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Citation
Journal of cell science (2010), 123(Pt 7): 1020-1030

Issue Date
2010-03-02

Type
Journal Article

URL
http://hdl.handle.net/2433/130601

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Molecular mechanisms underlying nucleocytoplasmic shuttling of actinin-4

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Accepted 19 December 2009
Journal of Cell Science 123, 1020-1030
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doi:10.1242/jcs.059568

Summary
In addition to its well-known role as a crosslinker of actin filaments at focal-adhesion sites, actinin-4 is known to be localized to the nucleus. In this study, we reveal the molecular mechanism underlying nuclear localization of actinin-4 and its novel interactions with transcriptional regulators. We found that actinin-4 is imported into the nucleus through the nuclear pore complex in an importin-independent manner and is exported by the chromosome region maintenance-1 (CRM1)-dependent pathway. Nuclear actinin-4 levels were significantly increased in the late G2 phase of the cell cycle and were decreased in the G1 phase, suggesting that active release from the actin cytoskeleton was responsible for increased nuclear actinin-4 in late G2. Nuclear actinin-4 was found to interact with the INO80 chromatin-remodeling complex. It also directs the expression of a subset of cell-cycle-related genes and interacts with the upstream-binding factor (UBF)-dependent rRNA transcriptional machinery in the M phase. These findings provide molecular mechanisms for both nucleocytoplasmic shuttling of proteins that do not contain a nuclear-localization signal and cell-cycle-dependent gene regulation that reflects morphological changes in the cytoskeleton.

Key words: INO80 complex, NOR, Actinin, Cell cycle, Nucleocytoplasmic shuttling

Introduction

α-Actinin is a well-known crosslinking protein that bundles actin filaments at focal-adhesion sites. Evolutionary analysis suggests that this protein best resembles the ancestral proteins of the spectrin superfamily, which is characterized by ubiquitously conserved spectrin repeats (SRs) and found in a large variety of taxa (Virel and Backman, 2004). In addition to its structural role as a crosslinker of actin filaments, α-actinin is known to serve as a platform for a number of protein-protein interactions within the cytoskeletal organization (reviewed in Otey and Carpen, 2004; Sjoblom et al., 2008).

α-Actinin-4 (hereafter referred to as actinin-4) was first described in 1998 as a novel isoform of non-muscle α-actinin (Honda et al., 1998). Four isoforms of α-actinin are expressed in a tissue-specific manner in humans. Actinin-2 and -3 are specifically expressed in muscle cells, whereas actinin-1 and -4 are ubiquitously expressed (Beggs et al., 1992; Millake et al., 1989; Youssoufian et al., 1990). The actinin molecule is divided into three functional subdomains: an N-terminal actin-binding domain, which is composed of two calponin homology domains (CH1 and CH2); the C-terminal EF-hands; and the central rod domain, which is composed of four spectrin repeats (SR1-SR4) (Davison and Critchley, 1988; Mimura and Asano, 1987). Antiparallel dimerization of the molecule results in the formation of a dimer with an actin-binding domain located at both ends. Dimer formation is required for its normal function as a crosslinker of the actin cytoskeleton (Ylanne et al., 2001). In addition to its homodimeric interaction, the α-actinin isoforms are also known to interact with numerous other factors involved in focal-adhesion formation. Triple-helical SR4 in α-actinin directly binds to the adhesion-complex protein vinculin (Bois et al., 2006). Microinjection of the vinculin-binding site (VBS) of α-actinin into 3T3 cells has been shown to inhibit the normal α-actinin–vinculin interaction and induces cytoskeletal collapse (Bois et al., 2006), suggesting that this molecular interaction plays a key role in the maintenance of focal adhesions.

Although the α-actinin isoforms are approximately 84% identical and share 90% similarity in their amino acid sequences, the two non-muscle isoforms of α-actinin, actinin-1 and -4, exhibit vastly different behaviors in vitro and in vivo. By contrast to the high sensitivity of actinin-1 to calcium concentration, actinin-4 is different in that its C-terminal EF-hands confer lower calcium sensitivity (Imamura et al., 1994; Nikolopoulos et al., 2000). Fluorescence ratio imaging analysis of highly motile cells, such as macrophages, has revealed that actinin-4 is more concentrated in the circular ruffles located on the dorsal surface, whereas actinin-1 is evenly distributed in all F-actin structures, such as the dorsal and peripheral edge ruffles, phagocytic cups and basal podosomes (Araki et al., 2000). These findings suggest that these two isoforms play an important role in distinct cytoskeletal functions and thus might be regulated in a different manner.

