Two Distinct Domains of Protein 4.1 Critical for Assembly of Functional Nuclei \textit{in Vitro}*

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Sharon Wald Krauss‡§, Rebecca Heald¶, Gloria Lee‡, Wataru Nunomura†, J. Aura Gimmi‡, Narla Mohandas‡, and Joel Anne Chasis‡

From the ‡Department of Subcellular Structure, Life Sciences Division, University of California, Lawrence Berkeley National Laboratory, Berkeley, California 94720, the ¶Department of Molecular and Cell Biology, Division of Cell and Developmental Biology, University of California, Berkeley, California 94720, and the †Department of Biochemistry, School of Medicine, Tokyo Women’s Medical University, Shinjuku, Tokyo 162-8666, Japan

Protein 4.1R, a multifunctional structural protein, acts as an adaptor in mature red cell membrane skeletons linking spectrin-actin complexes to plasma membrane-associated proteins. In nucleated cells protein 4.1 is not associated exclusively with plasma membrane but is also detected at several important subcellular locations crucial for cell division. To identify 4.1 domains having critical functions in nuclear assembly, 4.1 domain peptides were added to \textit{Xenopus} egg extract nuclear reconstitution reactions. Morphologically disorganized, replication deficient nuclei assembled when spectrin-actin-binding domain or NuMA-binding C-terminal domain peptides were present. However, control variant spectrin-actin-binding domain peptides incapable of binding actin or mutant C-terminal domain peptides with reduced NuMA binding had no deleterious effects on nuclear reconstitution. To test whether 4.1 is required for proper nuclear assembly, 4.1 isoforms were depleted with spectrin-actin binding or C-terminal domain-specific antibodies. Nuclei assembled in the depleted extracts were deranged. However, nuclear assembly could be rescued by the addition of recombinant 4.1R. Our data establish that protein 4.1 is essential for nuclear assembly and identify two distinct 4.1 domains, initially characterized in cytoskeletal interactions, that have crucial and versatile functions in nuclear assembly.

Protein 4.1R was classically defined as an ~80-kDa cytoskeletal protein of mature human red cells crucial for maintaining erythrocyte shape and mechanical stability (for reviews see Refs. 1 and 2). In the red cell membrane skeleton, protein 4.1R stabilizes junctional interactions in the spectrin-actin lattice. It also binds to cytoplasmic domains of several transmembrane proteins such as glycophorin C and Band 3. Thus 4.1R provides linkage between the red cell cytoskeletal network and the overlying plasma membrane. Defects in protein 4.1 are associated with hereditary red cell elliptocytosis characterized by membrane fragmentation (as reviewed in Ref. 3).

In nucleated cells, however, protein 4.1 is not exclusively associated with the plasma membrane-associated cytoskeleton. Protein 4.1 epitopes are detected at several important subcellular locations crucial to cell division (4–8). In particular, protein 4.1 is at intranuclear sites in the nuclear matrix/scaffold (6, 7), at centrosomes (8), at mitotic spindle poles, in perichromosomal regions (7, 9), and at the midbody at telophase (7). The complex localization patterns of 4.1 in nucleated cells may reflect that although red cells contain predominantly one 80-kDa isoform, nucleated cells generally express multiple 4.1 isoforms generated via alternative splicing, posttranslational modifications, and expression of multiple related genes (10–14).

Analysis of 4.1R has revealed several functional domains important for its interactions in red cells, but the potential roles of these domains in nucleated cells have not been completely determined. A 4.1 domain that specifically interacts to form ternary complexes with spectrin and actin was mapped to exons 16,17 (spectrin-actin-binding domain, SABD)\textsuperscript{1} (15–21) (see Fig. 1A). The 4.1 30-kDa/FERM (4.1-ezrin-radixin-moesin) domain (beginning within exon 4 and ending within exon 12; see Fig. 1A) was shown to interact with membrane proteins such as glycoporphin C, anion exchanger Band 3, a membrane-associated guanylate kinase (MAGUK/p55), a cell surface receptor promoting tumor growth (CD44), ICAM-2, a chloride channel regulator (pICln), and calmodulin (1, 22–25). In nucleated cells, both the FERM domain and the SABD interact with importin α for nuclear import of protein 4.1 (26). Additionally, exons 20,21 in the 4.1 C-terminal domain (CTD; see Fig. 1A) recently were shown to interact with the nuclear mitotic apparatus protein NuMA (27), ZO2 (28) and immunophilin-binding protein FKBP13 (29). Thus in nucleated cells these 4.1 functional domains may serve as important linkers to actin structures, membrane-associated proteins, or microtubule-based structures.

