ELSEVIER

FEMS Microbiology Letters 147 (1997) 215–220

FEMS MICROBIOLOGY LETTERS

Purification of a 47-kDa calmodulin-binding polypeptide as an actin-binding protein from *Neurospora crassa*

Nicolas Capelli [a], Francisco Barja [c], Diederik van Tuinen [b], Jean Monnat [c], Gilbert Turian [c], Ruben Ortega Perez [c,*]

[a] *Laboratory of Biochemistry and Plant Physiology, 3 Place de l'Université, University of Geneva, CH-1211 Geneva 4, Switzerland*
[b] *Laboratory of Phytoparasitology, I.N.R.A.-SGAP, BV 1540, 21034 Dijon Cedex, France*
[c] *Laboratory of General Microbiology, Sciences III, University of Geneva, CH-1211 Geneva 4, Switzerland*

Received 31 October 1996; revised 4 December 1996; accepted 6 December 1996

Abstract

We have enriched a 47-kDa polypeptide (p47) from *Neurospora crassa* on the basis of its affinity to calmodulin. The p47 was purified to homogeneity by chromatography on a Mono S cation exchange column and evidence is presented that the polypeptide co-sediments specifically with F-actin. The intracellular distribution of p47 and actin was also examined using indirect double immunofluorescence staining of cells at different stages of development. Our results suggest that by altering the conformation binding site of actin to p47, calmodulin could play a regulatory role in the polarized hyphal growth of *N. crassa*.

Keywords: *Neurospora crassa*; Calmodulin; Actin-binding protein; Cellular localization

1. Introduction

Microfilaments (MFs) are ubiquitous cellular structures that contribute to the determination of cell shape and provide the machinery for several aspects of cellular motility and morphogenesis. Associated with MFs are a wide spectrum of accessory proteins which regulate many physiological functions linked to the actin cytoskeleton.

These actin-binding proteins (ABPs), notably villin, gelsolin, severin, and fragmin, are believed to play a regulatory role in the cytoskeletal changes that occur during cell locomotion, secretion, cytoplasmic streaming and cytokinesis [1,2]. The properties of these ABPs have been reviewed in detail [3–5].

A number of proteins that interact with actin and alter the assembly state of the actin MFs in several models have been described in a wide variety of fungi, principally *Saccharomyces cerevisiae* [6], and also in the slime mold *Dictyostelium discoideum* [7].

Although actin has been purified and partially

* Corresponding author. Tel.: +41 (22) 702 6762; fax: +41 (22) 781 1747; e-mail: ortega@sc2a.unige.ch

Abbreviations: ABPs, actin-binding proteins; BSA, bovine serum albumin; CaM, calmodulin; DAB, 3,3′-diaminobenzidine tetrahydrochloride; DTT, 1,4-dithio-DL-threitol; EGTA, ethyleneglycol-bis(β-aminoethyl ether)*N*,*N*,*N*′,*N*′-tetraacetic acid; Ig, immunoglobulin; MFs, microfilaments; $PhMeSO_2F$, phenylmethylsulfonyl fluoride; SDS-PAGE, sodium dodecylsulfate polyacrylamide gel electrophoresis; TBS, Tris buffered saline

PII S0378-1097(96)00527-7

characterized in *Neurospora crassa* [8], no ABPs have been described yet in this filamentous fungus.

We report here the purification and localization of a 47-kDa peptide (p47) able to bind not only to actin but also to calmodulin (CaM), an intracellular calcium regulator that we had previously characterized in *N. crassa* [9,10]. Our results suggest that CaM can play a regulatory role by altering the binding of actin to p47, depending upon calcium concentrations. This mechanism may be involved in the polarized growth of the hyphae of *N. crassa*.

2. Materials and methods

2.1. Organism and growth conditions

Wild-type *N. crassa* strain St. Lawrence 74A (FGSC 262) was obtained from the Genetics Stock Center, University of Kansas Medical Center, Kansas City, KS, USA. Growth conditions and harvesting of the cells were as described in [9]. Vegetative cells were quickly frozen in liquid nitrogen after harvest and lyophilized.

