrd incorporation was detected (Fig 4C). Thus, nuclear actin is acquired when chromatin and nuclear pores begin to assemble and precedes lamina formation and DNA synthesis.

Acquisition of Nuclear Actin Relative to Protein 4.1. Since the actin binding capacity of protein 4.1 is crucial for assembly of functional nuclei (1), we investigated the incorporation of protein 4.1 into nascent nuclei relative to actin by immunostaining 4.1 SABD epitopes in nuclei isolated from *in vitro* reactions supplemented with fluorescent actin. SABD signals were first detected in assembling nuclei when diffuse actin signals were also detected (Fig 5). Initially the two signals appeared coincident (yellow coloration) but as nuclei matured, actin was detected both coincident with and also independent of 4.1 epitopes. Coincident areas had strong focal

staining and some diffuse staining. The larger toroidal structures (Fig 5, 60 minutes) where 4.1 and actin are coincident were previously shown to stain with antibodies against splicing factors (3).

Nuclear Actin is Essential for Nuclear Assembly. To test whether acquisition of nuclear actin is required for nuclear assembly, the actin inhibitor latrunculin A was added to *Xenopus* egg extract. Latrunculin A binds to G-actin at a site adjacent to the nucleotide-binding cleft (20-22), sequestering available G-actin monomers and preventing incorporation of actin into filaments. Latrunculin A at concentrations from 1mM to 0.05mM, completely prevented the proper assembly of nuclei *in vitro* but had little effect below 10 μ M (data not shown). The concentration of actin in *Xenopus* egg extracts has been estimated to be 15-25 μ M (23). The morphologically disrupted nuclei formed in extracts containing latrunculin A were small and distorted, with areas of condensed DNA (Fig 6A, DAPI). Nuclear actin was not detected by fluorescence microscopy in the latrunculin A-perturbed nuclear structures formed in assembly reactions containing rhodamine-labeled actin (Fig 6A, actin). Furthermore, the defective structures had only traces of irregularly distributed pores and lamin and were incapable of DNA synthesis (data not shown).

When normal nuclei assembled *in vitro* were analyzed by Western blotting, actin produced a strong signal in controls but was not detected in an equal number of perturbed nuclei isolated from reactions containing latrunculin A (Fig 6B, left panel) consistent with fluorescence data. Previously we showed that a peptide encoding the 4.1 SABD acts as a dominant negative when added to *Xenopus* egg extract nuclear assembly reactions, producing perturbed nuclei with no detectable 4.1 epitopes (3). When nuclei from 4.1 SABD peptide dominant negative reactions were Western blotted, actin also was not detected (Fig 6B, left panel). Furthermore, protein 4.1 epitopes could not be detected by Western blotting of nuclear structures from reactions inhibited by latrunculin A (Fig 6B, right panel). Thus, actin acquisition is necessary for proper nuclear assembly and inhibition of either actin or 4.1 prevents both actin and protein 4.1 from being incorporated into nuclei.

DISCUSSION

Compelling evidence that actin is a *bona fide* resident nuclear protein has steadily accrued and is now widely accepted. To provide further evidence for potentially important interactions of protein 4.1 and actin in nuclei, we initially investigated the relative localization of 4.1 and actin in nuclei. We report here, using independent approaches, that 4.1 is associated with actin in nuclei at interphase. At the level of resolution afforded by cell whole mount EM, actin and 4.1 epitopes localized on continuous nuclear filaments or were even intermingled. We also devised an assay using *Xenopus* egg extract supplemented with fluorescent actin to directly image nuclear actin under non-perturbing conditions so that the total population of nuclear actin is retained and is visualized *in situ* relative to intact chromatin. As best seen in the overlay of actin, DAPI, and BrdUrd signals, actin appeared in mature nuclei as a filamentous system in interchromatin regions.

Recent evidence suggests that nuclear actin may be predominately monomeric G-actin, short oligomeric or branched actin polymers (24, 25); reviewed by (26). Our EM and *in situ* fluorescent actin data both show actin in a filamentous pattern but do not address whether nuclear actin is F-actin or whether its filamentous pattern reflects association with a filamentous nuclear structure. While in *Drosophila*, the EAST protein controls an expandable nuclear endoskeleton that promotes accumulation of actin in extrachromosomal regions (27), filament

proteins in vertebrate nuclear matrix are not well characterized. When these are identified, we may better understand their relationship to nuclear actin.

