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Conformation-specific antibodies reveal distinct actin structures in the nucleus and the cytoplasm

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Abstract

For many years the existence of actin in the nucleus has been doubted because of the lack of phalloidin staining as well as the failure to document nuclear actin filaments by electron microscopy. More recent findings reveal actin to be a component of chromatin remodeling complexes and of the machinery involved in RNA synthesis and transport. With distinct functions for nuclear actin emerging, the quest for its conformation and oligomeric/polymeric structure in the nucleus has resumed importance. We used chemically cross-linked 'lower dimer' (LD) to generate mouse monoclonal antibodies specific for different actin conformations. One of the resulting antibodies, termed 1C7, recognizes an epitope that is buried in the F-actin filament, but is surface-exposed in G-actin as well as in the LD. In immunofluorescence studies with different cell lines, 1C7 selectively reacts with non-filamentous actin in the cytoplasm. In addition, it detects a discrete form of actin in the nucleus, which is different from the nuclear actin revealed by the previously described 2G2 [Gonsior, S.M., Platz, S., Buchmeier, S., Scheer, U., Jockusch, B.M., Hinssen, H., 1999. J. Cell Sci. 112, 797]. Upon latrunculin-induced disassembly of the filamentous cytoskeleton in Rat2 fibroblasts, we observed a perinuclear accumulation of the 1C7-reactive actin conformation. In addition, latrunculin treatment led to the assembly of phalloidin-staining actin structures in chromatin-free regions of the nucleus in these cells. Our results indicate that distinct actin conformations and/or structures are present in the nucleus and the cytoplasm of different cell types and that their distribution varies in response to external signals.

Keywords: Actin antibody; Actin conformation; Latrunculin; Nucleus; Nucleocytoplasmic transport

1. Introduction

Actin has been characterized as an abundant protein with a plethora of diverse functions in the cytoplasm, but it has also been described as a component of the nucleus for quite some time (Bremer et al., 1981; Gonsior et al., 1999; Grenklo et al., 2004; Jockusch et al., 1974; Scheer et al., 1984). The failure to detect bona fide filaments that stain with phalloidin in nuclei conferred to many that nuclear actin simply reflects a contamination from the cytoplasm. However, recently the longstanding argument over the true existence of nuclear actin has been settled by the emergence of specific functions. For instance, it has been demonstrated that nuclear actin contributes to transcription by associating with initiation complexes of all three RNA polymerases, i.e., polymerase I (Philimonenko et al., 2004), II (Egly et al., 1984; Kukalev et al., 2005; Percipalle et al., 2003; Scheer et al., 1984; Smith et al., 1979), and III (Hu et al., 2004). In addition, evidence is increasing that nuclear actin is involved in the processing and export of mRNAs (Hofmann et al., 2001; Sahlas et al., 1993) and in chromatin remodelling (Olave et al., 2002; Percipalle et al., 2002; Rando et al., 2000). Notably, there is no evidence that conventional actin filaments, so abundantly present in the cytoplasm, are the building blocks of a nuclear matrix in tissue forming cells. In *Xenopus* oocytes, actin has been identified

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as a component of a filamentous network that may be part of the nuclear matrix (Gonsior et al., 1999; Kiseleva et al., 2004). However, the conformation of actin in these structures is unknown.

Because of its diverse nuclear functions, actin cannot be considered a mere thermodynamic wanderer that transiently occupies the nucleus. Rather, the levels of actin in this cellular compartment have to be regulated. Although actin itself does not harbor a classical nuclear localization signal (NLS), some of the proteins that bind actin, for example cofilin, do. On the other hand, profilin and β4-thymosin that bind monomeric actin and are also present in the nucleus lack a classical NLS. With a molecular mass of 42,000 and a compact structure, actin, even in complex with small proteins like profilin, may well pass through the nuclear pores (Fahrenkrog and Aebi, 2003) by passive diffusion. With several active and passive pathways for entering the nucleus, it seems more likely that regulating its export from the nucleus controls the level of actin. Consistent with this notion, two conserved nuclear export signals (NESs) have been described for actin (Wada et al., 1998). More recently, exportin 6, a novel transport receptor of the importin-β superfamily, has been shown to mediate the nuclear export of profilin–actin complexes in higher eukaryotic cells (Stüven et al., 2003). Based on their findings, Stüven et al. suggested exportin 6 to function as a suppressor of actin polymerization in the nucleus.