2.2. Purification of the 47-kDa peptide

To prepare the 47-kDa peptide, 6 g of lyophilized mycelia were disrupted in a Waring blender and the dry powder resuspended in 100 ml of buffer A (20 mM Tris-HCl, pH 7.5, 5 mM $MgCl_2$, 3 mM EGTA, 10 mM benzamidine, 0.25 μg/ml pepstatin, 0.2 μM aprotinin, 0.5 μg/ml leupeptin, and 10 mM β-mercaptoethanol per gram of lyophilized mycelium). The homogenate was rapidly filtered through a cheesecloth and clarified by centrifugation at $38\,000 \times g$ for 30 min in a SS-34 rotor Sorvall RC-5B (Sorvall Instruments, Wilmington, DE). The resulting supernatant was mixed with 35 ml of CM-Sepharose Cl-6B (Pharmacia) resin pre-equilibrated in buffer B (buffer A, without protease inhibitors and β-mercaptoethanol). After washing with 25 bed volumes of buffer B, 47-kDa fractions were eluted with 500 mM NaCl prepared in the buffer described above (buffer C). The pH of the eluate was adjusted to 7.5 with 1 M Tris base, $CaCl_2$ was added to give a free final concentration of 1 mM, and the mixture was chromatographed on a 10 ml calmodulin-Sepharose 4B column (containing 7.5 mg of CaM/ml of resin), equilibrated with buffer C. After washing with the same buffer until the $A_{280\ nm}$ of the column effluent came down to lower than 0.02, the bound proteins were released by chelation of the calcium with buffer D (buffer C without calcium but containing EGTA instead). The eluted proteins thus obtained were pooled, dialysed overnight against 5 mM Tris-HCl, pH 6.9 containing 50 mM KCl, and filtered on a Millex-GV 0.22 μm membrane (Millipore) and loaded onto a Mono S cation exchange column. After washing with the same buffer, elution was performed with a discontinuous gradient of KCl 0.05–0.5 M using a FPLC system (Pharmacia). The protein content of the collected fraction (0.5 ml) was examined by SDS-PAGE.

2.3. Gel electrophoresis analysis

One-dimensional SDS-PAGE was performed according to Laemmli [11] using a resolving slab gel of 12.5% (w/v) acrylamide and stained as described by Zehr et al. [12]. Protein concentration was determined by the method of Spector [13], using BSA as standard.

47-kDa fractions eluted from the Mono S column with 0.48 M KCl were analyzed by two-dimensional, non-equilibrium pH-gradient electrophoresis (NEPHGE) [14].

2.4. Immunological techniques

Antisera against the 'PAGE-purified 47-kDa peptide' were prepared by standard methods followed by concentration of the IgG using protein A-Sepharose

Table 1
Summary of total protein recovery after different purification steps

Stage	Protein (mg)
Crude extract:	1557 ± 36.4
Elution from CaM column by 0.5 M NaCl:	49 ± 1.7
Elution from CaM column by Ca^{2+} chelation:	8.97 ± 0.5
Elution of the Mono S column by	
0.22 M KCl:	3.11 ± 0.06
0.33 M KCl:	1.35 ± 0.05
0.48 M KCl:	0.76 ± 0.06

Each value is the mean ± S.E.M. of three experiments.