Actinin-4 has also been shown to localize to the nucleus. Upon inhibition of phosphoinositide 3-kinase (PI3 kinase) or actin depolymerization, significant nuclear accumulation of actinin-4 was observed in several cancer cell lines (Honda et al., 1998). This nuclear accumulation was also observed in A431 cells treated with tumor necrosis factor (TNF) or epidermal growth factor (EGF) in association with the p65 subunit of nuclear factor (NF)-κB. These findings most probably indicate a specific role for actinin-4, as actinin-1 has not been detected in the nucleus to date (Babakov et al., 2008). Several proteins that play an important structural role in the cytoplasm are also localized to the nucleus, including SR-containing proteins such as the spectrins, nesprins, bullous pemphigoid antigen 1 (Bpag1) and nuclear mitotic apparatus protein (NuMA) (Young and Kothary, 2005). These proteins must
be controlled by an as-yet-identified nuclear-localization mechanism, as their large molecular size (greater than 70 kDa) renders them unable to diffuse through the nuclear pore complex (NPC). Although Bpag1 contains a conserved nuclear localization signal (NLS) within the SR domain (Young et al., 2003), the other SR-containing proteins lack an obvious NLS. It is not clear how these large non-NLS proteins become localized to the nucleus. Given its large molecular mass (105 kDa) and its apparent lack of NLS, the precise molecular mechanisms underlying nuclear transport of actinin-4 remain largely unknown.

In this study, we investigate the molecular mechanisms underlying nucleocytoplasmic shuttling of actinin-4 and reveal that the nuclear localization of actinin-4 alters during cell-cycle progression. We also identified a novel function for actinin-4 in cell-cycle-dependent transcriptional regulation in relation to the INO80 chromatin-remodeling complex and the mitotic-specific association of actinin-4 with the rRNA transcriptional machinery, suggesting multiple roles for this molecule in cell-cycle-dependent nuclear events.

Results

Subcellular localization of actinin-4

We generated a specific monoclonal antibody, #92B, from a set of antibodies raised against nuclear structural proteins in HeLa cells (web database: http://www.lif.kyoto-u.ac.jp/labs/chrom/abgallery/index.html). The epitope of this antibody was one of the triple helices of SR4 (amino acid residues 735-759) (Fig. 1A), which is known to serve as a VBS that swings out from the molecule to expose its hydrophobic face (Bois et al., 2005). We did not observe any obvious cross-reactivity between antibody #92B and other proteins using western blot analysis (Fig. 1B). The intensity of the protein band recognized by #92B and anti-actinin-4 polyclonal antibody decreased to a similar level when the cells were subjected to siRNA-directed knockdown of actinin-4 (Fig. 1C). By contrast, the amount of actinin-1 and α-tubulin remained unaffected. These results demonstrated that #92B reacts specifically with actinin-4.

The subcellular localization of actinin-4, detected by #92B, suggested that actinin-4 is present in several molecular forms. Anti-actinin-4 polyclonal antibody predominantly stained the focal

Fig. 1. Monoclonal antibody #92B recognizes vinculin-free forms of nuclear actinin-4. (A) Epitope mapping analysis by western blot. His-tagged full-length actinin-4 and GST-tagged fragments corresponding to the actin-binding domain (amino acid residues 1-286), central SR region (287-758), SR region ΔVBS (287-735) and C-terminal EF-hand region (759-911) were constructed and expressed in bacteria. Western blot analysis of each fragment was performed using #92B, anti-actinin-4 polyclonal antibody, and anti-His and anti-GST antibodies. (B) Western blot analysis of HeLa whole-cell lysate using #92B. The cells were lysed in 5% SDS buffer and subjected to SDS-PAGE following #92B immunoblot detection. (C) Western blot analysis of actinin-4-depleted HeLa whole-cell lysate. Cells transfected with siRNA directed against actinin-4 were subjected to western blotting. siRNA against luciferase was transfected as a control (non-targeting siRNA). Anti-actinin-1 blotting was performed to check the specificity of the siRNA and anti-α-tubulin serves as a loading control. Ctrl: control (non-targeting siRNA); NT: non-transfected control. (D) Immunostaining of HeLa cells and 3T3 cells with #92B, anti-actinin-4 polyclonal antibody and anti-β-actin antibody. Three different localization patterns were observed with #92B and are categorized as strong nucleoplasmic signals (arrows), weak cytoplasmic signals (closed arrowheads), and both nucleoplasmic and cytoplasmic staining (open arrowheads). Scale bars: 10 μm. (E) #92B blocking assay with an epitope peptide. HeLa cells were subjected to immunostaining with #92B in the presence of GST-tagged actinin-4 fragment (amino acid residues 287-758). The same concentration of GST peptide was mixed with the antibody as a control. Scale bars: 10 μm.
adhesions and colocalized with β-actin (Fig. 1D). By contrast, #92B did not colocalize with β-actin and the pattern of immunostaining differed among cells. Some cells demonstrated a strong nuclear signal, some exhibited a complete lack of nuclear signal, whereas others showed a similar level of fluorescent signal in both the nucleoplasm and cytoplasm (Fig. 1D, arrows). These results indicated that #92B recognizes specific forms of actinin-4 that are dissociated from the focal adhesion. When the #92B epitope peptide was used to block antibody binding, both the nuclear and cytoplasmic signals disappeared (Fig. 1E). GST-tagged peptide alone did not affect immunostaining, demonstrating that the immunofluorescent signals of #92B were derived from actinin-4. These results suggested that some actinin-4 is generally localized to the nucleus without any stimulation. Nuclear localization of actinin-4 was also observed in mouse 3T3 cells (Fig. 1D). Thus, it appears likely that nuclear localization of actinin-4 occurs universally among different cell types and species.

