Actin antibodies

Preparation and characterization of antibodies specific for smooth-muscle actin isoforms

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We have determined the specificity of sera elicited by glutaraldehyde-stabilized bovine aortic actin. This modification induces a high titre of antibodies directed against the *N*-terminal (residues 1–39) and the *C*-terminal region of smooth-muscle actins. The crude antisera were purified on peptide (corresponding to the 1–9 or 1–8 *N*-terminal sequences of smooth-muscle isoactins)–polyacrylic-resin columns. By fractionating the antisera we obtained oligoclonal antibody populations specific for each isoactin.

INTRODUCTION

Actin is the major protein in skeletal and smooth muscles. In mammalian smooth muscles, four isoactins are expressed (Vandekerckhove & Weber, 1979) and their distribution appears to be tissue-specific (Small & Sobieszek, 1983; Fatigati & Murphy, 1984). These isoactins show a very high degree of homology in their primary structure, except in the *N*-terminal region (Vandekerckhove & Weber, 1981). It is now accepted that actin in smooth muscles has contractile and cytostructural functions (Bagby, 1983). However, no correlation between a specific isoactin and a given function has been established. The development of antibodies specific for each isoform appears to be a necessary step in assigning a precise role to each isoform. Although isoactins are highly conserved during development, their immune tolerance can be broken by slight chemical modifications of actin (Benyamin *et al.*, 1983). Some of the antibodies elicited by glutaraldehyde-stabilized F-actin are directed against the *N*-terminal residues 1–28 sequence (Benyamin *et al.*, 1986). We have also reported the presence of two antigenic continuous epitopes in skeletal-muscle actin, corresponding to the sequences embracing residues 1–7 and 18–28 (Roustan *et al.*, 1986).

In the present paper we describe the preparation of antibodies elicited by immunization with glutaraldehyde-stabilized bovine aortic F-actin. The resulting polyclonal antiserum was fractionated on a column of polyacrylic resin bound to peptide corresponding to residues 1–9 or 1–8 of the *N*-terminal sequences of the smooth-muscle isoforms (SM α , SM γ , NM β , NM γ). These fractionations yielded recognition of the sequence comprising residues 1–7, which are specific for the isoactins.

MATERIALS AND METHODS

Actins were obtained from rabbit skeletal muscle, bovine cardiac muscle and scallop adductor muscle as described by Pardee & Spudich (1982). Actins from bovine aortic muscle and chicken-gizzard muscle were prepared as described by Cavadore *et al.* (1985a). A cellular extract of macrophages containing actin was

prepared by the method of, and was generously given by, Dr. M. Pacaud (Pacaud, 1986). F-actins were coupled to CNBr-activated Sepharose 4B (Pharmacia) after glutaraldehyde treatment (Herman & Pollard, 1979). Carboxymethylated actins were prepared as described by Konigsberg (1972) and coupled to CNBr-activated Sepharose. Actin peptides were obtained by proteolysis with *Staphylococcus aureus* V8 proteinase (Roustan *et al.*, 1985), which gave two major fragments, (1–226)-peptide (*N*-terminal sequence) and (227–375)-peptide (*C*-terminal sequence), and a minor *N*-terminal fragment with an apparent M_r of 21000 resulting from a secondary cleavage at residue 167. A thrombin digest of actin (Muszbek & Laki, 1974) gave three major peptides, (40–113)-, (114–375)- and (40–375)-peptide, designated 'M', 'L' and 'K' respectively, and two small peptides [(1–28)- and (29–39)-peptide].

Solid-phase synthesis of *N*-terminal (1–9)- or (1–8)-peptide of α and γ smooth-muscle isoactins (SM α and SM γ) and β and γ non-muscle isoforms (NM β and NM γ) was performed using polyacrylic resin (Calas *et al.*, 1985). *t*-Butoxycarbonyl protection was adopted for all amino acids used in these syntheses. The syntheses were monitored by ninhydrin assay (Kaiser *et al.*, 1970). The amount of peptide linked to the resin was determined, after acidic hydrolysis, by amino acid analyses on a Chromakon 400 apparatus (Kontron).