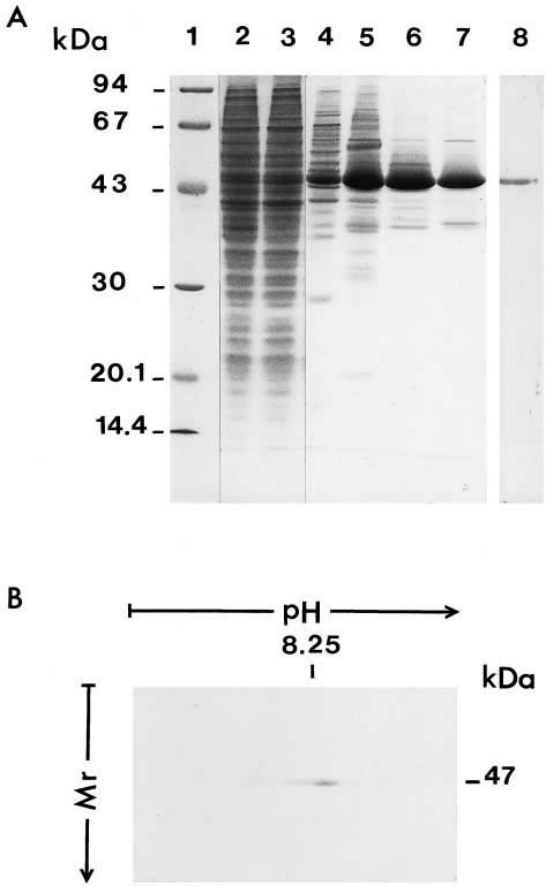

Fig. 1. Purification of p47 by calmodulin affinity chromatography and Mono S chromatography. A: SDS-PAGE analysis of proteins at different steps of the purification procedure. Lane 1, molecular mass markers (Sigma); lane 2, crude protein extract (35 μg); lane 3, CM-Sepharose Cl-6B flow-through (35 μg); lane 4, CM-Sepharose Cl-6B fraction (10 μg) eluted with buffer C; lane 5, 47-kDa pooled peak fractions (18 μg) eluted by calcium chelation from calmodulin-Sepharose affinity column; lanes 6–8, aliquots (20, 10, 2 μg) of peak fractions, after partial purification by Mono S cation exchange chromatography. B: Two-dimensional electrophoretic analysis of p47 (5 μg) eluted from the Mono S column with 0.48 M KCl.

(Millipore) [15] and further purification as described by Olmsted [16].

2.5. Western blot analysis

Following separation on SDS-polyacrylamide gels, total extracted proteins (40 μg/slot) from unstained gels were electrophoretically transferred onto BA 85 nitrocellulose membranes (Schleicher and Schuell GmbH, Dassel, Germany) using standard procedures [17,18]. The nitrocellulose paper was incubated with the anti-47-kDa IgG (1:400 dilution), for 3 h at room temperature, in TBS (25 mM Tris-HCl, pH 7.5, 150 mM NaCl) containing 0.5% (v/v) Tween 20 (Fluka Chemie AG, Buchs, Switzerland). The membranes were washed with three changes each after 15 min in TBS-Tween before incubation for 1 h in the same buffer containing a 1:2000 dilution of the donkey anti-rabbit IgG conjugated to horseradish peroxidase (Amersham, UK). The peroxidase activity was then revealed with 0.5 mg/ml of DAB (Fluka Chemie) in 100 mM Tris-HCl, pH 7.5 containing 0.03% H_2O_2. The reaction was stopped by thorough rinsing in distilled water.

2.6. Actin co-precipitation assay

The actin-binding assay was tested essentially as detailed by Nunnally et al. [19]. In brief, rabbit skeletal muscle actin (Sigma) was incubated in the presence or absence of p47 at 37°C for 3 h in 200 μl of the 'standard' buffer containing 5 mM Tris-HCl, pH 6.9, 100 mM KCl, 1 mM DTT, 1 mM $MgCl_2$, 0.2 mM $CaCl_2$ in the presence or absence of 5 mM ATP and in the presence of 5 μg CaM as indicated.

Samples were then centrifuged at $130\,000 \times g$ for 1 h in a TFT 80.2 rotor (Kontron). The resulting supernatant and pellet fractions of each were electrophoresed on SDS-PAGE and visualized by staining with Coomassie blue.

