Expression and characterization of Cys^{374} mutated human β-actin in two different mammalian cell lines: impaired microfilament organization and stability

A. Tsapara[a], D. Kardassis[a], A. Moustakas[a,1], A. Gravanis[b], C. Stournaras[a,c,*]

[a] *Department of Biochemistry, Division of Basic Sciences, School of Medicine, University of Crete, GR-71110 Heraklion, Greece*
[b] *Department of Pharmacology, Division of Basic Sciences, School of Medicine, University of Crete, GR-71110 Heraklion, Greece*
[c] *Max-Planck-Institute for Cell Biology, Rosenhof, D-68526 Ladenburg, Germany*

Received 9 June 1999

Abstract Previous studies have demonstrated that addition of glutathione at the penultimate Cys^{374} residue of actin results in filaments with diminished mechanical stability. In the present work substitutions introducing a negatively charged (Asp and Glu) or a neutral (Ala) amino acid at position 374 of the human β-actin and tagged at the N-terminus with the flag epitope were studied by transient transfections into Ishikawa human endometrial and opossum kidney cells. Immunofluorescence revealed that microfilaments which incorporated negatively charged mutants were partially to severely disorganized when compared to the almost well-formed actin-Ala^{374} filaments or the wild type actin filaments. Furthermore, microfilaments containing either negatively charged mutant were more sensitive to the destabilizing action of cytochalasin B. In addition, Triton fractionation revealed a considerable reduction of flag-actin content in the Triton insoluble fraction for cells expressing Asp^{374} or Glu^{374} mutant compared to wild type actin. These results demonstrate that negatively charged amino acid residues at the exposed C-terminal tail strongly affect actin microfilament organization and dynamics in vivo.

Key words: β-Actin-Cys^{374} mutant; Flag-tagged actin; Microfilament organization

1. Introduction

Actin is a highly conserved ubiquitous cytoskeletal protein, essential for multiple cellular functions including cytokinesis, cell morphology, cell motility, and cell growth [1]. In non-muscle cells rapid changes of the assembly and organization of the microfilaments seem to be regulated by various extracellular stimuli, indicating participation of actin dynamics in receiving and mediating signals [2–8]. Actin's key role in these processes has been greatly advanced by the elucidation of the atomic structure of monomeric G-actin in complex with different actin binding proteins [9–11], as well as by the construction of an atomic model predicting the orientation and the interactions between subunits within the F-actin filament [12–15]. In all known actin sequences the three C-terminal residues (Lys^{373}Cys^{374}Phe^{375}) are conserved pointing to a possible functional importance of this segment. However, the exact location of this segment in both the monomeric and polymer structure is still uncertain. In the case of G-actin the C-terminal segment is believed to be exposed on the surface of the molecule, accessible to solvent and highly mobile. Upon polymerization it adopts a different conformation which leaves Cys^{374} exposed on the surface of the filament [13,16] while Phe^{375} has been proposed to participate in one of the actin monomer-monomer contact interfaces [12].

The detailed role of the C-terminal tail in actin polymerization dynamics in vitro has been studied previously using actin modified at the penultimate Cys^{374} residue with thiol compounds [17–20], C-terminally truncated actin [20–22], and mutant human β-actin in which Cys^{374} was substituted by serine [23]. In all cases, the properties of filaments assembled from modified actin were changed, including reduced stability, enhanced ATPase activity and increased critical concentration. The in vivo importance of the C-terminal segment for actin assembly has been studied in the yeast *Saccharomyces cerevisiae* by successive deletion of the three last amino acid residues [24]. Deletion of all three residues was lethal, while cells expressing deletions of Phe^{375} or Cys^{374}Phe^{375} were temperature sensitive, with loss of filamentous actin structures and randomly distributed actin patches at the non-permissive temperature. In contrast, yeast cells expressing actin point mutants (Cys^{374} → Ala or Lys^{373} → Met) exhibited no phenotypic alterations. Combined together these structural, biochemical and genetic data stress the importance of the C-terminal segment in polymerization dynamics and filament stabilization.

In order to investigate more precisely the importance of the C-terminal tail in actin microfilament polymerization and stabilization in vivo, we focused on the role of Cys^{374}. For this purpose three different mutations, introducing either a negative (Asp or Glu) or a neutral (Ala) side chain charge in place of the sulfhydryl side chain of cysteine, were prepared. The negatively charged substitutions were designed such that they would chemically mimic the previously characterized glutathionyl-actin [18,19,25]. These actin mutants were expressed in two different mammalian cell lines, Ishikawa human endometrial adenocarcinoma cells and opossum kidney cells. Here we report studies on the effect of the exogenous mutant actins on the organization and overall integrity of the actin cytoskeleton and on the state of polymerization of the mutant actins.

2. Materials and methods

2.1. Materials

Restriction enzymes and DNA polymerases were obtained from

*Corresponding author. Fax: (30) (81) 394.530.
E-mail: cstourn@med.uoc.gr

[1] Present address: Ludwig Institute for Cancer Research, Box 595, S-751 24 Uppsala, Sweden.