Here we report that 4.1 is essential for proper assembly of nuclei, involving interactions of two distinct 4.1 domains. We have used \textit{Xenopus} egg extracts, a very powerful \textit{in vitro} experimental system that mimics \textit{in vivo} events such as assembly of nuclei and spindles, semiconservative DNA replication, and cell cycle regulation (30–35). When incubated with interphase \textit{Xenopus} egg extract, demembranated sperm DNA decondenses and recruits chromosomal and scaffold proteins to form mature nuclei containing a double membrane with pores, nuclear lamina, and a perinuclear centro-

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§ To whom correspondence should be addressed: Univ. of California, Lawrence Berkeley National Lab., 1 Cyclotron Rd., MS 74-157, Berkeley, CA 94720. Tel.: 510-486-4073; Fax: 510-486-6746; E-mail: sakrauss@lbl.gov.

† The abbreviations used are: SABD, spectrin-actin-binding domain; CTD, C-terminal domain; PIPES, 1,4-piperazinediethanesulfonic acid; DAPI, 4,6-diamino-2-phenylindole.
some derived from the sperm basal body. The reconstituted nuclei replicate DNA and import proteins bearing appropriate nuclear localization signals. Using this system we established that 4.1 is essential for nuclear assembly. Depletion of 4.1 from extracts prevents nuclear assembly, which is restored by addition of recombinant 4.1R. We identified two 4.1R domains critical to nuclear assembly in assays utilizing dominant negative peptides corresponding to protein 4.1 domains. This strategy has several advantages because peptides can be added in controlled concentrations to reactions, may mediate effects by competitive binding to 4.1 substrates, and may circumvent issues of steric hindrance in antibody inhibition experiments by antibodies bound to endogenous 4.1 proteins. One 4.1 domain, the SABD, appears to require interaction with actin to have a critical role in nuclear assembly. The other domain critical for nuclear assembly, the CTD, was shown previously to interact with protein 4.1 proteins and 15% glycerol and 1% Triton X-100 were spun through 25% glycerol-BrBB80 cushions onto coverslips. For Western blots, extract first was cleared of nonspecific aggregates by centrifugation at 1500 × g prior to incubation with sperm. After nuclear assembly, the reactions were diluted, and the nuclei were pelleted through cushions by centrifugation at 1500 × g in Eppendorf tubes. The nuclear pellets were rinsed with BrBB80 and dissolved in SDS-PAGE loading buffer. Aliquots of resuspended pellets fixed on coverslips produced only intranuclear immunofluorescence when probed with 4.1 antibodies. Although a range of 1–8 μg was tested for each peptide, the data presented are from experiments using 8 μg of the indicated peptide.

**Indirect Immunofluorescence—**In vitro assembled nuclei on coverslips were fixed in −20 °C methanol and probed by immunofluorescence as described (38). The concentrations of primary antibodies were: SABD IgG, 5 μg/ml; CTD IgG, 10 μg/ml; monoclonal antibody 414, 1:2500 dilution; L46F7, 1/10 dilution; and Y12, 1 μg/ml. Secondary fluorescent antibodies were used at a 1:100 dilution. The samples probed without primary antibody or with equal amounts of control nonimmune IgG or sera showed no fluorescent patterns. Images were captured using a Nikon Eclipse E600 microscope equipped with a CCD camera and processed using Adobe Photoshop.

**Expression and Purification of Histagged Proteins—**Protein 4.1-related peptides were expressed as His6 fusion proteins in BL21/DE3, pLysS grown at 30 °C and induced for 3 h with 1 mM isopropyl-1-thio-β-D-galactopyranoside. His6-tagged peptides were purified by nickelagarose chromatography (Qiagen), then dialyzed into XB buffer (0.1 M KCl, 1 mM MgCl2, 0.1 mM CaCl2, 50 mM sucrose, and 10 mM HEPES, pH 7.1), and analyzed by Western blot for both protein 4.1 and His6 tag epitopes.