**Actinin-4 acts as a nucleocytoplasmic shuttling molecule and contains a functional nuclear export signal**

HeLa cells for immunostaining were pretreated with 5 ng/ml leptomycin B (LMB), a specific inhibitor of chromosome region maintenance-1 (CRM1). Under these experimental conditions, nuclear signals were detected when using the anti-actinin-4 polyclonal antibody, but there were no signals in non-treated cells (Fig. 2). Control immunostaining of β-actin (positive) and keratin-8 (negative) was performed to ensure successful LMB treatment (supplementary material Fig. S1). This result indicates that actinin-4 serves as a shuttling molecule between the nucleoplasm and cytoplasm, and that the amount of nuclear actinin-4 is significantly smaller than the amount present in the actin cytoskeleton.

CRM1 is known to mediate nuclear export, which requires the nuclear-export signal (NES). A search for the NES consensus sequence – L-x(2,3)-(L, I, V, F, M)-x(2,3)-(L, I)-x-(L, I) (Bogerd et al., 1996; la Cour et al., 2003) – in the entire actinin-4 molecule revealed two putative NESs at amino acid residues 141-152 and 523-532 (Fig. 3A). The former site is located in the middle of the two CH domains, whereas the latter is positioned in the middle of the SR region (Fig. 3B). To investigate whether these putative NESs are functional, a set of GFP-fused deletion constructs including or excluding the NES was generated (Fig. 3C). Interestingly, the GFP-fused SR region (287-758) dominantly localized to the nucleus, suggesting that this region possesses the potential to migrate into the nucleus. Construct region 152-758 was also dominantly localized to the nucleus, whereas construct 141-758 was found to be successfully excluded from the nucleus. Furthermore, LMB treatment resulted in the nuclear accumulation of full-length and the 141-758 fragment of actinin-4, indicating that the NES at amino acid residues 141-152 is functional (Fig. 3D). These findings were consistent with a previous report demonstrating that a splicing variant of actinin-4 that lacked amino acid residues 89-478 was predominantly localized to the nucleus (Chakraborty et al., 2006). Thus, the nuclear export of actinin-4 appears to be regulated by the NES-dependent, CRM1-mediated nuclear export pathway.

**Fig. 2. Actinin-4 accumulates in the nucleus following treatment with LMB.** Immunostaining of LMB-treated HeLa cells. The cells were incubated with 5 ng/ml LMB for 24 hours and subjected to immunostaining by anti-actinin-4 polyclonal antibody. The same volume of ethanol (vehicle) was incubated as a control. Scale bars: 10 μm.

