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Smooth muscle actin isoforms: A tug of war between contraction and compliance

Richard Arnoldi[a], Anita Hiltbrunner[a], Vera Dugina[a,b], Jean-Christophe Tille[c], Christine Chaponnier[a,*]

[a] Department of Pathology and Immunology, Faculty of Medicine, University of Geneva, Geneva, Switzerland
[b] Belozersky Institute of Physico-Chemical Biology, Lomonosov Moscow State University, Moscow, Russia
[c] Division of Clinical Pathology, Faculty of Medicine, University of Geneva, Geneva, Switzerland

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ABSTRACT

In higher vertebrates, smooth muscle (SM) contains two tissue-specific actin isoforms: α-SMA and γ-SMA, which predominate in vascular and visceral SM, respectively. Whether α-SMA has been extensively studied and recognized for its contractile activity in SM and SM-like cells such as myofibroblasts, myoepithelial and myoid cells, the distribution and role of γ-SMA remained largely unknown. We developed a new specific monoclonal antibody against γ-SMA and confirmed that γ-SMA predominates in the visceral system and is minor in the vascular system, although more expressed in highly compliant veins than in stiff arteries. Contrary to α-SMA, γ-SMA is absent from myofibroblasts *in vitro*, and in fibrotic diseases *in vivo*. We raised the hypothesis that, whereas α-SMA is responsible for the "contractile" activity, γ-SMA would be involved in the "compliance" of SM and SM-like cells. Several models support this hypothesis, namely veins *vs.* arteries and the physiological modifications occurring in the uterus and mammary glands during pregnancy and lactation. Our results suggest that, in addition to enteric smooth muscles, γ-SMA is expressed in all the tissues submitted to an important dilation including veins, gravid uterus, and lactating mammary glands. The hypothesis of two complementary mechanical roles for the two SMA isoforms is sustained by their different intracellular distributions and by functional assays.

Introduction

Smooth muscles (SMs) are formed by non-striated mononucleated fusiform cells generally disposed as dense layers in the walls of blood vessels and most hollow organs. Their involuntary contraction and relaxation is therefore critical to the vascular, digestive, respiratory and urogenital systems. Although SMs are usually classified into visceral and vascular SMs and show similar morphologic features, the variety of physiological roles played by SM cells (SMCs) in the body explains the great heterogeneity of their functional and anatomic features. Mechanically, in addition to contraction, SMCs also have an important contribution to the organ resistance to dilation. Since actin is a major component of the contractile apparatus, it is not surprising that SMCs are relatively heterogeneous in their content of actin isoforms (Gabbiani et al., 1984; Skalli et al., 1987; Vandekerckhove and Weber, 1981).

Actin is the most abundant moonlighting protein in a vast majority of eukaryotic cells. Apart of its specialized role in muscle contraction, actin is present in all muscle and nonmuscle cells, where it plays a variety of fundamental roles thanks to its ability to assemble and disassemble depending on cell requirements (reviewed in Pollard and Cooper, 2009). In higher vertebrates, actin is present with six highly conserved isoforms encoded by six different genes (Gunning et al., 1983): two of them are ubiquitously expressed, *i.e.* β- and γ-cytoplasmic actins (β- and γ-CYAs), whereas four are considered tissue-specific, *i.e.* α-skeletal actin (α-SKA) in skeletal muscle, α-cardiac actin (α-CAA) in cardiac muscle, and α- and γ-smooth actins (α- and γ-SMAs) in smooth muscle (Vandekerckhove and Weber, 1978). Although encoded by a set of structurally related genes that probably evolved from a common ancestor (Hightower and Meagher, 1986), actin isoforms have highly conserved primary amino acid sequences with their differences lying predominantly in a cluster of acidic residues within their N-terminus (Vandekerckhove and Weber, 1978).