2.7. Fixation, wall digestion, membrane permeabilization

Germlings (3–8 h growth) were fixed first for 15 min at 37°C and then for 45 min at room temperature with 3.7% (w/v) paraformaldehyde in 10 mM Imidazol buffer (pH 7) containing 2 mM $MgCl_2$, 2 mM EGTA, 2 mM ATP, 1 mM $PhMeSO_2F$, and 20 μM leupeptin.

For partial cell wall digestion, the cells were incubated with 5 mg/ml lysing enzymes (Sigma) in 66.6 mM Sörensen's phosphate buffer, pH 6.8, for 30 min at 40°C. The digestion was stopped by rinsing for 5 min in the above buffer. The cell membrane of digested cells was permeabilized with 0.7% Triton

X-100 in TBS for 15 min at room temperature. The detergent was removed by washing with TBS (5 times for 3 min each) and the cells used for immunofluorescence microscopy.

2.8. Indirect immunofluorescence microscopy

Hyphae were immobilized on coverslips pretreated with poly-L-lysine (M_r 393 000; Sigma). For immunocytochemistry, cells were first incubated for 4 h at 37°C with a final 1:16 dilution of anti-p47 polyclonal antibody and then with a 1:1000 dilution of monoclonal anti-actin antibody (Amersham N350). The cells were rinsed at least 5 times in TBS and then labelled for 2 h at 37°C in the dark with the second antibodies, fluorescein isothiocyanate (FITC)-conjugated anti-mouse IgM (Sigma) for actin detection and rhodamine (TRITC)-conjugated to goat anti-rabbit immunoglobulin (Nordic Biogenzia, Lemania, Lausanne, Switzerland) for 47-kDa peptide detection at a final concentration of 1:80, then rinsed as above.

After this treatment, the germlings were embedded in 90% glycerol with *p*-phenylenediamine [20] and preparations were examined in a Leitz Orthoplan epi-illumination microscope (Ernst Leitz, Wetzlar, Germany) equipped with fluotar optics and selective filter combination. Photographs were taken on HP 5 Ilford black and white film (Ilford, Basel, Switzerland) rated at 1600 ASA. Controls were performed by using only the secondary antibodies.

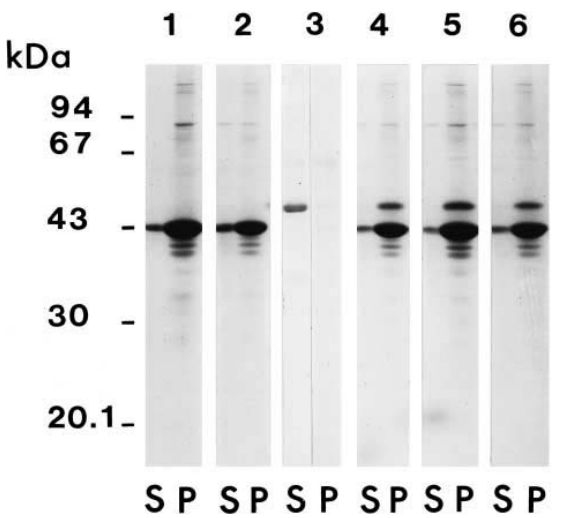

Fig. 2. Co-sedimentation of p47 with F-actin. The pellets (P) and an equivalent volume of the high-speed supernatants (S) of the actin precipitation assays were electrophoresed on SDS-PAGE and staining with Coomassie blue. Lane 1, actin+ATP; lane 2, actin−ATP; lane 3, p47+ATP; lane 4, p47+actin+ATP; lane 5, p47+actin+ATP+Ca^{2+}+CaM; lane 6, p47+actin+ATP+CaM without Ca^{2+}; lane 7, p47+actin+ATP+Ca^{2+} without CaM.