PII: S0014-5793(99)00848-0

New England Biolabs (Beverly, MA, USA) or Gibco-BRL (Life Technologies, Inc., Inchinnan, UK). All oligonucleotides were from the Microchemical Facility of the IMBB (Heraklion, Greece). The TNT T7 Quick coupled transcription/translation system was from Promega (Madison, WI, USA). The ECL Western blotting kit, anti-actin antibody, Hyperfilm-ECL films, Sequenase v2 kit and ^{35}S-labeled methionine were from Amersham Corp. (Arlington Heights, IL, USA). The anti-flag M2 monoclonal antibody was from IBI/Kodak (New Haven, CT, USA) and the anti-mouse IgG antibody conjugated to fluorescein isothiocyanate (FITC) from Chemicon (Temecula, CA, USA). Rhodamine-phalloidin and slow fade reagent were from Molecular Probes Inc. (Eugene, OR, USA). All other chemicals were obtained from usual commercial sources at the purest grade available.

2.2. Construction of mutant actin cDNAs

All DNA manipulations were performed according to standard procedures [26]. Actin point mutations were introduced by PCR. The human β-actin cDNA sequence, bp 34–1184 (including the whole protein encoding sequence (bp 42–1167)), cloned into the *Eco*RV site of pBluescript SK (pBS-hβ-actin, a generous gift from Dr. G. Mavrothalassitis) served as template. For the generation of point mutants $Cys^{374} \rightarrow Ala^{374}$, Asp^{374} or Glu^{374} appropriate 3′ primers (5′ GGC TCG AGG ATC CGC CTA GAA **GGC** TTT GCG GTG GAC 3′, 5′ GGC TCG AGG ATC CGC CTA GAA **GTC** TTT GCG GTG GAC 3′, 5′ GGC TCG AGG ATC CGC CTA GAA **TTC** TTT GCG GTG GAC 3′, where bold letters indicate the mutated codons) and a common 5′ primer (5′ GGC TCG AGA AGC TTC ACC ATG GAT GAT GAT ATC GCC GC 3′) were designed and used. In the case of $Cys^{374} \rightarrow Asp^{374}$ or Glu^{374} mutants, the PCR product was digested with *Hin*dIII and *Bam*HI and cloned into the mammalian expression vector pCB6. The two pCB6 constructs were digested with *Eco*RV and *Bam*HI and the insert (actin sequence, bp 54–1174, lacking the first three amino acid residues) was transferred to *Eco*RV/*Bam*HI digested pSG5-flag vector (a generous gift from Dr. E. Hatzivassiliou, Harvard Medical School, USA), downstream of the flag epitope sequence and in the same frame with it. The resulting constructs pSG5-flag-actin-Asp^{374} and pSG5-flag-actin-Glu^{374} express an N-terminal flag-tagged actin mutant, where the eight residue flag epitope sequence is linked, through a six amino acid long linker to actin amino acids 4–375, mutated at position 374. To generate pSG5-flag-actin-Ala^{374} the PCR product was digested with *Eco*RV and *Bam*HI and the resulting fragment was inserted to *Eco*RV/*Bam*HI digested pSG5-flag vector. Finally, for construction of the pSG5-flag-actin-Cys^{374} (wt) control plasmid, the *Eco*RV/*Xho*I actin insert from pBS-hβ-actin was subcloned into pSG5-flag vector. All mutations introduced by PCR and the in-frame fusion of the flag epitope with the actin N-terminus were verified by DNA sequencing.

2.3. Cell culture and transient transfections

Ishikawa cells were established as a permanent cell line from a well-differentiated endometrial adenocarcinoma [27]. Opossum kidney cells (OK cells) provided by the American Type Culture Collection were studied between passages 40 and 50. Both cell lines were maintained as monolayers in regular growth medium as previously described [4,27]. For immunofluorescence and Western analysis experiments, Ishikawa or OK cells plated at $2–2.5 \times 10^5$ per 35 mm diameter dish were transiently transfected with 6 μg of pSG5-flag-actin-X^{374} construct (where X = Cys(wt), Ala, Asp or Glu) by the calcium-phosphate protocol [28]. After overnight incubation with the DNA-calcium phosphate co-precipitate, cells were washed and cultured in regular growth medium for 24 h. For immunofluorescence experiments cells were then trypsinized and plated in new 35 mm dishes with glass coverslips and were incubated overnight in regular growth medium.

2.4. Fluorescence microscopy

For morphological observations by fluorescence microscopy, transiently transfected Ishikawa or OK cells were cultured for 24 h on glass coverslips in regular growth medium, followed by 12 h incubation in medium lacking serum. In the appropriate experiments Ishikawa cells were incubated for 1 h with increasing concentrations of cytochalasin B (0.5–7.5 μM) or with 1 μM phallacidin. Control cells were treated with the same amount of ethanol. Cells were fixed with 3.7% v/v formaldehyde in phosphate buffer saline (PBS) and subsequently permeabilized with acetone. Specimens were incubated with anti-flag M2 monoclonal antibody (1:100 dilution in PBS containing 1.5% FCS) at 4°C for 1 h (indirect fluorescence), followed by incubation with secondary goat anti-mouse IgG antibody conjugated to fluorescein isothiocyanate (FITC) at 4°C for 1 h. Finally specimen were mounted on glass slides with slow-fade. For double fluorescent experiments, rhodamine-phalloidin staining followed the indirect immunofluorescence step. Cells were incubated with 0.25 μM rhodamine-phalloidin in a humid chamber for 40 min at room temperature (direct fluorescence). The coverslips were observed using either a confocal laser scanning module as previously described [5] or an Olympus BH-2 microscope equipped with epifluorescent illumination and photographed with a 35 mm Olympus (C-35AD-4) camera on Kodak P3200 black and white film.