**In Vitro Protein Binding Assay Using Resonant Mirror Detection—**Interactions between NuMA Tail 1 peptide and 4.1R CTD and CTDmut3Y were measured using the IasyTM system (Affinity Sensors) and FASTfitTM software as detailed (22, 39). Purified peptides were dialyzed into phosphate-buffered saline prior to binding reactions at 25 °C.

**BrdUrd Labeling of Nuclei Assembled in Vitro—**The nuclei were assembled in interphase Xenopus egg extract made 20 μM in BrdUrd and fixed in cold methanol. DNA was denatured in 2 M HCl/0.5% Triton X-100 and then neutralized with 0.1 M borate, pH 8.5, and the coverslips were probed with 10 μg/ml aBrdUrd for indirect immunofluorescence.

**Immunodepletion and Rescue—**For depletion of 4.1 from Xenopus extracts, protein G-coupled magnetic beads (Dynal) from 100 μl of slurry were mixed with 15 μg of 4.1R SABD or CTD IgGs or nonimmune rabbit IgG for 1 h at 4 °C; the beads washed twice with 0.1 M sodium phosphate, pH 7.5, and resuspended in 50 μl of kinase buffer. For rescue, 20 μM of recombinant His6-tagged peptides was incubated with protein G-coupled magnetic beads (Dynal) from 100 μl of slurry.
protein 4.1 epitopes in *Xenopus* sperm and nuclei assembled in vitro detected by immunofluorescent microscopy. Localization of DNA was by DAPI. A, left, protein 4.1 signals (green) in sperm at the basal body region, adjacent to the sperm pronuclear chromatin (blue). Middle, nuclear SABD and CTD signals (green) in *Xenopus* nuclei assembled in vitro. Right, a confocal micrograph of αSABD-stained toroids at the midsection of a nucleus. Bar, 10 μm. B, double label immunofluorescent microscopy of a representative *Xenopus* nucleus assembled in vitro and stained with αSABD (green) and αSm (red). The yellow signals in the merged image indicate colocalization of SABD and Sm epitopes.

In vitro 4.1 epitopes. In vitro assembled nuclei stained with αCTD produced a diffuse intranuclear immunofluorescent pattern, whereas αSABD staining revealed a diffuse pattern as well as larger intranuclear circular or toroidal structures (Fig. 2A). Sperm basal bodies, precursors to mitotic spindle poles, also displayed 4.1 epitopes in *Xenopus* (Fig. 2A) as well as in murine and porcine samples (41). To further explore the nature of the nuclear toroidal structures detected with αSABD, reconstituted nuclei were probed with a variety of antibodies against splicing factors because 4.1 epitopes previously were observed to colocalize with splicing factors (7, 42, 43). Double label experiments revealed a strong coincidence of immunofluorescent signals for Sm antigens (monoclonal Y12) with SABD epitopes in intranuclear toroids along with additional coincidence in some of the smaller more diffuse intranuclear foci (Fig. 2B). In confocal sections, larger toroidal structures in *Xenopus* nuclei appeared to be composed of multiple smaller domains (Fig. 2A), consistent with mammalian cells (44). Thus *Xenopus* extracts appear to be a valuable and appropriate experimental system for deciphering 4.1 functions.

**RESULTS**

**Localization of 4.1 in Nuclei Reconstituted in Xenopus Egg Extracts Is Similar to That in Mammalian Cells—**To dissect protein 4.1 function in nuclear assembly, we performed experiments using *Xenopus* egg extracts. Our previous work in cultured mammalian cells established that nuclear protein 4.1R epitopes are distributed throughout non-nuclear nuclear domains (7). Comparisons of regions of *Xenopus* 4.1 sequence with those of mammalian family members revealed strong conservation in the SABD as well as the CTD (Fig. 1B). In studies exploring the evolutionary conservation of 4.1 function, a recombinant glutathione S-transferase fusion protein encoding the *Xenopus* SABD specifically bound to and mechanically stabilized 4.1-deficient human erythrocyte membranes (40), providing a precedent of functional domain interchange between *Xenopus* 4.1 SABD and mammalian 4.1R.