**Fig. 3. Actinin-4 contains a functional NES.** (A) Comparison of known NES and two putative NES sequences from actinin-4. The NES consensus amino acids are indicated in capital letters. (B) Schematic model of truncated actinin-4 mutants. Four constructs were designed as follows: amino acids 1-911 (full length); 141-758 (contains two NES sequences); 152-758 (contains one NES); and 287-758 (whole SR region). The two putative NES sequences are indicated by the black boxes. (C) Subcellular localization of GFP-fused truncated mutants. HeLa cells were transfected with the constructs and incubated for 24 hours following microscopic observation. Scale bars: 10 μm. (D) Nuclear accumulation of GFP-fused full-length actinin-4 and fragment 141-758 in LMB-treated cells. HeLa cells transfected with the GFP constructs were treated with 5 ng/ml LMB for 24 hours and then subjected to microscopic observation. Scale bars: 10 μm.
Actinin-4 interacts with the NPC and independently migrates through the nuclear membrane

Although actinin-4 was found to function as a shuttling molecule, the nuclear-import mechanisms remain unknown. To investigate the molecular mechanism underlying the nuclear import of actinin-4, nuclear-transport assays were performed using semi-intact HeLa cells. These cells were first treated with digitonin, a chemical that exhibits a high affinity for cholesterol and selectively permeabilizes the cell membrane. The cells were then incubated with florescence-fused proteins of interest. Using this system, the addition of IgG conjugated to Alexa-Fluor-568 to the observation buffer labels the nucleus as black spots and indicates whether the nuclear membrane is intact. GFP-fused proteins with NLSs are expected to pass through the NPC. We found that the GST-NLS-GFP protein was transported into the nucleus when incubated with the transport components importin-α, importin-β, RanGDP and the ATP-regeneration system, but not without sufficient transport components (Fig. 4A). By contrast, the GFP-fused actinin-4 SR region (287-758), which has a molecular mass of 87 kDa and shows nuclear localization in vivo (Fig. 3C), exhibited nuclear localization in the presence and absence of transport components (Fig. 4B).

The molecular mechanisms underlying importin-dependent nuclear transport are known to rely on the hydrophobic interaction between the NPC and its associated proteins. Importin-β contains numerous hydrophobic helices and two consecutive α-helices form several pockets that directly interact with the phenylalanine-glycine (FG)-motif-containing proteins (or FG-Nups) in the NPC (Otsuka et al., 2008; Patel et al., 2007). This direct interaction between the hydrophobic helix and the FG-motif is thought to be responsible for the migration of this molecule through the NPC. The importin-β fragment containing amino acids 45-462 has been shown to lack both the N-terminal Ran-binding site and the C-terminal cargo-binding site, binds tightly to the NPC, masks the interactive FG-motif of the FG-Nups and thus inhibits FG-mediated nuclear transport (Kutay et al., 1997). When the semi-intact cells were pretreated with the importin-β 45-462 fragment, nuclear accumulation of the GFP-fused actinin-4 SR region was effectively inhibited (Fig. 4C). Western blot analysis of the transported materials also demonstrated that the levels of transported actinin-4 peptide were significantly decreased in the presence of the importin-β fragment (Fig. 4D). Given these results, we conclude that the nuclear import of actinin-4 is mediated by its inner SR region and the molecule clearly passes through the NPC.

Localization and biochemical properties of actinin-4 are significantly altered during cell-cycle progression

HeLa cells were synchronized using the double-thymidine block method and subjected to immunostaining with #92B at specific time intervals following release (Fig. 5A). We counted more than 600 cells and classified them into three localization pattern categories: nucleoplasmic localization; cytoplasmic localization; and localization at both sites (Fig. 1, arrows). The histogram illustrates the alterations in the nucleoplasm-localized population during the cell cycle (Fig. 5B). Approximately 10% of the nuclear population was found to be in the S phase. This number increased significantly to 80% in the G2 phase, before gradually decreasing in the G1 phase. Synchronization was assessed by means of changes in cyclin E levels and the total amount of actinin-4 remained constant throughout the cell cycle (Fig. 5C). Given that there were no obvious band shifts in the western blot using #92B and the anti-actinin-4 polyclonal antibody, the changes in localization were most probably not due to either protein digestion or degradation.

The subcellular fractionation technique was developed to separate proteins based on their biochemical properties (Fey et al., 1986). When immunostaining and immunoblotting analyses were undertaken in combination with the fractionation technique, we observed two distinct populations of actinin-4 molecules: a population eluted by high-salt buffer and a highly insoluble population (Fig. 6A,B). Lamin B2 and α-tubulin immunoblotting was performed to assess fractionation. The nuclear actinin-4 recognized by #92B was eluted following high-salt buffer treatment (Fig. 6A), indicating that nuclear actinin-4 possesses different biochemical properties to the highly insoluble cytoskeletal forms of the molecule. Thus, it is suggested that nuclear actinin-4 might play a different role to actinin-4 located at the actin cytoskeleton. When the high-salt extraction was performed on the synchronized G2-phase cells and subjected to western blot analysis, significantly larger amounts of actinin-4 were detected in the extracted fraction compared to the non-synchronized cells (Fig. 6C). The intensity of the non-synchronized bands compared with that of G2-phase cells.
was estimated at 1:2.5 (Fig. 6D). LMB treatment also resulted in an increase in the high-salt-soluble population (Fig. 6D). As the total actinin-4 levels were unchanged throughout the cell cycle, the increase in the nuclear population was probably due to an increase in the levels of high-salt-soluble actinin-4 in the G2 phase. It is reported that the proteins in different fractions participate in the formation of different orders of architecture in both nucleus and cytoplasm (Yoshimura et al., 2003). Therefore, it is suggested that highly soluble nuclear actinin-4 is not involved in the bundling of actin filaments, as in the cytoplasm, and that actinin-4 is a multifunctional molecule.