2.5. Western analysis

2.5.1. Total flag-actin determination. For detection of flag-actin, Ishikawa cells were cultured for 24 h following transfection in regular growth medium. Cells were washed with ice-cold PBS and immediately scraped into 100 μl of lysis buffer (0.9% NaCl, 20 mM Tris-HCl, 0.5% Triton X-100, 400 μM PMSF, 10 μM leupeptin, pH 7.4). Equal amounts of total protein (50 μg) of the cell extracts were subjected to SDS-PAGE and the resolved proteins were transferred to nitrocellulose membranes, using an LKB electroblot apparatus (LKB, Bromma, Sweden). Nitrocellulose blots were incubated with monoclonal mouse anti-flag M2 and anti-actin antibodies, followed by incubation with the appropriately labelled secondary antibody, by using the ECL Western blotting kit.

2.5.2. Flag-actin determination in detergent extracts. For measurements of monomeric (Triton soluble) and polymeric (Triton insoluble) actin levels, Ishikawa and OK cells were cultured for 24 h following transfection in regular growth medium, followed by 12 h incubation in medium lacking serum. In the appropriate experiments, Ishikawa cells were incubated for 30 min with 10^{-7} M dexamethasone. Control cells were incubated with the same amount of vehicle. After treatment, Triton X-100 fractionation was performed as previously described [29]. Equal volumes of each fraction were subjected to SDS-PAGE and Western analysis as above. Band intensities were quantified by the Molecular Analyst Software (Bio-Rad, Palo Alto, CA, USA).

2.6. Co-assembly of wild type and mutant flag-actins with carrier actin

Wild type or mutant flag-actins were transcribed/translated from the pSG5-flag-actin-X^{374} constructs in the presence of ^{35}S-labeled methionine, using the TNT T7 Quick coupled transcription/translation system according to the manufacturer's instructions. The final volume of each transcription/translation reaction was 25 μl. Rabbit skeletal muscle actin was isolated from acetone powder and used for the co-assembly assays to a final concentration of 22 μM in G-buffer (Tris HCl 2 mM, ATP 0.2 mM, pH 7.4). 20 μl of the transcription/translation reaction mixture was added to G-actin and polymerization was started by addition of $MgCl_2$ to a final concentration of 2 mM. After 1 h incubation at room temperature, assembled actin was pelleted by centrifugation at $150\,000 \times g$ in a Beckman TLS 55 rotor (Beckman Instruments, Palo Alto, CA, USA) at 4°C for 15 min. Pellets were resuspended in Tris-SDS buffer (Tris-HCl 0.625 M pH 7.4, 2% SDS and 10% glycerol) and equivalent supernatant and pellet volumes were analyzed by SDS-PAGE. After electrophoresis the gel was stained with Coomassie brilliant blue, destained, vacuum-dried and exposed to Hyperfilm-ECL film for a minimum of 18 h.

3. Results and discussion

3.1. Construction of point substitution mutants of Cys^{374} in human β-actin and expression of mutant actins in mammalian cells

Three mutations in human β-actin were generated by substitution of Cys^{374} with Ala^{374}, Asp^{374} or Glu^{374}. Wild type (wt) and mutant actins were tagged with the flag epitope. The resulting pSG5-flag-actin constructs, when transfected to mammalian cells, should express actin with the flag epitope tagged at the N-terminus through a six amino acid long linker (Fig. 1A) and thus be easily distinguished from endogenous cellular β-actin. Indeed, immunoblot analysis of total cell extracts from Ishikawa cells transiently expressing all four con-