Besides β- and γ-CYAs, α- and γ-SMAs are present in different proportions in different animals and organs. Albeit differing in their sequence by only three amino acids, seminal studies described α-SMA as the predominating variant in vascular and airway SMs and γ-SMA as the main isoform in visceral SMs (Fatigati and Murphy, 1984; Gabbiani et al., 1981; McHugh et al., 1991; Sawtell and Lessard, 1989; Vandekerckhove and Weber, 1978, 1981). This heterogeneity, which seems to represent a general

* Corresponding author. Tel.: +41 223795766; fax: +41 223795746.
E-mail address: christine.chaponnier@unige.ch (C. Chaponnier).

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pattern of SM isoactin expression in warm-blooded vertebrates, has been recently confirmed in multiple human tissues microarrays, where γ-SMA expression is roughly limited to blood vessels, gastrointestinal and urogenital systems (Kilpinen et al., 2008; http:\\ist.genesapiens.org).

These subtle variations are probably functionally relevant (Khaitlina, 2001; Perrin and Ervasti, 2010) and the modulation of actin isoform expression is often characteristic of different pathological conditions in several animal and human tissues (Chaponnier and Gabbiani, 2004; Lambrechts et al., 2004). According to the results on experimental KO mice for actins, each isoactin displays specialized functions (reviewed in Tondeleir et al., 2009). Although viable, α-SMA$^{-/-}$ mice have been reported to display major defects in vascular contractility and blood pressure regulation (Schildmeyer et al., 2000), decreased bladder contractility (Zimmerman et al., 2004), altered retinal vascular permeability (Tomasek et al., 2006), and impaired myoepithelial (MC) contraction in mammary glands (Haaksma et al., 2011; Weymouth et al., 2012). Moreover, thanks to the "anti-αsm-1 antibody", developed in our laboratory (Skalli et al., 1986), α-SMA has been extensively studied and recognized for its "contractile" activity in several cell types including SM, myofibroblasts, and MCs. Based on these studies, the current paradigm states that α-SMA is responsible for contraction in all SM and SM-like cells. Whether γ-SMA is its equivalent in the enteric system still remains highly speculative. Since no null mice and no specific antibody were available for this isoform yet, it has never been possible to discriminate between α-SMA and γ-SMA at the protein level and its approximate known distribution is mainly based on studies at mRNA level (McHugh et al., 1991; Szymanski et al., 1998).

We developed the first γ-SMA specific monoclonal antibody (mAb) and we performed successively several experiments in order to highlight its localization in different tissues. Since the relative abundance of γ-SMA in various hollow organs including esophagous, stomach, intestine, bladder, and uterus may be related to stronger mechanical constraints supported by these organs, we hypothesized a new paradigm in which α-SMA is responsible for contraction and γ-SMA for compliance. In order to validate our hypothesis we investigated the distribution and amount of the two isoforms in SM and SM-like cells within several contractile and/or dilatable systems. Our results were confirmed by a collagen lattice contraction assay and by the selective depletion of both isoforms.

Materials and methods

Production and selection of a new monoclonal antibody

Six nona- or decapeptides representing the acetylated NH_2-terminal sequence of the six actin isoforms were synthesized by Affiniti Research Products Ltd. (Exeter, Devon, UK) (Fig. 1B). To ensure chemical linkages, peptides were synthesized with an additional cysteine at their C-terminus. The peptides were coupled through their cysteine with maleimide-activated keyhole limpet hemocyanin (KLH; Pierce, Thermo Fisher Scientific, Wohlen, Switzerland) or bovine serum albumine (BSA, Pierce) for immunization and ELISA experiments respectively. BALB/c mice were immunized with KLH-conjugated γ-SMA peptide (Ac-EEETTALVC-COOH) following the Repetitive Immunizations at Multiple Sites Strategy (RIMMS) (Dugina et al., 2009; Kilpatrick et al., 1997). Isolated lymph node lymphocytes were fused with an NSO murine myeloma cell line. Hybridomas were screened by triple solid-phase ELISA using 96-well plates coated with γ-SMA, α-SMA or γ-CYA BSA-conjugated peptides. Selected hybridomas were cloned twice by limited dilution and characterized by immunofluorescence staining of rat intestine sections and immunoblotting of various tissue extracts. In addition, the six isoform peptides coupled with maleimido-benzoyl-N-hydroxysuccinimide (MBS)-activated BSA, as previously described (Chaponnier et al., 1995), were subjected to SDS-PAGE, stained with Coomassie blue (CB) and immunoblotted with anti-γ-SMA. A clone (20 D2) secreting γ-SMA-specific antibodies was finally selected. For specific experiments, anti-γ-SMA has been coupled with biotin (21217; Pierce, Thermo Fisher Scientific).