Actinin-4 interacts with the INO80 chromatin-remodeling complex

The INO80 chromatin-remodeling complex is a member of the SWI/SNF2 family of ATP-dependent chromatin-remodeling complexes and is composed of Ino80 protein, actin, actin-related protein (Arp) 4, Arp5, Arp8 and RuvB ATPases (Cai et al., 2007; Harata et al., 2000; Jin et al., 2005; Shen et al., 2003). Because the INO80 complex contains many actin cytoskeletal proteins, the molecular interactions and functional relationship between actinin-4 and this complex were analyzed to understand the role of actinin-4 in the nucleus. Full-length cDNA of the human Ino80 protein was cloned and expressed in HeLa cells in a GFP-fused form. The fluorescent signal was mainly observed in the nucleus, demonstrating that the GFP tag did not result in the abnormal localization of Ino80 (supplementary material Fig. S2A). Endogenous Arp5 was also found ubiquitously in the nucleus, whereas nuclear actinin-4 was found in some of the cells, depending on the cell-cycle phase, as we revealed previously (Fig. 7A). Precipitation assays were then undertaken against GFP-Ino80 and the coprecipitated proteins were analyzed by western blot. The coprecipitated fraction contained significant amounts of actinin-4, Arp5 and β-actin (Fig. 7B). The same coprecipitated material immobilized on beads was subjected to binding assays with the bacterially expressed GST-fused actinin-4 fragments. The full-length

Fig. 5. Nuclear localization of actinin-4 is regulated in a cell-cycle-dependent manner. (A) HeLa cells were synchronized to the G1-S phase by double-thymidine block and subjected to immunostaining with #92B at specific time intervals after release. Scale bars: 20 μm. (B) Statistical analysis of the signal patterns observed by #92B immunostaining. More than 600 cells in each phase were counted and classified according to the distribution patterns indicated in Fig. 1D. NP: strong nucleoplasmic signal, CP: cytoplasmic signal, both: both nucleoplasmic and cytoplasmic signal. n=638 (0 hours), 746 (2 hours), 705 (4 hours), 477 (6 hours), 601 (7 hours), 648 (8 hours), 766 (9 hours), 626 (11 hours), 709 (12 hours), 657 (14 hours), 603 (16 hours). (C) Western blot analysis of synchronized HeLa cell lysates. Whole-cell lysates from each indicated cell phase were subjected to western blot analysis using #92B, anti-actinin-4 polyclonal antibody, anti-cyclin E and anti-β-actin.

Fig. 6. Localization-dependent and cell-cycle-dependent alteration in the biochemical properties of actinin-4. (A) HeLa cells were sequentially treated with detergent buffer (buffer A), high-salt buffer (buffer B) and DNase buffer (buffer C), and subjected to immunostaining with #92B and anti-actinin-4 antibody. Scale bars: 10 μm. (B) Western blot analysis of the extracted proteins in each buffer treatment. Actinin-4 was mainly detected in the high-salt buffer fraction and the pellet fraction. Successful fractionation was monitored by anti-lamin B2 and anti-α-tubulin blotting. WC: whole cell, A,B,C: fractions eluted by each buffer treatment, ppt: pellet after the sequential treatment. (C) Western blot analysis of proteins extracted by high-salt buffer. The following phases of the cells were directly subjected to buffer B extraction: G2, synchronized to G2 phase by double-thymidine block (8.5 hours after release); NS, non-synchronized control; and LMB, treated with LMB for 24 hours. Both extracted and pellet fractions were subjected to the #92B western blot. The amounts of lamin B2 and α-tubulin were monitored as loading controls. (D) Statistical analysis of the western blots. The intensities of the actinin-4 bands were measured and calibrated by the lamin B2 and tubulin bands. Error bars indicate ± s.d. (n=3).
molecule and the actin-binding domain of actinin-4 were successfully pulled down by the INO80 complex, whereas the central SR region and the GST tag alone were not detected (Fig. 7C). The actin-binding region is responsible for the actinin-4–INO80 complex interaction, a process that might be mediated by β-actin and Arps.