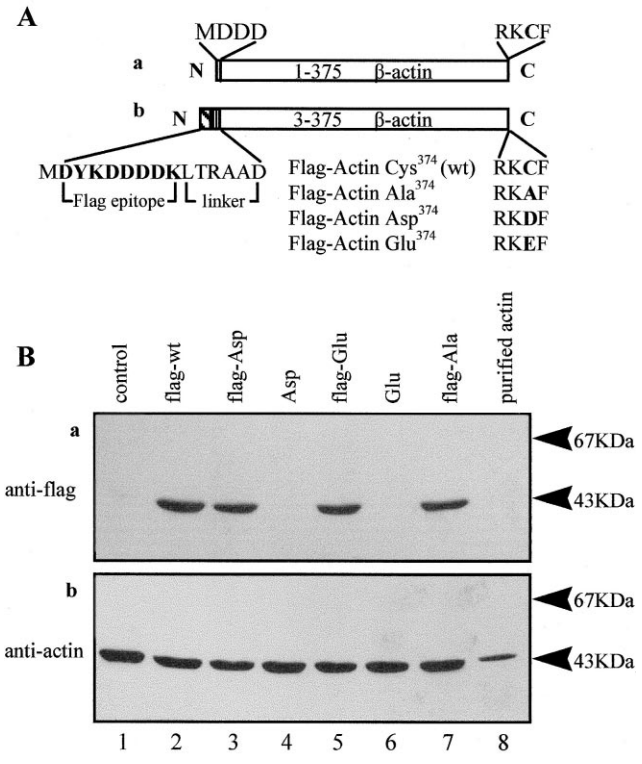

Fig. 1. A: Schematic representation of (a) β-actin and (b) wt and mutant flag-actin amino acid sequences. The β-actin sequence is represented as white box and the number of amino acid (aa) residues in each protein is indicated. Amino- and carboxy-terminal aa sequences are shown with residue 374 in bold. In (b) the flag epitope is represented as a diagonally hatched box (bold aa sequence) and the linker segment as a vertically hatched box. The nomenclature of the four flag-actins used in this study is listed along their corresponding carboxy-terminal aa sequence. B: Expression of flag-actin constructs in Ishikawa cells. Immunoblot analysis of equal amounts of total cellular protein from Ishikawa cells transfected with different actin constructs using monoclonal antibodies against (a) the flag epitope and (b) actin. Lane 1: non-transfected Ishikawa cells; lanes 2–7: Ishikawa cells transfected with pSG5-flag-actin-Cys^{374} (wt) (lane 2), pSG5-flag-actin-Asp^{374} (lane 3), pCB6-actin-Asp^{374} (lane 4), pSG5-flag-actin-Glu^{374} (lane 5), pCB6-actin-Glu^{374} (lane 6), pSG5-flag-actin-Ala^{374} (lane 7); lane 8: purified rabbit skeletal muscle actin. Arrowheads indicate the protein molecular marker positions.

structs (wt and the three mutants) showed that the anti-flag antibody detected a protein of the appropriate molecular size (Fig. 1Ba, lanes 2,3,5,7). Furthermore, the electrophoretic mobility of this band was identical to that of cellular or purified rabbit skeletal muscle actin recognized by a monoclonal anti-actin antibody (Fig. 1Bb, lanes 1–8). The specificity of the anti-flag antibody was evident from the fact that in non-transfected Ishikawa cells or in cells transfected with the non-flag-actin constructs no signal was observed (Fig. 1Ba, lanes 1,4,6).

3.2. *Effect of flag-actin expression on the actin cytoskeleton architecture – Mutant actin protein incorporation in microfilaments in vivo*

Ishikawa cells transfected with flag-actins were indirectly stained with the anti-flag antibody and analyzed with confocal laser scanning microscopy. For flag-actin-Cys^{374} (wt) well organized microfilament structures, including stress fibers, were observed (Fig. 2A) and the overall cell morphology was very similar to the actin cytoskeleton of non-transfected cells when stained directly with rhodamine-phalloidin. Thus, introduction of exogenous actin into mammalian cells did not cause any major disturbance in the organization of the actin cytoskeleton, although downregulation of β and/or γ actin expression cannot be excluded [30,31]. In addition, the presence of the N-terminally tagged flag epitope which resulted in sequence alterations of this segment of the molecule (Fig. 1A and Section 2) did not result in aberrant behavior of the actin molecule at least at the level of incorporation into functional microfilaments and cytoskeletal organization. The mutations at position 374 exhibited various effects on the actin cytoskeleton organization. The Ala^{374} mutant performed essentially like the wt actin as judged by immunofluorescence studies (Fig. 2B). In contrast, the effect of flag-actin-Asp^{374} or flag-actin-Glu^{374} expression was very severe, ranging from partial disruption to complete disorganization of microfilament structures. In most specimens diffuse staining with simultaneous intense peripheral submembranous fluorescence, probably due to cortical flag-actin, was observed (Fig. 2C,D). In some cases shorter filamentous structures and even shorter stress fibers were seen (Fig. 2, C4 and D4). A positive correlation between the intensity of fluorescence of each transfected cell with the level of cytoskeletal disorganization was observed indicating that the diversity of the effect may be due to differences in the expression levels of the mutant actins obtained at individual cells.

When flag-actin constructs were transfected to OK cells, expression of flag-wt or flag-Ala^{374} mutant had no effect on microfilament organization, in agreement with the results obtained for Ishikawa cells. The actin cytoskeleton architecture was well preserved as was revealed by indirect anti-flag staining, as well as by double staining. A well organized array of stress fibers and a bright staining of leading lamellae and membrane ruffles were observed by indirect immunofluorescence (Fig. 3a,b). In contrast, expression of negatively charged Asp^{374} and Glu^{374} mutants did not produce the extensive deleterious effect obtained in Ishikawa cells. In the majority of specimens stress fibers were still observed, albeit in many cases less extended as compared to flag-wt expressing cells. Further-

Table 1
Quantification of the distribution of flag-actin into Triton X-100 fractions

Cells transfected with flag-actin	Ishikawa cells			OK cells		
	% TI/TS+TI		% wt	% TI/TS+TI		% wt
Wild type (Cys^{374})	59.15 ± 4.8	(n = 5)	100	68.0 ± 7.1	(n = 10)	100
Mutant Ala^{374}	48.00 ± 4.6**	(n = 7)	81.1	51.2 ± 5.9**	(n = 10)	75.3
Mutant Asp^{374}	40.10 ± 2.6**	(n = 3)	67.8	28.4 ± 10.4**	(n = 11)	41.7
Mutant Glu^{374}	39.10 ± 11.4*	(n = 5)	66.1	37.9 ± 11.2**	(n = 10)	55.7

Ratio of Triton insoluble (TI) to total (Triton soluble and insoluble, TS+TI) cellular flag-actin from transiently transfected Ishikawa and OK cells. Values are mean ± S.D. from n distinct experiments. Significance levels (defined by unpaired Student's t-test): *$P < 0.05$; **$P < 0.01$.