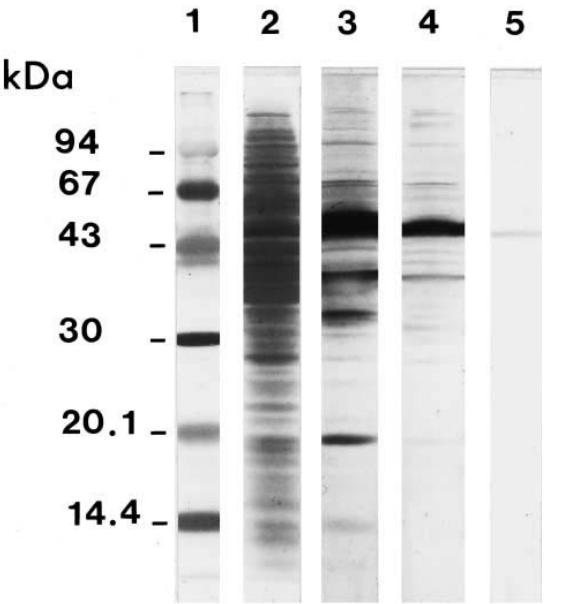

Fig. 3. Immunoblot analysis of crude cytosolic proteins from *N. crassa*. Polyclonal antibody raised against p47 was tested after different purification steps, on Western blots of total protein (40 μg) extracts from germlings of *N. crassa*. Molecular weight markers (1); Ponceau S staining of nitrocellulose after the protein transfer (2); immunostaining with p47 antiserum (3), with anti-p47 eluted from protein A-Sepharose (4), and with affinity-purified polyclonal antibody raised against p47 (5).

3. Results and discussion

The organization of the actin cytoskeleton in non-muscle cells is a subject of great interest in biological research. Several ABPs have been described in fungi, mainly in *Saccharomyces cerevisiae* [6]. However, in *Neurospora*, a fungus with a strong background of classical genetics, no ABPs are known. In order to analyze how calmodulin can regulate the in vitro assembly of actin in MF, our experimental approach was to search for CaM target proteins able to interact with actin. Approximately 18% of the basic solu-

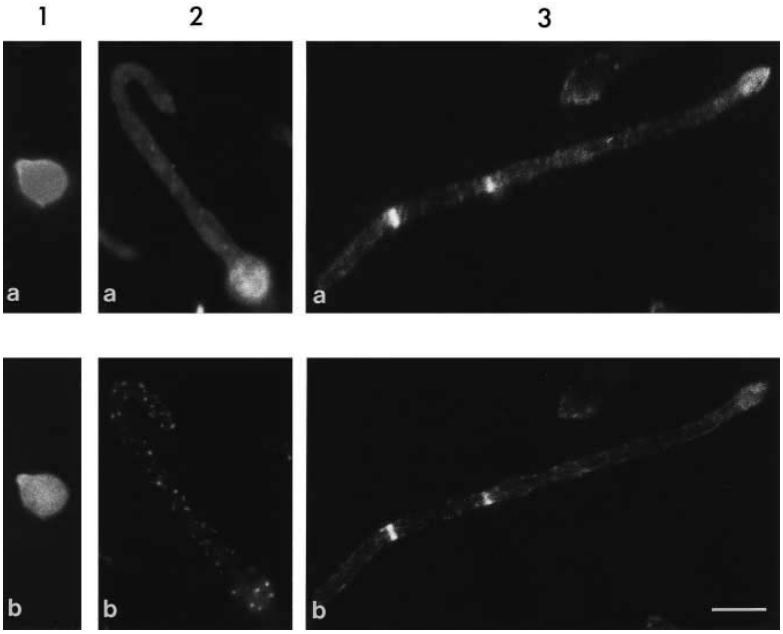

Fig. 4. Indirect double immunofluorescence staining of *N. crassa* cells at different stages of development. Intracellular distribution of p47 (a) and actin (b), in partially digested and permeabilized hyphae, was examined by indirect immunofluorescence as indicated in Section 2. Hyphae growth for 3, 6, and 8 h (1, 2, 3), respectively. The bar in b3 applies to all panels and represents 10 μm.