The functional relationship between actinin-4 and the INO80 complex was then analyzed by focusing on cell-cycle-dependent transcriptional regulation. This complex is known to regulate as much as two-thirds of the total gene expression in yeast, including cell-cycle-related genes such as cyclins and cell-cycle checkpoint factors (Shimada et al., 2008). Total RNA was extracted from HeLa cells 12 or 48 hours following transfection with small interfering RNAs (siRNAs) directed against actinin-4 or Ino80. The effect of this RNAi system on target mRNA levels was analyzed by quantitative PCR.

The actinin-4 gene (ACTN4) was effectively suppressed both 12 and 48 hours following incubation with actinin-4 siRNA (relative score: 0.05 and 0.04), whereas Ino80 knockdown resulted in only an approximately 70% reduction in INO80 expression (0.31 and 0.24) (Fig. 7D). Although the mRNA level of ACTN4 was slightly affected by Ino80 knockdown, the protein level and the nuclear localization of actinin-4 were not affected within this timeframe (supplementary material Fig. S2B,C). Relative mRNA levels of a set of cell-cycle-related genes were then analyzed, including CDC6, which is known to be regulated by the INO80 complex in human cells (Cai et al., 2007). The results of these experiments suggested the involvement of actinin-4 in the expression of cell-cycle-dependent genes. Knockdown of Ino80 resulted in the suppression of CyclinB1 (CCNB1), CDC6, and CDK4 (Fig. 7E). Interestingly, actinin-4 knockdown significantly affected CCNB1 gene expression, but did not affect CDC6 and CDK4 after 12 hours, suggesting that actinin-4 affects a subset of INO80-dependent gene expression. After a 48-hour incubation, the vast majority of target genes, except CDK6, were negatively regulated by both knockdowns. This result might reflect indirect knockdown effects, including cell-cycle defects. Actinin-4 appeared to regulate specific gene expression in conjunction with the INO80 complex in a cell cycle-dependent manner. CCNB1 is one of the target genes of the actinin-4-containing INO80 complex.

**Actinin-4 associates with the rRNA transcriptional machinery during mitosis**

Nuclear actinin-4 exhibited distinct and dynamic behavior during mitosis. HeLa cells stained with #92B demonstrated strong signals located on the condensed mitotic chromosomes (Fig. 8A). It is well established that various rRNA transcription factors, such as RNA polymerase I and upstream binding factor (UBF), form foci on rDNA regions termed nucleolar organizer regions (NORs) of
specific mitotic chromosomes (Roussel et al., 1996). When the UBF was expressed in a GFP-fused form, the #92B signals completely overlapped with the GFP-UBF foci located on the mitotic chromosomes (Fig. 8B). These results revealed that the actinin-4 foci correspond to the NORs. Both actinin-4 and UBF accumulated in the NORs before prophase and were equally divided into the two daughter cells. When the cells entered telophase, the actinin-4 signals were reduced and more dispersed, whereas the UBF signals remained localized to the NORs until the early G1 phase. The association of actinin-4 with UBF was also assessed via coprecipitation analysis (Fig. 8C). Significant amounts of actinin-4 were coprecipitated with UBF from colcemid-arrested mitotic cell lysate, whereas protein bands were not detected in S-phase or non-synchronized samples. Thus, actinin-4 interacts with the rRNA transcriptional machinery in a mitotic-specific manner.

The suppressive effect of siRNA directed against UBF was monitored by western blot analysis of HeLa cells stably expressing GFP-UBF. Compared to the protein level of α-tubulin, the GFP-UBF protein level decreased to 0.27 (Fig. 8D). The endogenous UBF would also be suppressed to the same level. Although the protein level of actinin-4 detected by the western blot remained unaffected (1.02), the actinin-4 signal localized to the NORs disappeared following siRNA knockdown of UBF (Fig. 8E). Therefore, the localization of actinin-4 on mitotic NORs was UBF dependent. The rRNA transcriptional machinery is known to contain nuclear actin (Philimonenko et al., 2004), suggesting that the association of actinin-4 with this complex might also be mediated by actin or actin-related molecules, as in the case of the INO80 complex.