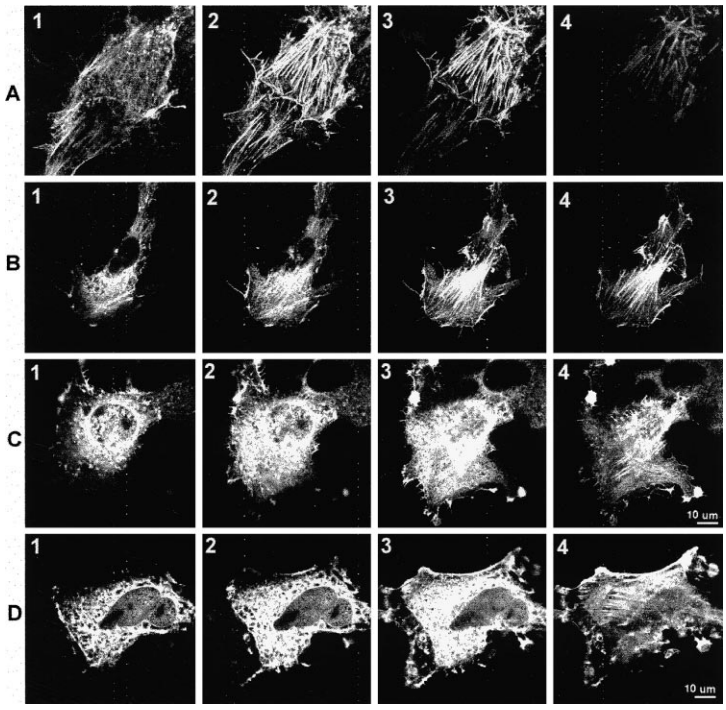

Fig. 2. Confocal laser scanning analysis of Ishikawa cells expressing flag-actins stained indirectly with anti-flag antibody. Ishikawa cells expressing (A) flag-actin-Cys^{374} (wt), (B) flag-actin-Ala^{374}, (C) flag-actin-Asp^{374} and (D) flag-actin-Glu^{374}. 1–4 represent scanning sections from the upper cytoplasmic region towards the attachment site of the cells. The step size of the optical sections was adjusted to 0.5 μm (bar: 10 μm).

more cells completely lacking filamentous structures were very rare. The most pronounced effect, besides partial stress fiber disruption, was the increase in the intensity of the leading lamellae and membrane ruffle staining. This was often accompanied by the appearance of intensely stained submembranous extensions with different morphology from lamellipodia, filopodia or membrane ruffles (Fig. 3c,d). The different behavior of the two cell lines may be due to the better formed and more extended stress fiber organization in OK cells compared to the microfilament architecture of Ishikawa cells. Additionally, the observed subcellular localization of the negatively charged mutants being incorporated primarily into highly motile regions in OK cells, while appearing rather diffuse in Ishikawa cells, may also contribute to the difference in the intensity of microfilament disorganization observed between these two cell lines.

The differences observed in the actin cytoskeletal organization of cells expressing actin mutants could not be attributed to the partitioning of the actin mutants into monomeric pools or loss of their ability to interact with endogenous actin, since double staining revealed identical fluorescence images for both indirect anti-flag/FITC and direct rhodamine-phalloidin staining (data not shown). The fact that microfilament structures which contain only endogenous cellular actin were not detected suggests that exogenous negatively charged actin molecules can be recognized by the cell as interchangeable units capable of actively participating in polymerization dynamics. Similarly, wt and Ala^{374} mutant were always incorporated into microfilament structures together with endogenous cellular actin. No segregation of the transfected flag-actins from the cellular actin was obvious. The observed structures must result from the active incorporation of flag-actins to the en-

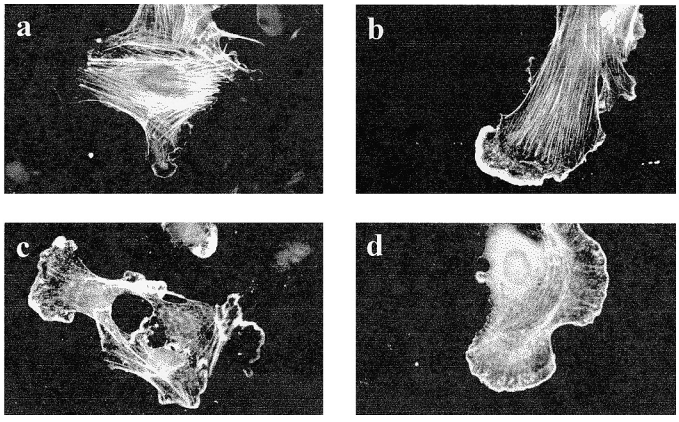

Fig. 3. Fluorescence analysis of OK cells expressing flag-actins stained indirectly with anti-flag antibody. OK cells expressing (a) flag-actin-Cys^{374} (wt), (b) flag-actin-Ala^{374}, (c) flag-actin-Asp^{374} and (d) flag-actin-Glu^{374}.

dogenous dynamic actin cytoskeleton and not from self-polymerization.