Initially we verified that nuclei of cultured *Xenopus* fibroblasts as well as *Xenopus* nuclei assembled in vitro contained 4.1 epitopes. In vitro assembled nuclei stained with αCTD produced a diffuse intranuclear immunofluorescent pattern, whereas αSABD staining revealed a diffuse pattern as well as larger intranuclear circular or toroidal structures (Fig. 2A). Sperm basal bodies, precursors to mitotic spindle poles, also displayed 4.1 epitopes in *Xenopus* (Fig. 2A) as well as in murine and porcine samples (41). To further explore the nature of the nuclear toroidal structures detected with αSABD, reconstituted nuclei were probed with a variety of antibodies against splicing factors because 4.1 epitopes previously were observed to colocalize with splicing factors (7, 42, 43). Double label experiments revealed a strong coincidence of immunofluorescent signals for Sm antigens (monoclonal Y12) with SABD epitopes in intranuclear toroids along with additional coincidence in some of the smaller more diffuse intranuclear foci (Fig. 2B). In confocal sections, larger toroidal structures in *Xenopus* nuclei appeared to be composed of multiple smaller domains (Fig. 2A), consistent with mammalian cells (44). Thus *Xenopus* extracts appear to be a valuable and appropriate experimental system for deciphering 4.1 functions.

**Dominant Negative 4.1 Peptides Distort Nuclear Formation in Vitro—**To dissect 4.1 function in nuclei, we added bacterially expressed peptides encoding 4.1 domains to nuclear assembly reactions and then observed and quantified the morphological characteristics of the resulting structures. For these experiments, His<sub>6</sub>-tagged peptides were constructed based on their relationship to either exons 16,17 (amino acids 644–705) in the 4.1R SABD or exons 20,21 (amino acids 800–858) of the 4.1R CTD (Fig. 3), because these domains already have important defined functions and are highly conserved between frog and mammals (44). For purposes of simplicity, these domains will be referred to as SABD or CTD peptides. As controls for the 4.1R SABD peptide, we used a variant 4.1N SABD peptide with low amino acid sequence homology to the 4.1R SABD peptide, we used a variant 4.1N SABD peptide with low amino acid sequence homology to the 4.1R peptide (Fig. 3). As a second control, we expressed a 4.1R SABDΔN peptide with a deletion of two amino acids within its actin-binding domain. This mutant SABD cannot bind actin but retains spectrin binding (45). Importantly, both of these control peptides are incapable of forming ternary complexes with spectrin and actin (45). As control for the 4.1R CTD peptide, a peptide was engineered containing the 4.1R CTD sequence, except that three valines were mutated to alanines (Fig. 3) based on a preliminary report that these residues are part of the 4.1R NuMA-binding site (46). In experiments measuring the relative affinities of 4.1R to NuMA Tail I peptides (47) containing the 4.1 binding site (9), we determined that the binding affinity of...
the mutated 4.1R CTD peptide is decreased about 60-fold compared with the unmutated peptide sequence (Table I). Interestingly, the dissociation constants of the CTD peptide and the 80-kDa red cell 4.1 for Tail I are similar, implying that other 4.1 domains do not significantly contribute to NuMA Tail I binding.²

The addition of SABD peptides produced nuclei that were smaller, multilobed, and surrounded by disorganized microtubules relative to control nuclei (Fig. 4A, top row). Both nuclear membrane pores and the underlying lamina network were disorganized in nuclear structures assembled in reactions containing the 4.1R SABD peptides (Fig. 4A, middle and bottom rows). By contrast, a deletion mutant in the 4.1R SABD (ANF) did not affect nuclear assembly because the nuclei were normal in size, had continuous rims of pores and lamina, and displayed microtubule arrays similar to control nuclei (Fig. 4A). A variant SABD from 4.1N (neuronal) had only a very weak effect, suggesting that this domain in a 4.1N isoform does not have a similar function to that of 4.1R SABD in the nucleus. In fact, in extracts of murine fibroblast nuclei we did not detect 4.1N by Western blot analysis, consistent with our observations that 4.1N peptides had little effect on nuclear assembly in reactions containing either 4.1R SABD or 4.1R CTD peptides, these data support the hypothesis that 4.1-NuMA interactions may be critical for nuclear assembly.