Discussion

Multifunctional actinin-4 at focal adhesions and the nucleus

In this study, we demonstrated that actinin-4 was localized to the focal adhesions, cytoplasm and nucleoplasm. In addition, polyclonal antibody raised against actinin-4 recognized focal adhesions and the nucleus of LMB-treated cells (Fig. 2). We hypothesized that the actinin-4 polyclonal antibody might recognize all subcellular actinin-4 and therefore reflect the relative levels of the molecules in a cell. The vast majority of the actinin-4 molecules were located at the focal-adhesion sites, whereas reduced levels were identified in the cytoplasm and nucleoplasm. By contrast, monoclonal antibody #92B did not recognize the focal-adhesion form of the actinin-4 molecule (Fig. 1). It is reasonable to assume that the epitope of #92B was masked by factor(s) in the focal adhesion. The #92B epitope exactly matches the VBS, which forms a distinct helix and can bind vinculin both in vivo and in vitro (McGregor et al., 1994; Zhang et al., 2009). A three-dimensional model of the binding of actinin and vinculin showed that the VBS is completely covered by the vinculin molecule (Bois et al., 2005). Combining our results with previous findings, it is likely that vinculin binding masks the #92B epitope and therefore #92B specifically recognizes the vinculin-free form of actinin-4. Alternatively, masking of the #92B epitope might not necessarily be done by vinculin. Although vinculin is the most probable candidate for this, it might also be possible that other factors affect #92B binding to actinin-4, such as dimerization or the binding of other proteins.

Proteomic analysis of the various subcellular structures has been undertaken previously to identify the precise composition of these elements. Three different types of proteomic analyses of the nuclear matrix, nucleolus and mitotic chromosomes revealed that actinin-4 was located within the nuclear matrix and in mitotic chromosomes, but was not found in the nucleolus (Andersen et al., 2002; Hirano et al., 2009; Ishii et al., 2008; Scherl et al., 2002; Uchiyama et al., 2005). These findings are consistent with our observation that
actinin-4 was localized to the NORs during mitosis (Fig. 8), but was not identified within the nucleolus at any stage of interphase cells. Therefore, the association of actinin-4 with the rRNA transcriptional machinery might represent a mitotic-specific event. Nuclear actin and myosin I are known to be associated with various transcriptional elements related to RNA polymerases I, II and III (Hofmann et al., 2004; Hu et al., 2004; Philimonenko et al., 2004). Actinin-4 might exhibit correlative functions with these factors, although its precise role on the NORs remains unclear.

The SR region of actinin-4 possesses the potential to migrate into the nucleus

In this study, we demonstrated that the SR region of actinin-4 itself possesses the potential to migrate into the nucleus through the NPC. Nuclear-transport assays showed that the nuclear import of this protein was mediated by its inner SR region, independent of transport mediators (Fig. 4). We hypothesize that the hydrophobic helices located in the central SR region directly interact with FG-Nups in the NPC. It has been reported previously that binding pockets consisting of two hydrophobic helices present in importin-β directly interact with the FG-motifs of the NPC (Otsuka et al., 2008). Given that the surface-charge-potential distribution of the actinin SR region revealed numerous hydrophobic areas on the surface (Ylanne et al., 2001), the SR region could interact with the NPC in the same manner as importin-β. This leads to our hypothesis that the molecular mechanisms underlying nuclear localization of actinin-4 are dependent on a direct interaction with FG-Nups. The molecular mechanisms underlying karyopherin-independent nuclear import of cytoskeletal proteins over 100 kDa in size have not been reported to date. These mechanisms will also be relevant to other SR-containing structural proteins that are localized to the nucleus.

Molecular mechanisms controlling nuclear levels of actinin-4

Nuclear localization of actinin-4 is thought to be regulated by the balance between nuclear import and export (Fig. 9A). The molecular mechanisms of nuclear import and export have not been elucidated for all kinds of proteins. The rate of nuclear import and export should vary depending upon the protein, and the cytoplasmic and nuclear distribution of a particular protein should be determined from the balance of nuclear import and export. In this sense, it is interesting and important to consider the following facts. First, in the case of focal adhesion kinase (FAK, a 122 kDa protein that contains both NLS and NES), most of the molecules are localized to the focal adhesion, suggesting that the NES is much more powerful than the NLS in this case (Lobo and Zachary, 2000; Ossovskaya et al., 2008; Stewart et al., 2002). Second, the GFP-fused actinin-4 141-758 fragment (which contains NES and SR) did not localize to the nucleus (Figs 3, 4). When the cells were treated with LMB for 4 hours, significant accumulation of the FAK protein in the nucleus was observed. By contrast, it was difficult to detect a nuclear signal under the same conditions following incubation with the anti-actinin-4 polyclonal antibody (data not shown). These findings suggest that the SR-dependent nuclear import of actinin-4 appeared to be very slow compared to NLS-dependent transport, and the NES-dependent nuclear export of actinin-4 is much more powerful than nuclear import. Alternatively, it is also possible that actinin-4 exhibits a high affinity for actin-related cytoskeletons and has more limited opportunities to reach the NPC.