ble proteins, representing 3% of total soluble proteins from a *N. crassa* crude extract, bound to the CaM-Sepharose 4B column in the presence of calcium and 0.5 M NaCl. After elution by Ca^{2+} chelation, we observed the presence of a major polypeptide corresponding to M_r of 47 kDa, termed p47. Chromatography on the Mono S cation exchange column allowed further purification of p47 to homogeneity (Table 1, Fig. 1A). The relative isoelectric point of 8.25 of the purified protein as determined by NEPGHE (Fig. 1B) indicated clearly that it is a basic protein.

The interaction of p47 to actin was studied by cosedimentation assay as described in Section 2. The SDS-PAGE analysis of both supernatant and pellet fractions after ultracentrifugation showed that (Fig. 2) in the absence of actin, no p47 could be detected by Coomassie blue staining in the pellet lane (Fig. 2, lane 3P), while in the presence of actin both proteins were found in the pellet (Fig. 2, lane 4P), indicating clearly that p47 is an ABP. Aliquots of p47-actin complex were also examined by electron microscopy (data not shown) and under the condition of actin polymerization, i.e. ATP, $MgCl_2$, KCl and low $CaCl_2$, a network of microfilaments was observed. Even if actin precipitates in the absence of ATP (Fig. 2, lane 2P), when analyzed by EM no MF could be detected and only aggregates of actin were visible.

Antibodies, either in the serum or in the affinity purified form, were tested for specificity in immunoblotting reaction with crude extract of germlings and during different steps of its purification. With the immune serum few bands of proteins were revealed in the crude extract (Fig. 3, lane 3) but after their immunopurification, the anti-p47 antibodies reacted exclusively with a single band corresponding to M_r of 47 kDa (Fig. 3, lane 5) indicating the specificity of our antibody. Having confirmed the properties of our polyclonal antibodies, we investigated the cellular distribution of the p47 and actin.

We looked for p47 distribution in germlings and compared it with localization of actin in the same material using immunofluorescence technique. The results indicated that the p47 polypeptide was localized in the cytoplasm and along the cytoplasmic membrane (Fig. 4). The fluorescence patterns of p47 and actin in germ tubes, revealed with antibod-

ies, were found to be virtually identical (Fig. 4). Actin and p47 stained with antibodies were found to be concentrated in the tip of the normally single outgrowing germ tube (Fig. 4a,b) as previously shown for actin alone [8]. After 6 h of germination, actin appeared as homogeneously distributed diffuse spots and patches (Fig. 4, 2b). With anti-p47, however, strong additional staining of the septum of young mycelium was visible (Fig. 4, 3a). The colocalization of both proteins by this technique was, thus, clearly demonstrated. In control experiments the hyphae were labelled with the conjugates only and no detectable staining could be observed (data not shown).

The p47 and actin distribution, visualized by indirect immunofluorescence presented in this report, corroborate the results previously described in *N. crassa* for the actin localization [8] and demonstrate that p47 and actin show the same intracellular distribution.

Our results suggested that p47 might belong to the actin monomer binding protein family [21] and that by its possible dual, active interaction with both actin and calmodulin, it is implicated in the mechanism of polarized growth of *Neurospora* hyphae.

Acknowledgments

We are indebted to M. Ojha for critically reading the manuscript, M.-L. Chappuis and B. Swiderski for excellent technical assistance. We also express our gratitude to the Academic Society of Geneva (F.B.) for financial contribution.