Protein concentrations were determined by the method of Spector (1978) with globulins as the standard or by using the following absorbance values: actin, $A_{290}^{0.1\%} = 0.63$; IgG, $A_{280}^{0.1\%} = 1.5$ (Benyamin *et al.*, 1985). Polyacrylamide-gel electrophoresis was carried out in the presence of 0.1% SDS as described by Laemmli (1970), using the discontinuous buffer system and 15% (w/v) polyacrylamide.

Sheep were immunized by multi-site intradermal injections with 2 mg (per injection) of glutaraldehyde-stabilized aortic F-actin emulsified in Freund's complete adjuvant. Booster injections were given 15 days after the first injection and then monthly with antigen in Freund's incomplete adjuvant. Blood (250 ml) was taken from the jugular vein 10 days after every booster injection.

The sera obtained were purified on a glutaraldehyde-stabilized aortic F-actin column. Antibodies were eluted

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from the immunoabsorbent using 10% dioxan/20 mM- Na_3PO_4 , pH 12 (Andersson *et al.*, 1978). The eluate was immediately neutralized to pH 7.5 and concentrated by vacuum dialysis against phosphate-buffered saline (1.4 mM- KH_2PO_4 /8.1 mM- Na_2HPO_4 /136 mM- NaCl /2.7 mM- KCl , pH 7.4) containing 10^{-4} M-merthiolate. These anti-actin antibody populations were then fractionated on (1-9)- or (1-8)-peptide-resin columns corresponding to the *N*-terminal sequences of $\text{SM}\alpha$, $\text{SM}\gamma$, $\text{NM}\beta$ and $\text{NM}\gamma$ isoactins. The columns were equilibrated with a phosphate-buffered saline solution and eluted as described above.

RESULTS

The filamentous aortic F-actin stabilized by glutaraldehyde treatment elicited anti-actin antibodies with a high titre (0.8 mg of anti-actin antibody/ml of serum) after 45 days and remained quite stable during the following days. The titre was estimated by the specific binding of the antibodies to an aortic F-actin column. The characteristics of the sera were analysed in terms of the ability of the antibodies to bind with filamentous or unfolded monomeric forms of immobilized actins (Table 1).

A major fraction of the elicited antibodies (60%) interacted with unfolded carboxymethylated actin, which selects antibodies directed against sequential or continuous epitopes. The antibodies specific for conformational epitopes selected by the filamentous form of actin represented 40%. The differential binding to the filamentous form of skeletal-muscle and aortic actins indicated that about 30% of the antibodies recognized conformational structures specific for smooth-muscle actins. The crude antiserum populations purified on aortic F-actin columns were further purified on columns of resin to which was bound peptide corresponding to the 1-9 or 1-8 *N*-terminal sequence of isoactins. These fractionations were used to determine the distribution of the purified antibodies among the various 1-9 or 1-8 sequences (Table 2).

The location of the antigenic sites along the primary aortic actin structure was determined by using Western blots of the proteolytic fragments of aortic actin (Fig. 1). The purified whole antiserum strongly interacted with the thrombin K fragment and to a lesser extent with the thrombin L, but not with the M, fragment (Fig. 1*b*, lane 2). The largest interaction with the *Staphylococcus* V8 proteinase fragments took place with the *N*-terminal fragment (1-226)-peptide. However, *C*-terminal (227-375)- and *N*-terminal (1-167)-peptide were also

Table 2. Reactivity of antibodies purified against aortic F-actin, towards *N*-terminal peptides of actin isoforms coupled to polyacrylic resin

Peptide residues are indicated by the one-letter amino acid notation. Abbreviation used: Ac, acetyl.

<i>N</i> -Terminal peptide-resin	Antibodies bound to insolubilized peptide (%)
AcEEEDSTALV-resin ($\text{SM}\alpha$)	24.0
AcEEETTALV-resin ($\text{SM}\gamma$)	5.2
AcDDDIAALV-resin ($\text{NM}\beta$)	3.7
AcEEEEIALV-resin ($\text{NM}\gamma$)	10.2
Not bound to the four resin-peptide columns	57%

reactive (Fig. 1*b*, lane 1). These results show that the whole antiserum contains both antibodies directed against the *N*- and *C*-terminal regions. Coomassie Blue staining (Fig. 1*a*, lane 1) shows that 227-375 (*C*-terminal) and 1-116 (*N*-terminal) fragments are present in equal amounts. In contrast, immunostaining with the whole antiserum displays (Fig. 1*b*, lane 1) marked differences in the intensity of staining. In addition the antibodies that did not bind to any of the (1-9)- or (1-8)-peptide-resin columns still interacted with both *N*- and *C*-terminal fragments. However, the *C*-terminal fragment appeared to be more reactive with the latter antibody population than with the whole antiserum (results not shown).