Cell-free *Xenopus* egg extracts are a powerful *in vitro* experimental system that recapitulates *in vivo* events of nuclear assembly and DNA replication. Nuclear reconstitution using *Xenopus* egg extracts and demembranated sperm has been characterized as proceeding through discrete intermediate steps (28, 29): DNA forms nucleosomes and scaffold proteins bind and organize chromatin; vesicles and pores bind to chromatin; lamins polymerize to form a lamina; membrane vesicles fuse to form a double membrane envelop; chromatin decondenses and DNA replication centers form as nuclei further mature.

We first detected diffuse actin during initial stages of chromatin assembly in egg extracts. Actin continued to accumulate as nuclei assembled and became organized into a discrete network during lamina and pore formation, prior to DNA synthesis. Therefore, acquisition of nuclear actin appears to be an early event. This observation is supported by our finding that latrunculin A inhibition of nuclear assembly resulted in small irregular nuclear structures often with highly condensed DNA. These morphologically abnormal structures had only traces of detectable pores and lamin and were defective in synthesizing DNA suggesting that nuclear actin may even be a prerequisite for organization of functional pores, lamina and DNA replication centers. In a previous preliminary report also using *Xenopus* egg extracts, the actin polymerization inhibitor gelsolin S1 inhibited nuclear formation and DNA replication. (Zhang et al, 1996; Mol. Biol. Cell 7, 477a, abstract). The latrunculin A nuclear phenotype we observe is quite similar to that resulting from 4.1 depletion or by dominant negative 4.1 peptides (3) and distinct from the effects of lamin depletion (30) or inhibition of nuclear pores (31) again suggesting that inhibition of actin acquisition interferes with nuclear assembly at a very early stage.

The function of actin in the nucleus is an area under intense investigation. Actin has been shown to be integral to a number of chromatin modifying complexes having ATPase, helicase and acetylase activities (10). Actin has also been reported to complex with members of the A/B-group hnRNP proteins (32). A nuclear complex has been identified containing RNA polymerase II, a U5 spliceosome snRNP, NDH II and several (33). Multiple lines of evidence implicate actin in mRNA processing and export: rapidly labeled RNAs associate with actin in nuclear matrix (34), cytochalasin B causes selective release of precursor mRNA (35), actin localizes adjacent to snRNP aggregates (36), and actin associates with intron-containing HIV-1 gag mRNA (37) and is essential for rev-mediated mRNA export (38).

What roles might 4.1 play in the nucleus in conjunction with actin? Our data show that actin and 4.1 both become diffusely associated with nascent chromatin in the early phases of nuclear assembly. Actin contains two nuclear export signals but no nuclear localization signal so it is unclear how actin becomes nuclear. Protein 4.1 contains an actin binding site distinct from its nuclear localization signal (39, 40). It is possible that one means for actin to become nuclear is via 4.1 binding, a hypothesis to be tested in the future. At later stages of nuclear assembly, 4.1 remains extensively colocalized with actin in foci although actin also forms an extensive intranuclear network. Some areas of 4.1-actin colocalization may be mRNA splicing centers since 4.1 was shown to colocalize with splicing centers in mammalian cells (1, 41) and in *Xenopus* nuclei assembled *in vitro* (3) 4.1 coimmunoprecipitates with splicing factors, and depletion of 4.1 inhibits RNA splicing *in vitro* (42). Other colocalization signals may detect complexes of 4.1, actin, and actin binding proteins. For example, we have preliminary evidence that 4.1 can bind the BAF chromatin remodeling complex (Krauss, unpublished observations).

Additionally, we have found that incubation of mature nuclei with latrunculin A causes nuclear structures to become distorted and chromatin to collapse (Krauss, unpublished observations). This suggests that nuclear actin is essential for the maintenance of interphasic nuclear structure as well as for nuclear assembly. It is also interesting that mammalian cells cease dividing when actin and 4.1 are overexpressed by co-transfection or overexpressed individually (Krauss, unpublished observations). Perhaps relative levels of nuclear 4.1 and actin are highly regulated during normal cell cycling.

Previously we showed an intact actin binding site in protein 4.1 is essential for nuclear assembly and here we report that sequestration of actin monomers with latrunculin A both prevented 4.1 incorporation into nuclei and inhibited nuclear assembly. Currently we are testing whether latrunculin A directly prevents actin-4.1SABD binding. Protein 4.1 interactions with actin in the nucleus may recruit, target and/or modulate functions of various actin-containing complexes, a role reminiscent of its well-established adaptor function in the erythrocyte membrane skeleton.