3.3. Quantitation of flag-actin subcellular distribution by Triton X-100 fractionation

The incorporation of flag-actins into microfilaments in Ishikawa and OK cells was analyzed by quantitative immunoblot determination of the Triton soluble (TS) and insoluble (TI) cellular flag-actin content. As calculated from the relative band intensities, in both cell lines wt flag-actin was detected at a relative distribution of 30–40% soluble and 60–70% insoluble (Table 1) which falls well within the range of distribution of the endogenous cellular actin of these two cell lines [4,6]. In the case of Ishikawa cells the TI/TI+TS ratio for actin-Ala^{374} exhibited a statistically significant decrease of 18.9% compared to that for wt actin. However, the TI/TI+TS ratio was decreased by 32.2% for actin-Asp^{374} and by 33.9% for actin-Glu^{374} (Table 1). Surprisingly, considering the differences observed in the immunofluorescence images, similar or rather more pronounced results were obtained for OK cells. The TI/TI+TS ratio decreased by 24.7% for actin-Ala^{374} compared to wt actin, by 58.3% for actin-Asp^{374} and by 44.3% for actin-Glu^{374} (Table 1).

3.4. In vitro incorporation of mutant actin protein in microfilaments

Flag-actins, wt and the three mutants were transcribed/translated in the presence of ^{35}S-labeled methionine and their ability to co-polymerize with purified rabbit skeletal muscle actin in vitro was studied. When transcription/translations were added to carrier actin and allowed to polymerize all four flag-actins (wt and the three mutants) were co-sedimented with carrier actin. In each case the majority of the labeled actin was detected in the pellet (Fig. 4B, lanes 2,4,6,8). Some unpolymerized flag-actin was also detected in the supernatant (Fig. 4B, lanes 1,3,5,7). This result does not indicate a reduced ability of flag-actin to polymerize, but it rather suggests that the in vitro transcribed/translated actin follows the distribution of carrier actin (Fig. 4A). Furthermore, no difference in the amount of pelleted flag-actin between wt and mutants was observed indicating that mutants

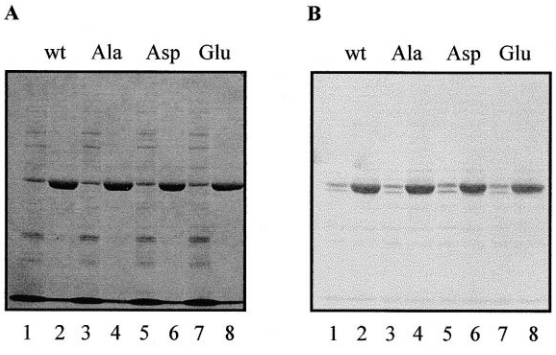

Fig. 4. Co-polymerization of flag-actin and skeletal muscle actin. Flag-actins, wt or mutants, were in vitro transcribed/translated in the presence of ^{35}S-labeled methionine, co-assembled with carrier rabbit skeletal muscle actin and precipitated by ultracentrifugation. A: Coomassie brilliant blue stained SDS-polyacrylamide gel of supernatants (lanes 1,3,5,7) and pellets (lanes 2,4,6,8). B: Autoradiography of the same gel. Carrier actin co-assembled with wt (lanes 1 and 2) or Ala^{374} (lanes 3 and 4), Asp^{374} (lanes 5 and 6) and Glu^{374} (lanes 7 and 8) mutants.

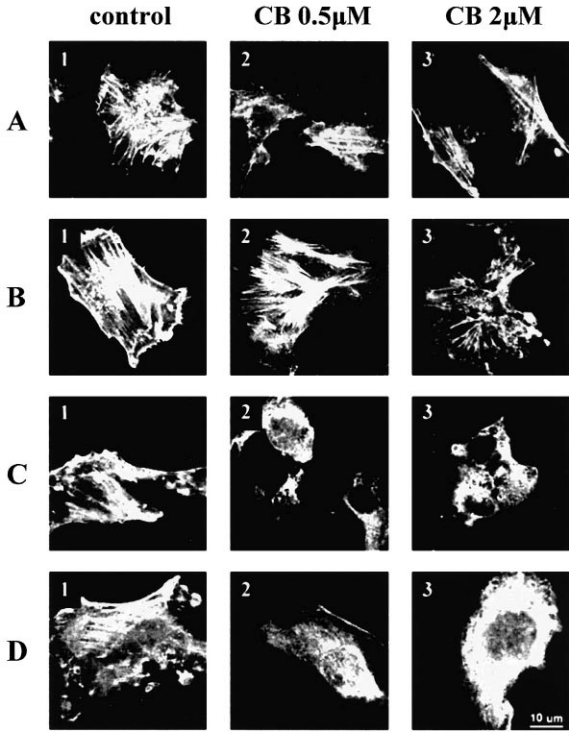

Fig. 5. Effect of cytochalasin B on the actin microfilament organization of Ishikawa cells expressing flag-actins. Confocal laser scanning micrographs of Ishikawa cells expressing (A) flag-actin-Cys^{374} (wt), (B) flag-actin-Ala^{374}, (C) flag-actin-Asp^{374} and (D) flag-actin-Glu^{374} stained indirectly with anti-flag antibody. Cells were incubated for 1 h with cytochalasin B 0.5 μM or 1 μM or with the corresponding volume of ethanol (control).