The antigenic reactivity of various actins with the different antibody populations was also tested on Western blots (Fig. 2). When tested against vertebrate or invertebrate actins, the purified antiserum recognized macrophage, cardiac muscle, gizzard muscle and aortic-muscle actins. Scallop-adductor and skeletal-muscle

Fig. 1. Antigenic reactivity of electrophoretic blotting from digest of aortic actin with *S. aureus* V8 proteinase (1) and thrombin (2)

(a) Shows the Coomassie Blue-stained polyacrylamide gel after electrophoresis; (b) the electrophoretic transfers of SDS/polyacrylamide gels (15%, w/v) were revealed with anti-actin antiserum. For experimental details, see the text. *N* and *C* represent the *N*-terminal and *C*-terminal peptides of aortic actin obtained with *S. aureus* V8 proteinase. K, L and M represent the peptides obtained with thrombin.

Table 1. Reactivity of bovine aortic actin antibodies towards chemically modified actins coupled to Sepharose 4B

Coupled antigen	Antibodies bound on column (mg/ml of serum)
Glutaraldehyde-stabilized aortic F-actin	0.87
Carboxymethylated aortic G-actin	0.52
Glutaraldehyde-stabilized skeletal-muscle F-actin	0.24

Fig. 2. Antigenic reactivity of various actins with the whole antiserum and specific anti-actin antibody populations

Actins were purified, as described in the Materials and methods section, from rabbit alveolar macrophage (M), scallop adductor muscle (P), bovine cardiac muscle (C), chicken gizzard (G), rabbit skeletal muscle (Sk) and bovine aorta (A). These actins were transferred from SDS/polyacrylamide gels to nitrocellulose by Western blotting. The immunoreaction of actins with the whole antiserum (100 µg/ml; lane 1) and the anti-actin antibody populations SMα, SMγ, NMβ, NMγ (10 µg/ml; lanes 2, 3, 4 and 5) were revealed by antibody coupled to peroxidase.

actins were poorly reactive (Fig. 2, lane 1). In contrast with the antiserum, the antibody populations purified on the (1-9)- or (1-8)-peptide-resin columns (SMα, SMγ, NMβ and NMγ) were able to discriminate the various actins.

Table 3 displays the degree of reactivity of the different antibody populations with various actins.

DISCUSSION

The purification of smooth-muscle isoactins under non-denaturing conditions is, to our knowledge, not yet

feasible. Consequently, their (possibly different) properties and roles in the cell are unknown.

The aim of the present work was to elicit, purify and characterize specific antibodies against smooth muscle (SMα,γ) and non-muscle (NMβ,γ) isoactins, in order to study further their immunolocalization in smooth-muscle cells and to determine their role in contractile or cytostructural functions.

The 1-17 sequences in smooth-muscle actins are the regions where most of the sequence changes are concentrated. They are also the most mobile (Barden & Dos Remedios, 1983) and one of the most hydrophilic regions (Roustan *et al.*, 1986). These two characteristics, mobility and hydrophilicity, have been reported as being correlated with antigenicity (Westhoff *et al.*, 1984; Van Regenmortel, 1986). It has been shown that a chemical modification of skeletal-muscle actin breaks the immune tolerance of the actin and induces a preferential production of antibodies directed against the N-terminal region (Benyamin *et al.*, 1986). Our results confirm that injections of glutaraldehyde-stabilized F-actin elicit preferential production of antibodies reacting with the N-terminal sequence (Fig. 1), and extend the results obtained with skeletal-muscle actin to aortic actin.