To further analyze the extent of nuclear assembly perturbation produced by SABD and CTD peptides, we assayed the capacity to replicate DNA. In extracts supplemented with 20 μM BrdUrd, nucleotide analogue incorporation into DNA by control nuclei was readily detected by immunofluorescence, whereas aberrant nuclei from reactions containing either 4.1R SABD or CTD peptides had no or very low amounts of detectable BrdUrd (Fig. 5B). Thus in addition to gross morphological abnormalities observed in nuclear structures formed in the presence of either 4.1R SABD or 4.1R CTD peptides, these data demonstrate that the aberrant nuclei are metabolically incapacitated.

To test whether dominant negative effects of SABD or CTD peptides were due to incorporation of SABD or CTD peptides into abnormal nuclear structures, we probed isolated assembly products with CTD and SABD IgGs. We observed that aberrant nuclei had very reduced or no detectable immunofluorescent signals (Fig. 5C). Thus it appears that aberrant nuclei were incorporated and also that endogenous Xenopus 4.1 was displaced from abnormal nuclear structures.

Defective Nuclear Assembly in 4.1-immunodepleted Extracts Can Be Rescued by Recombinant 4.1R—To confirm an essential role for 4.1 in nuclear assembly and the importance of the SABD and CTD domains, we depleted 4.1 from Xenopus egg extracts using 4.1 domain-specific IgGs bound to protein G

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² S. W. Krauss and W. Nunomura, unpublished observations.
magnetic beads. Although normal nuclei assembled in control extracts, a dramatic morphologic disruption of nuclear assembly was apparent in extracts depleted with either SABD or CTD IgGs (Fig. 6A). In general, 80–95% of nuclei from SABD-depleted extracts were small and irregularly shaped, whereas those from CTD-depleted extracts were highly condensed and often bean-shaped. By immunofluorescence (Fig. 6B) and by Western blotting (data not shown), aberrant nuclei from depleted extracts had no detectable SABD or CTD epitopes. Nuclear pore and lamin epitopes were irregularly distributed, similar to perturbed nuclei assembled in the presence of SABD and CTD peptides (Fig. 6B). Western blotting showed that Xenopus extracts and isolated nuclei contain protein bands from ~47 to 110 kDa detected by IgGs against 4.1 SABD and CTD (Fig. 6, C and C’), which could be effectively reduced by 50–100% following three rounds of depletion (Fig. 6C’). Therefore, even incomplete removal of 4.1 proteins containing SABD and CTD regions dramatically inhibited nuclear assembly in vitro. Defects in nuclear reconstitution observed in 4.1-depleted extracts or upon addition of 4.1 SABD or CTD peptides could be due to functional disruption of either 4.1 or a 4.1 binding partner essential for nuclear assembly. To directly test whether 4.1 itself is critical for nuclear assembly, purified recombinant 80-kDa 4.1R was preincubated with depleted extracts. Nuclear assembly was completely restored in both α-SABD and α-CTD-depleted extracts, producing regularly shaped nuclei with decondensed DNA comparable in size with controls (Fig. 6A’) and with a normal distribution of pores and lamina (data not shown). Restoration of normal nuclear assembly by recombinant 80-kDa 4.1R, containing both the SABD

**FIG. 4. Effects of 4.1 peptides on assembly of nuclei in vitro in Xenopus interphasic egg extracts.** A, the products of assembly reactions with the indicated His6 peptides were visualized by immunofluorescence. The reactions were spiked with fluorescent bovine tubulin (top; red) or probed with monoclonal antibody 414 against nuclear pores (middle; red) or antibody Lp46F7 against lamin (bottom; red). DNA (blue) was stained with DAPI. All of the images within the horizontal register are at equal magnification. Bar, 10 μm. B, quantitation of nuclear assembly perturbation by 4.1R SABD and 4.1R CTD peptides. Control reactions included buffer alone, variant 4.1N SABD peptides, or 4.1R SABD, NF peptides. The total number of structures counted is indicated below. The data from reactions containing 4.1R CTDmut3V peptides are not presented because perturbation was less dramatic and thus more difficult to accurately score.
Protein 4.1 Domains Critical for Nuclear Assembly