are capable of polymerization, although changes in polymerization dynamics, such as on and off rates for barbed and pointed filament ends or critical concentration, cannot be excluded.

3.5. Morphological and quantitative analysis of the stability and dynamics of mutant actin cytoskeleton

The effect of cytochalasin B and phallacidin on cells transfected with flag-actin was studied by indirect immunofluorescence and confocal laser scanning microscopy. At relatively low cytochalasin B concentrations (0.5–2 μM) the microfilament organization in flag-actin-wt and flag-actin-Ala^{374} expressing cells was not altered. In contrast, in flag-actin-Asp^{374} and -Glu^{374} expressing cells the short filamentous structures were completely disrupted and actin accumulated into intensely fluorescent patches, accompanied sometimes with changes in cell shape (Fig. 5). In the presence of 1 μM phallacidin no change in the filamentous flag-actin (wt or mutant) organization was evident. The effect of phallacidin was further studied by performing transfection and subsequent culture in the continuous presence of 0.1 μM phallacidin. Immunofluorescence analysis as well as Triton X-100 fractionation pointed to a slightly impaired microfilament organization even for wt actin (data not shown).

Finally the effect of dexamethasone on the dynamics of exogenous flag-actin was studied by measuring the TI/TI+TS ratio after treatment of Ishikawa cells for 30 min with 10^{-7} M dexamethasone, conditions previously shown

to induce rapid actin polymerization [6,7]. For all three mutants, the TI/TI+TS ratio exhibited a small increase, albeit not statistically significant, comparable to that for wt actin (data not shown), indicating that differential actin cytoskeleton organization and stability do not necessarily inhibit functionality and the ability to respond to extracellular signals by modulating the polymerization state.

4. Conclusion

The effect of the expression of the actin mutants studied here cannot be solely attributed to the removal of the sulfhydryl group of Cys^{374} since the Ala^{374} mutant behaved similarly to the wt. Rather, the effect must be due to a change in the chemical environment of the C-terminal segment which is introduced by the negative charge or the hydrogen bonding ability of the Asp or Glu side chains. Although Cys^{374} is not directly implicated in the actin monomer to monomer contacts in the filament it has been proposed that actin polymerization results in a chemical change of the Cys^{374} environment which is important for filament assembly and function [16,32]. In addition, the neighboring Phe^{375} is proposed to be involved in actin monomer to monomer contacts [12]. Furthermore, it is already known that the bottom edge of the actin molecule and especially subdomain 1, where the C-terminal segment is located, is the binding site for different actin binding proteins [33]. Changes at or near these binding sites are expected to destroy or attenuate the interactions between actin and actin binding proteins, leading eventually to disorganization of the microfilaments, since it is through their association with actin that actin binding proteins modulate the actin polymerization dynamics, thus allowing remodeling of actin cytoskeleton in response to intracellular or extracellular stimuli [34–37]. It is tempting to speculate that the effect of the negatively charged mutants is produced by the combination of filament structural destabilization and inhibition of actin binding protein function, leading to reduced actin polymerization and establishment of a steady state displaced towards monomeric actin. This difference in the steady state would result in altered cytoskeleton morphology but not necessarily in loss of the ability to respond to extracellular stimuli, as our preliminary dexamethasone results suggest.

In conclusion, this study provides evidence about the importance that the specific chemical environment around the C-terminus of the actin molecule can have in the state of polymerization and the overall cellular integrity of the actin cytoskeleton. This is the first attempt to approach this problem in mammalian cells and the results obtained are in agreement with previously published in vitro studies on the role of Cys^{374} in actin polymerization dynamics. Finally, the system of transfected flag-actins presented here provides a useful tool in handling questions of regulation of microfilament assembly by structural determinants of the actin molecule itself or by environmental factors under in vivo conditions.

Acknowledgements: The authors thank Prof. Dr. Peter Traub and the Max-Planck-Gesellschaft zur Förderung der Wissenschaften for their generous support of this project. We would also like to thank Dr. R. Hartig (Max-Planck-Institute for Cell Biology, Ladenburg) for assistance with confocal laser microscopy. This work was further supported in part by the Biomedical Research Committee of the Greek Ministry of Health and the Greek Secretariat of Research and Technology (GGET).