Considering the molecular mechanisms underlying nuclear import and export, there are two possible ways in which the nuclear localization of actinin-4 is regulated. One way is through the regulation of CRM1 activity levels. As actinin-4 is exported in an NES-dependent manner, CRM1 might directly regulate the nuclear localization of actinin-4. It has been reported that the expression of mammalian CRM1 is initiated in the late G1 phase and reaches its peak at the G2-M phase. However, the total protein levels remain unchanged throughout the cell cycle (Kudo et al., 1997). This result suggests that the overall activity of CRM1 does not change during the cell cycle and thus nuclear export of actinin-4 would remain constant. Another way in which nuclear localization of actinin-4
might be regulated is through the release of actinin-4 molecules from the cytoskeleton. As the actinin-4 molecule itself possesses nuclear-import potential, an increase in the level of free molecules might directly accelerate nuclear import. In combination with our cell-cycle observations and high-salt extraction analyses, the nuclear population in the G2 phase reasonably becomes greater because of an increase in the number of free molecules during this phase (Fig. 9B). It has been reported that the amount of the focal-adhesion protein paxillin is significantly reduced during mitosis, whereas the amount of other focal-adhesion proteins remained constant. That is, focal-adhesion components appear to behave differently during mitotic events (Yamaguchi et al., 1997). As actinins function to anchor F-actin to the focal adhesions, it is likely that degradation of various focal-adhesion components might induce the release of actinin-4 from the cytoskeletal architecture and result in an increase in nuclear actinin-4 levels.

Actinin-4 as a link between cytoskeleton and gene expression

Our studies revealed the molecular mechanisms underlying the nucleocytoplasmic shuttling of actinin-4 and its role in the expression of cell-cycle-dependent genes. We hypothesize that this molecular shuttling serves as a link between morphological changes in the cytoskeleton and cell-cycle-dependent gene expression, and is important for the orchestration of cell activity (Fig. 9). Nuclear actin and Arps have been identified in several chromatin-remodeling complexes, where they are thought to play key roles in the transcriptional regulation of numerous genes (Hofmann et al., 2004; Hu et al., 2004). The rRNA transcriptional machinery also contains nuclear actin and myosin I (Philimonenko et al., 2004). It therefore seems likely that nuclear transcriptional events are dynamically regulated by cytoskeletal components. Actinin-4 might also play an important role in transcriptional regulation. Indeed, a splicing variant of actinin-4 that lacks amino acid sequence 89-478 is predominantly localized to the nucleus and mediates the transcription of the TAF55 gene, which is regulated by myocyte enhancer factor-2 (Chakraborty et al., 2006). In this study, we showed that nuclear actinin-4 associates with the INO80 chromatin-remodeling complex via its actin-binding domain, an association that directs the expression of a subset of cell-cycle-dependent genes. It appears reasonable that actinin-4 is associated with various chromatin-remodeling complexes, as all of the SWI/SNF-like complexes examined to date contain at least one actin or Arp isoform (Szerlong et al., 2008). These findings suggest a more general role for nuclear actinin-4 in transcriptional regulation, in addition to other actin cytoskeletal proteins in the remodeling complex.

Because the expression of the M-phase cyclin CCNB1 gene was rapidly suppressed in actinin-4 knockdown cells, CCNB1 might serve as a direct target gene of the actinin-4-containing INO80 complex. A global gene expression analysis of a yeast strain lacking INO80 revealed that various cell-cycle-related genes are regulated by the INO80 complex (van Attikum et al., 2004). In this strain, the expression of the M-phase cyclins CLB1-CLB4 was most significantly affected among all the cyclin genes, a finding that was consistent with our results in human cells. Considering the facts that nuclear actinin-4 was observed only in particular cells, whereas Ino80 and Arp5 were localized to the nucleus ubiquitously, some cells are likely to contain actinin-4-containing INO80 complexes, others actinin-4-depleted INO80 complexes (Fig. 9B). We hypothesize that the actinin-4-containing INO80 complex is responsible for specific gene expression in late-G2 to early-G1 phase.
Nucleocytoplasmic shuttling of actin-4