References

[1] Pollard, T.D. (1990) Actin. Curr. Opin. Cell Biol. 2, 33–40.
[2] Kreis, T. and Vale, R. (1993) in: Guidebook to the Cytoskeletal and Motor Proteins (Kreis, T. and Vale, R., Eds.) pp. 15–97, Oxford University Press, New York.
[3] Stossel, T.P., Chaponnier, C., Ezzell, R.M., Hartwig, J.H., Janmey, P.A., Kwiatkowski, D.J., Lind, S.E., Smith, D.B., Southwick, F.S., Yin, H.L. and Zaner, K.S. (1985) Nonmuscle acting-binding proteins. Annu. Rev. Cell Biol. 1, 353–402.
[4] Pollard, T.D. and Cooper, J.A. (1986) Actin and actin-binding proteins. A critical evaluation of mechanisms and functions. Annu. Rev. Biochem. 55, 987–1035.
[5] Vandekerckhove, J. and Vancompernolle, K. (1992) Structural relationships of actin-binding proteins. Curr. Opin. Cell Biol. 4, 36–42.
[6] Drubin, D.G., Miller, K. and Botstein, D. (1988) Yeast actin-binding proteins: evidence for a role in morphogenesis. J. Cell Biol. 107, 2551–2561.
[7] Yang, F., Demma, M., Warren, V., Dharmawardhne, S. and Condeelis, J. (1990) Identification of an actin-binding protein from *Dictyostelium* as elongation factor 1a. Nature 347, 494–496.
[8] Barja, F., Nguyen Thi, B.-N. and Turian, G. (1991) Localization of actin and characterization of its isoforms in the hyphae of *Neurospora crassa*. FEMS Microbiol. Lett. 77, 19–24.
[9] Ortega Perez, R., van Tuinen, D., Marmé, D., Cox, J.A. and Turian, G. (1981) Purification and identification of calmodulin from *Neurospora crassa*. FEBS Lett. 133, 205–208.
[10] Capelli, N., van Tuinen, D., Ortega Perez, R., Arrighi, J.-F. and Turian, G. (1993) Molecular cloning of a cDNA encoding calmodulin from *Neurospora crassa*. FEBS Lett. 321, 63–68.
[11] Laemmli, U.K. (1970) Cleavage of structural proteins during the assembly of the head bacteriophage T4. Nature 227, 680–685.
[12] Zehr, B.D., Savin, T.J. and Hall, R.E. (1989) A one-step, low background Coomassie staining procedure for polyacrylamide gels. Anal. Biochem. 182, 157–159.
[13] Spector, T. (1978) Refinement of the Coomassie blue method of proteins quantification. Anal. Biochem. 86, 142–146.
[14] O'Farrell, P.Z., Goodman, H.M. and O'Farrell, P.H. (1977) High resolution two-dimensional electrophoresis of basic as well as acidic proteins. Cell 12, 1133–1142.
[15] Hudson, L. and Hay, F.C. (1976) in: Practical Immunology, pp. 205–207, Blackwell Scientific, Oxford.
[16] Olmsted, J.B. (1986) Analysis of cytoskeletal structures using blot purified monospecific antibodies. Methods Enzymol. 134, 467–472.
[17] Towbin, H., Staehelin, T. and Gordon, J. (1979) Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets. Procedure and some applications. Proc. Natl. Acad. Sci. USA 76, 4350–4354.
[18] Burnette, W.N. (1981) Western blotting: electrophoretic transfer of proteins from sodium dodecyl sulfate-polyacrylamide gels to unmodified nitrocellulose and radiographic detection with antibody and radio-iodinated protein A. Anal. Biochem. 112, 195–303.
[19] Nunnally, M.H., Powell, L.D. and Craig, S.W. (1981) Reconstitution and regulation of actin gel-sol transformation with purified filamin and villin. J. Biol. Chem. 256, 2083–2086.
[20] Johnson, D.G and de C. Nogueira Araujo, G.M. (1981) A simple method of reducing the fading of immunofluorescence during microscopy. J. Immunol. Methods 43, 349–350.
[21] Sun, H.-Q., Kwiatkowska, K. and Yin, H.I. (1995) Actin monomer binding proteins. Curr. Opin. Cell Biol. 7, 102–110.