The aberrant nuclear structures had little endogenous *Xenopus* 4.1 or exogenous 4.1 peptides. This might implicate either disruption of 4.1 function directly or functional inhibition of key 4.1 partners, sequenced by 4.1 peptides. For example, replication defective nuclei are observed when lamin function is perturbed or absent (50–52). The absence of 4.1 in defective nuclei may indicate a disruption of importin pathways because the SABD contains a nuclear localization signal that binds importin α (26). Compromised import could have major effects on nuclear assembly, including DNA decondensation and DNA replication.

In depletion/add-back experiments, markedly abnormal nuclei formed in depleted extracts, but the addition of purified recombinant 80-kDa 4.1R protein restored normal nuclear assembly. Thus 4.1R alone was sufficient for rescue of nuclear assembly, even though extracts were not entirely devoid of detectable 4.1 by Western blot analysis. Residual 4.1 in extracts was resistant to complete removal by further rounds of depletion, perhaps because of denaturation or complexes rendering 4.1 epitopes inaccessible.

**4.1-Actin Binding Capacity Is Necessary for Nuclear Assembly**—One of the 4.1R dominant negative peptides (SABD) contains all of the amino acids necessary for binding of spectrin (1–21 of exon 16 and 27–43 of exon 17) (16, 19, 53, 54) as well as the binding site for actin (amino acids 19–26 of exon 17) (45).

Two variant SABD peptides, both incapable of forming ternary complexes with spectrin/actin, did not perturb nuclear assembly in *in vitro*, even though one peptide binds spectrin but not actin. Thus we conclude that the actin binding capacity of 4.1 SABD is crucial for proper nuclear assembly.

It is likely that there are multiple binding partners of nuclear 4.1 and actin. Although reports of nuclear actin have long been controversial, mounting evidence now includes identification of two nuclear export sequences in actin, characterization of numerous actin-binding proteins in nuclei, ultrastructural localization of intranuclear actin, and cross-linking of actin to DNA (reviewed in Ref. 55). Recent direct evidence that the BAF (BRG- or Brm-associated factors) chromatin remodeling complex contains a functional β-actin subunit (56) and that nuclear DNA helicase II binds actin and is detected adjacent to nuclear actin filaments (57) has implicated essential functions for nuclear actin. Additionally, subnuclear localization of actin adjacent to spliceosomes has been reported (58). Protein 4.1 epitopes are detected at spliceosomes in mammalian cells (7, 42) and in *Xenopus* nuclei as reported here. By analogy to 4.1 function as an adaptor within the plasma membrane cytoskeleton, during nuclear assembly 4.1 may act to recruit factors or modulate multi-protein interactions crucial for proper nuclear formation.

**4.1-NuMa Interaction and Nuclear Assembly**—At least one interaction of 4.1R CTD critical for proper reconstitution of *Xenopus* nuclei appears to be NuMA binding because a 4.1R peptide with mutations decreasing its affinity for NuMA about 60-fold had a minor impact on nuclear assembly relative to CTD peptides with high NuMA binding. Furthermore, the addition of NuMA Tail I peptide containing 4.1 binding sequences severely perturbed nuclear formation.