References

[1] Mitchison, T.J. and Cramer, L.P. (1996) Cell 84, 371–379.
[2] Cantiello, H.F., Prat, A.G., Bonventre, J.V., Cunningham, C.C., Hartwig, J.H. and Ausiello, D.A. (1993) J. Biol. Chem. 268, 4596–4599.
[3] Theodoropoulos, P.A., Stournaras, C., Stoll, B., Markogiannakis, E., Lang, F., Gravanis, A. and Häussinger, D. (1992) FEBS Lett. 311, 241–245.
[4] Papakonstanti, E.A., Emmanouel, D.S., Gravanis, A. and Stournaras, C. (1996) Biochem. J. 315, 241–247.
[5] Papakonstanti, E.A., Bakogeorgou, E., Castanas, E., Emmanouel, D.S., Hartig, R. and Stournaras, C. (1998) J. Cell. Biochem. 70, 60–69.
[6] Koukouritaki, S.B., Theodoropoulos, P.A., Margioris, A.N., Gravanis, A. and Stournaras, C. (1996) J. Cell. Biochem. 62, 251–261.
[7] Koukouritaki, S.B., Margioris, A.N., Gravanis, A., Hartig, R. and Stournaras, C. (1997) J. Cell. Biochem. 65, 492–500.
[8] Moustakas, A. and Stournaras, C. (1999) J. Cell Sci. 192, 1169–1179.
[9] Kabsch, W., Mannherz, H.G., Suck, D., Pai, E.F. and Holmes, K.C. (1990) Nature 347, 37–44.
[10] McLaughlin, P.J., Gooch, J.T., Mannherz, H.G. and Weeds, A.G. (1993) Nature 364, 685–692.
[11] Schutt, C.E., Myslik, J.C., Rozycki, M.D., Goonesekere, N.C. and Lindberg, U. (1993) Nature 365, 810–816.
[12] Holmes, K.C., Popp, D., Gebhard, W. and Kabsch, W. (1990) Nature 347, 44–49.
[13] Milligan, R.A., Whittaker, M. and Safer, D. (1990) Nature 348, 217–221.
[14] Egelman, E.H. and Orlova, A. (1995) Curr. Opin. Struct. Biol. 5, 172–180.
[15] Steinmetz, M.O., Stoffler, D., Hoenger, A., Bremer, A. and Aebi, U. (1997) J. Struct. Biol. 119, 295–320.
[16] Orlova, A. and Egelman, E.H. (1995) J. Mol. Biol. 245, 582–597.
[17] Stournaras, C., Merkler, I. and Faulstich, H. (1988) Biochem. Biophys. Res. Commun. 155, 962–970.
[18] Stournaras, C., Drewes, G., Blackholm, H., Merkler, I. and Faulstich, H. (1990) Biochim. Biophys. Acta 1037, 86–91.
[19] Drewes, G. and Faulstich, H. (1990) J. Biol. Chem. 265, 3017–3021.
[20] Drewes, G. and Faulstich, H. (1993) Eur. J. Biochem. 212, 247–253.
[21] Mossakowska, M., Moraczewska, J., Khaitlina, S. and Strzelecka-Golaszewska, H. (1993) Biochem. J. 289, 897–902.
[22] Strzelecka-Golaszewska, H., Mossakowska, M., Wozniak, A., Moraczewska, J. and Nakayama, H. (1995) Biochem. J. 307, 527–534.
[23] Aspenstrom, P., Schutt, C.E., Lindberg, U. and Karlsson, R. (1993) FEBS Lett. 329, 163–170.
[24] Johannes, F.J. and Gallwitz, D. (1991) EMBO J. 10, 3951–3958.
[25] Theodoropoulos, P.A., Gravanis, A., Tsapara, A., Margioris, A.N., Papadogiorgaki, E., Galanopoulos, V. and Stournaras, C. (1994) Biochem. Pharmacol. 47, 1875–1881.
[26] Sambrook, J., Fritsch, E.F. and Maniatis, T. (1989) Molecular Cloning: A Laboratory Manual, 2nd edn., Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.
[27] Gravanis, A. and Gurpide, E. (1986) J. Clin. Endocrinol. Metab. 63, 356–359.
[28] Graham, F.L. and van der Eb, A.J. (1973) Virology 52, 456–467.
[29] Golenhofen, N., Brian Doctor, R., Bacallao, R. and Mandel, L.J. (1995) Kidney Int. 48, 1837–1845.
[30] Ng, S.Y., Erba, H., Latter, G., Kedes, L. and Leavitt, J. (1988) Mol. Cell. Biol. 8, 1790–1794.
[31] Lloyd, C., Schevzov, G. and Gunning, P. (1992) J. Cell. Biol. 117, 787–797.
[32] Brauer, M. and Sykes, B.D. (1986) Biochemistry 25, 2187–2191.
[33] Rozycki, M.D., Myslik, J.C., Shutt, C.E. and Lindberg, U. (1994) Curr. Opin. Cell. Biol. 6, 87–95.
[34] Safer, D.A. and Cooper, J.A. (1995) Annu. Rev. Cell. Biol. 11, 497–518.
[35] Fechheimer, M. and Zigmond, S.H. (1993) J. Cell. Biol. 123, 1–5.
[36] Carlier, M-F. and Pantaloni, D. (1997) J. Mol. Biol. 269, 459–467.
[37] Welch, M.D., Mallavarapu, A., Rosenblatt, J. and Mitchison, T.J. (1997) Curr. Opin. Cell. Biol. 9, 54–61.