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Abbreviations: SABD, spectrin-actin binding domain; EM, electron microscopy

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Figure Legends

Fig. 1. Colocalization of actin and 4.1 in human diploid fibroblasts (A) Double label immunofluorescence of 4.1 SABD epitopes (GREEN) and actin (RED) in WI38 cells. Cells were

extracted *in situ* to prepare nuclear matrix, fixed and probed with antibodies. The merged image shows a high degree of coincidence (YELLOW) at this level of resolution. (B) Transient co-transfection of WI38 cells with actin tagged with YFP (RED) and 4.1R 80kD tagged with CFP (GREEN). Cells were imaged 4-72 hrs post-transfection. The image presented at 24 hours shows expression of YFP-actin in both the cytoplasm and nucleus while expression of CFP-4.1 is exclusively nuclear generating coincident nuclear signals (YELLOW). (Bar= 10µm)

Fig. 2. Immunolocalization of actin and 4.1 SABD epitopes by double label immunoelectron microscopy of human fibroblasts. In cell whole mounts of WI38 cells, actin epitopes (10nm beads) and 4.1 SABD epitopes (5nm beads) decorated fibrous structures near dense structures in nuclear matrix. At the top is a stereo pair image of a nuclear matrix region with boxes (A, B, C) indicating areas presented as stereo pairs at higher magnification below. Arrow (C) points to intermingled 5 and 10nm beads.

Fig. 3. Acquisition of actin during nuclear assembly *in vitro*. Actin was imaged by fluorescent microscopy of samples from a *Xenopus* egg extract nuclear assembly reaction containing rhodamine labeled actin (RED). Times after initiation of nuclear assembly by addition of demembrated sperm (DAPI, BLUE) are indicated for a representative experiment (n=4). Diffuse actin was detected only after the first 30 minutes of incubation. As mature nuclei assembled (50-70 minutes), an intranuclear actin network became apparent.

Fig. 4. Acquisition of actin relative to nuclear pores, lamina formation and DNA synthesis during nuclear assembly *in vitro*. Nuclei isolated from *Xenopus* egg extract reactions spiked with rhodamine labeled actin (RED) were fixed and probed with monoclonal antibody 414 against nuclear pore proteins (A, GREEN) or antibody L₀46F7 against lamin (B, GREEN). DNA synthesis was detected by probing nuclei assembled in extract containing BrdUrd as well as rhodamine labeled actin using antibody against BrdUrd (C, GREEN) as described previously (3). Control samples with BrdUrd omitted had no detectable green signals. Note that in the merged images of mature nuclei, BrdUrd signals are coincident with DNA as expected (AQUA) and actin appears noncoincident. Times after initiation of nuclear assembly by addition of sperm (DAPI, BLUE) to parallel reactions are indicated below. Diffuse actin and irregularly distributed pores are detected at 30 minutes, lamin epitopes at 40 minutes and BrdUrd at 50 minutes in this experiment. Times of nuclear assembly vary with extract but the relative sequence of actin acquisition, pore assembly, lamina formation and DNA synthesis was maintained in three independent experiments.

Fig. 5. Acquisition of actin relative to 4.1 SABD epitopes during nuclear assembly *in vitro*. Nuclei isolated from *Xenopus* egg extract reactions spiked with rhodamine labeled actin (RED) were fixed and probed with antibody against the 4.1 SABD (GREEN). The images presented are from reactions using the same extract as in Fig. 4 although the experiment was performed three times with independent extracts. Times after initiation of nuclear assembly by addition of sperm (DAPI, BLUE) are indicated beneath the images. Diffuse actin and SABD epitopes detected at 30 minutes in the merged images of actin and SABD signals were largely coincident (YELLOW). At later times coincidence of actin with 4.1 foci was apparent along with some areas of non-coincidence.

Fig. 6. Latrunculin A inhibition of nuclear assembly *in vitro*. Nuclei were assembled in *Xenopus* egg extracts containing rhodamine labeled actin (RED) and latrunculin A as indicated. There was no effect of adding an equal amount of DMSO (latrunculin vehicle) on nuclear assembly. (A) The products from assembly reactions with latrunculin A had highly aberrant chromatin morphology (DNA, BLUE) and actin was not detected by fluorescence microscopy of the

perturbed nuclear structures. At 0.1mM latrunculin A, 94% of the structures were abnormal as judged by DAPI staining. Control nuclei are shown at the left. (B) By Western blot analysis of proteins from equal amounts of nuclei assembled in reactions with latrunculin A or dominant negative 4.1 SABD peptides, actin was not detected whereas it was readily detected in control nuclei (left panel). Western blotting of perturbed nuclei from assembly reactions with latrunculin A also had no detectable 4.1 epitopes when probed using antibodies against the spectrin actin binding domain α SABD) or the C terminus (α CTD) of 4.1 (right panel).