As the data described above point to multiple, seemingly unrelated functions within the nucleus, nuclear actin might comprise actin in a number of different conformations. The association with RNA polymerases II and III is apparently restricted to β-actin (Hofmann et al., 2004; Hu et al., 2004) but there is no information on the other actin isoforms with respect to nuclear functions. The notion that nuclear actin does not exist in a filamentous, phalloidin-binding form prompted the speculation that it exists either as G-actin monomers or in unconventional conformations, such as very short polymers not containing the 'F-actin'-conformation, that specifically occur in the nucleus and are distinct from those found in conventional actin filaments in the cytoplasm.

To challenge this possibility, we have started to generate a panel of monoclonal antibodies recognizing specific actin conformations. Hereby, we consider even subtle spatial rearrangements of the actin molecule to represent a distinct conformation. We present comparative data on two of these antibodies, describing their reactivity and usefulness as tools to localize nuclear actin. Our data support the hypothesis that more than one form of actin exists in the nucleus of cultured cells.

2. Materials and methods

2.1. Cells

HeLa and Rat2 fibroblasts (Leavitt et al., 1985) were grown in Dulbecco's minimal essential medium supplemented with 2 mM L-glutamine, 100 IU/ml penicillin, 100 μg/ml streptomycin, and 10% FCS (DFCS) at 37 °C, in a humidified atmosphere containing 5% CO_2. Cells were subcultured weekly up to 10 passages.

2.2. Reagents

Latrunculin-A (LatA; Calbiochem) was stored as a 2 mM stock solution in DMSO at −70 °C. LatA was diluted to a final concentration of 5 μM in DFCS prior to use. Alexa Fluor 568-phalloidin was purchased from Molecular Probes. DRAQ5 cell permeant DNA probe was obtained from Alexis Biochemicals.

2.3. Antigens and antibodies

Purified actin isolated from skeletal rabbit muscle was covalently cross-linked at the onset of KCl-induced polymerization using homobifunctional 1,4-penylenebis maleimide (PBM; Sigma). A freshly prepared 5 mM PBM stock solution in dimethylformamide was diluted to 12 μM in 10 mM sodium borate, pH 9.2, containing 20 mM $CaCl_2$ immediately prior to use. For cross-linking, an equal volume of freshly diluted PBM was added to 24 μM G-actin solution in buffer A (2.5 mM imidazole, 0.2 mM $CaCl_2$, 0.2 mM DTT, 0.2 mM ATP, and 0.005% NaN_3, pH 7.4) immediately after the initiation of polymerization by 20 mM $CaCl_2$ to achieve a final molar ratio of 0.5:1 PDM/actin. After 15 min incubation at room temperature, further cross-linking was blocked by the addition of a tenfold molar excess of β-mercaptoethanol. After extensive dialysis against buffer A, cross-linked lower dimer (LD) was separated from monomeric actin by gel filtration chromatography using a Superdex 200 HiLoad column (Amersham Biosciences). Fractions containing <95% LD were pooled and concentrated by flow filtration.

The monoclonal antibody 1C7 was generated by immunizing mice with LD following a standard immunization protocol. After hybridization and cloning, antibody producing hybridoma cells were screened for their binding to LD and G-actin by ELISA. Isotype analysis of the strongly reactive clone 1C7 revealed it to be an IgG1 subtype. Supernatants were grown either according to standard protocols or in serum-free medium in the presence of 500 mg/L albumin and 10 mg/L transferrin. 1C7 was used as culture supernatant or purified by immunoaffinity chromatography using anti-mouse IgG-agarose (Sigma).

Production and purification of monoclonal anti-actin 2G2 (IgM) has been described previously (Gonsior et al., 1999).

2.4. Dot blot analysis

Serial dilutions of purified protein (rabbit skeletal muscle G-actin, LD, BSA) in buffer A were applied onto a PVDF immobilon-P membrane by vacuum filtration in a