In mammalian smooth muscle, four isoactins are expressed. The amounts of each of these isoforms depend both on the muscle and species types (Vandekerckhove & Weber, 1981; Small & Sobieszek, 1983; Fatigati & Murphy, 1984). Actin purification from bovine aorta yields a mixture of at least four isoactins (Cavadore *et al.*, 1985a).

Two isoforms are typical of smooth muscles (SMα, SMγ) and two isoforms (NMβ, NMγ) are present in both smooth-muscle and non-muscle cells. However, the SMγ and NMγ isoforms are not identical and differ in their N-terminal primary sequence (Vandekerckhove & Weber, 1981). The presence of non-muscle isoforms of actin in smooth muscle was reported as being attributable to differential genomic expression. Injections of aortic F-actin elicit antibodies against all the isoforms present in the injected material, suggesting that all the isoforms possess similar antigenicity. The elicited antibodies recognize both the monomeric and the F-conformation of aortic actin (Table 1).

The differential binding of the antibodies elicited by aortic F-actin with skeletal-muscle and aortic F-conformations of actin confirms our previous results showing the existence of structural differences between skeletal-muscle and aortic actins (Cavadore *et al.*, 1985b).

Table 3. Reactivity of the anti-actin antibody populations towards various actins

Antibody population	Actin . . .	Reactivity					
		Macrophage	Scallop adductor muscle	Cardiac muscle	Gizzard muscle	Skeletal muscle	Aortic muscle
Whole antiserum		+++	0	++	++++	0	++++
Affinity-purified antibody population							
SMα		0	0	0	++++	0	++++
SMγ		++	+	++	++++	+	+++
NMβ		+++	0	+	+++	+	+++
NMγ		+++	+	+	+++	0	++

Western blots of aortic actin fragments obtained by proteinase cleavage show that the whole antiserum contains antibody populations that are essentially directed towards the 1–39 *N*-terminal region. The whole antiserum strongly interacts with (1–226)- and (1–167)-peptide and to a lesser extent with (40–375)- and (114–375)-peptide. However, the M fragment 40–113 does not interact at all. These results suggest that the major antigenic part of actin is concentrated in the *N*-terminal region 1–40.

The whole antiserum recognizes smooth-muscle, cardiac-muscle and macrophage actins. The cross-reactivity with actin is expected, since macrophages belong to the non-muscle-cell family, which express non-muscle isoforms identical with those expressed in smooth muscles. Mammalian cardiac muscle is abundantly vascularized, and actin preparations from this muscle could be contaminated by the presence of smooth-muscle actin isoforms(s). This could be a possible explanation for the observed cross-reactivity. In contrast with the whole antiserum, purified antibody populations discriminate the different actins. The SM antibody population only recognizes smooth-muscle actins. More intriguing is the cross-reactivity of SM γ antibody population with macrophage actin, which supposedly only contains NM β and NM γ isoforms. However, the primary structure of the actin(s) of the macrophage is unknown and may share common epitope(s) with smooth-muscle actins, a possibility worth investigating. Alternatively, muscular and non-muscular cells could express, in addition to the predominant isoform, a low level of other isoactin(s). These possibilities have already been demonstrated in cardiac muscle, where cardiac actin α and skeletal actin α isoforms are simultaneously expressed (Vandekerckhove *et al.*, 1986).

The specificity of our antibody populations results from both chemical actin modification and affinity-purification procedures. The affinity chromatography on F-actin selects antibody populations directed towards all the smooth-muscle isoforms. The second affinity chromatography on peptide-resin (the peptide corresponding to the 1–9 or 1–8 *N*-terminal sequence of the various isoactins) permits the final purification. The second chromatographic procedure takes advantage of the properties of polyacrylic resin, which swells considerably in aqueous solutions, making it usable as an immunosorbent, in contrast with the polystyrene resin conventionally used in solid-phase peptide synthesis. The latter cannot be used in this way, since it is a very hydrophobic support with little tendency to swell in aqueous medium. The use of actin peptides linked to a polyacrylic resin made it possible to fractionate our polyclonal antibody to give an oligoclonal antibody. Our specific antibody

populations, SM α , SM γ , NM β and NM γ , will now permit one to determine whether isoactins are present as individual microfilaments in the cell and provide a general approach to probing specialized functions and localizations of isoactins in smooth-muscle cells.

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