Antibodies

Proteins from cells/tissues were identified with the following primary mAbs: anti-α-SMA (anti-αsm-1, 1A4, IgG2a; Skalli et al., 1986), newly developed anti-γ-SMA (20D2, IgG1), anti-total-actin (pan-actin, 1C4, IgG1; Chemicon, Temecula, CA), anti-α-tubulin (B-5-1-2, IgG1; Sigma–Aldrich, Buchs, Switzerland), anti-vimentin (V9, IgG1; Dako, Copenhagen, Denmark) or anti-vinculin (HVIN-1; IgG1; Sigma–Aldrich). The following secondary antibodies were used: FITC-conjugated goat anti-mouse (GAM) IgG1, TRITC-conjugated GAMs IgG1, IgG2a, IgG2b, IgG3, IgM (SouthernBiotech, Birmingham, AL), Alexa-Fluor-488-conjugated GAM IgG, and Alexa-Fluor-488-conjugated streptavidin (Molecular Probes, Life Technologies, Luzern, Switzerland). DAPI (Sigma–Aldrich) was used for nuclear staining.

Immunofluorescence and immunohistochemistry

Cryopreserved tissues were sliced (3 μm) with a microtome cryostat (Cryo-Star HM 560M, Microm International GmbH, Walldorf, Germany). Sections were disposed on glass slides, fixed with 1% PFA for 30 min at RT, permeabilized with MeOH −20 °C for 3 min, and incubated with primary and secondary antibodies at appropriate dilutions. In rat tissues, normal rat serum (1:50) was used to block non-specific sites, and DAPI was used for nuclear staining. Cells cultured in 60-mm culture dishes (Nunc; Thermo Fisher Scientific) or on glass coverslips were fixed with 1% PFA in DMEM (37°) for 30 min, permeabilized with MeOH −20 °C for 3 min as previously described (Dugina et al., 2009), and processed following the procedure described for tissues. Analysis was performed with a Zeiss Axiophot photomicroscope (Zeiss, Oberkochen, Germany) equipped for epifluorescence with appropriate excitation and emission filters, using plan apochromatic 10×, 20×, or 40× objectives. Confocal images were acquired using a confocal microscope (LSM510, Zeiss) equipped with oil-immersion Plan-Neofluar 63×1.4 objective (Zeiss). For serial optical sections stacks with z-step of 0.3 μm were collected. Images were processed using Adobe Photoshop software. Total internal reflection fluorescence (TIRF) microscopy was performed using a microscope (Axiovert 100M; Zeiss) equipped with a combined epifluorescence/TIRF adapter (TILL Photonics) and a 100×1.45 objective (Zeiss) controlled by Openlab software (PerkinElmer, Waltham, MA, USA). Images were processed and colocalization analysis conducted with Fiji software (http://fiji.sc/Fiji; Schindelin et al., 2012). Manders' and Pearson's colocalization coefficients (MCC and PCC) were used to define the level of colocalization between the SMA fibers and FAs (Dunn et al., 2011). Paraffin sections of formol-fixed rat, porcine and human tissues were also used. After alcohol deparaffination and dehydration, sections were stewpan heated for 3 min in citrate buffer (10 mM, pH 6). After preincubation with 5% PBS-BSA, tissue sections were successively incubated with primary antibody, biotinylated Rabbit Anti-Mouse Ig F(ab) (E041301-2; 1:250; Dako), and streptavidin–peroxidase complex (K037711; Dako), revealed with DAB, and counter-stained with Mayer's hemalun. Immunofluorescence was also performed on paraffin sections as previously described (Orlandi et al., 2009). Briefly, paraffin sections were dewaxed, rinsed twice in H_2O and microwave treated in 0.01 M Tris,