The role of NuMA, well defined in organizing and stabilizing mitotic spindles (47, 59–61), has remained enigmatic in nuclei. NuMA was proposed to be a structural component of nuclei on the basis of its association with nuclear matrix (62–64), its localization on a subset of nuclear filaments (63), and its capacity to form ordered lattices during overexpression (65, 66). Formation of micronuclei after NuMA antibody microinjection or overexpression suggested that NuMA plays a role in nuclear assembly after mitosis (61, 67, 68). However, NuMA may be nonessential for nuclear structure because nuclei of several cell types are formed in the absence of NuMA.
types are NuMA negative, particularly certain highly differen-
tiated cells (47, 69). Furthermore, Merdes and Cleveland (70)
reported that Xenopus extracts depleted of NuMA by anti-
NuMA form nuclei around human sperm with apparently nor-
mal chromatin and intact nuclear membrane structures. This
finding may not be inconsistent with our observations that
4.1CTD or NuMA Tail I peptides are deleterious to nuclear
assembly in egg extracts. First, antibody depletion of endoge-
 nous NuMA may not exhaustively deplete NuMA-interacting
proteins present in excess, some of which may be essential for
nuclear assembly. Second, NuMA Tail I peptides may have
broader accessibility for binding NuMA substrates relative to
NuMA-anti-NuMA complexes. Third, 4.1R CTD peptides might
be targeting other proteins critical for nuclear assembly that
share the NuMA-binding site on protein 4.1CTD. These possi-
bilities will be addressed directly in future experiments ana-
yzing the proteins that associate with various CTD and CTD-
mutated peptides as well as with Tail I and Tail I mutant
peptides.

There is now a growing roster of classically categorized cy-
toskeletal structural proteins also identified in nuclei: for ex-
ample, actin, myosin, tubulin, spectrin, and 4.1R (71–75). Sev-
eral of these proteins belong to complex superfamilies. Within
the 4.1 family, both the SABD and CTD of 4.1 R and 4.1G are
highly homologous, and potentially 4.1G also could function in
nuclear processes. Therefore, it will be critical in future studies
to identify family affiliations and exonic compositions of 4.1
proteins critical for nuclear assembly. This should aid in ulti-

FIG. 6. Aberrant nuclear assembly in 4.1 depleted extracts and rescue of defective nuclear assembly by the addition of recombi-
nant 80-kDa 4.1. A, the products of nuclear assembly reactions in Xenopus extracts depleted using SABD IgG (△SABD) or CTD IgG (△CTD)
relative to nuclei assembled in control extracts (IgG) were imaged with DAPI (blue). A′, nuclei assembled after the addition of recombinant 80-kDa
4.1R (+4.1) to depleted extracts. With the addition of 4.5 μg of 80-kDa 4.1 (4.4 μM), the nuclei were ~50–80% the size of mock-depleted or control
nuclei, whereas with 9 μg of 80-kDa 4.1 (8.8 μM), the nuclei were similar in size to controls (as shown). DNA was imaged with DAPI (blue). B, double
label immunofluorescence of abnormal nuclei assembled in vitro probed with SABD and CTD IgGs. Immunoreactive bands 1 and 3–6 are detected both in nuclei and extract,
whereas bands 2 and 7 are detected in extract only. The epitopes detected are: bands 1, 2, and 6, SABD and CTD; bands 3–5 and 7, CTD only. No
significant bands were detected when nuclei were probed with an equal amount of nonimmune IgG or when proteins from mock assembly reactions
without sperm were probed with SABD or CTD IgGs. C, Western blot analysis of Xenopus egg extract and isolated nuclei assembled in vitro probed with SABD and CTD IgGs. Immunoreactive bands 1 and 3–6 are detected both in nuclei and extract,
whereas bands 2 and 7 are detected in extract only. The epitopes detected are: bands 1, 2, and 6, SABD and CTD; bands 3–5 and 7, CTD only. No
significant bands were detected when nuclei were probed with an equal amount of nonimmune IgG or when proteins from mock assembly reactions
without sperm were probed with SABD or CTD IgGs. C′, Western blot analysis of equal amounts of extracts depleted with SABD or CTD IgGs or
incubated with control IgG. In extracts depleted with SABD IgG, bands 1 and 6 appear fully depleted, whereas band 2 is 52% depleted relative
to a mock depleted (control IgG) lane by densitometry measurements. In extracts depleted with CTD IgG, band 1 and bands 5–7 are 72 and 69%
depleted, respectively. Bands 3 and 4 in CTD depleted extracts were too faint to be accurately measured and thus are estimated to be more than
~70% depleted. The red brackets indicate areas scanned by densitometry. The arrow (C′) indicates a nonspecific band seen in some experiments.
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