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Brigitte M. Jockusch *Editor*

The Actin Cytoskeleton

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The Actin Cytoskeleton

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This book is devoted to the memory of Klaus Weber (1936–2016).
His work on actin's expression and evolution stimulated cell biological research for decades.

Preface

This volume comprises 12 articles on the various functional tasks of actin. This protein, identified almost 160 years ago as specifically engaged in muscle contraction (see Mommaerts 1992 and Szent-Gyorgyi 2004), has made a dramatic career and is now considered a universal player in almost every motility phenomenon in all eukaryotic kingdoms. The history of actin research contains many interesting, even amusing details that are reported in Schleicher and Jockusch (2008) and Jockusch and Graumann (2011).

One of the turning points in considering the role of actin in life came in 1973 when Hugh Huxley, previously convinced that actin is a structural component specifically operating in the contractile machinery of striated muscle, was confronted with data on the cross-reactivity of muscle myosin with filamentous components in nonmuscle cells. In a well-received lecture, he then suggested that actomyosin components are generally responsible for cellular motility (Huxley 1973). One year later, it was shown that antibodies elicited against muscle actin decorate prominent filament bundles in nonmuscle cells (Lazarides and Weber 1974). The next decade revealed that most organisms contain not one, but many different actin genes leading to proteins with slightly different functions (see for example Vandekerckhove and Weber 1978) and actin genes then rapidly evolved in structure and number (Vandekerckhove and Weber 1984).

The chapters compiled in this volume shed light on the present state of the art in understanding the stunning functional versatility of actin—a small, rather compact protein of approximately 42 kDa. The introductory article by Ampe and Van Troys, revealing the present knowledge on actin isoforms, their differential expression and mutations, is followed by a detailed description of the precise organization of actomyosin filaments, the myofibrils, into sarcomeres, the functional units in striated muscle (Sanger JW et al.). Muscle myosin, the other prominent protein also originally identified in striated muscle (cf. Szent-Gyorgyi 2004)}, belongs to a large and very diverse family, many members of which show special adaptations to execute various motility processes in conjunction with actin (Masters, Kendrick-Jones and Buss). The conservation of the original architectural building plan of actomyosin filaments, modified by a large panel of actin binding partners, is highlighted in the article on the different cytoskeletal structures in migrating vertebrate cells (Lehtimäki, Hakala and Lappalainen).

Another spotlight is then switched on by the contribution by Steffen, Stradal and Rottner, who emphasize the many proteins involved in signaling cascades that mediate between membranes and the actin cytoskeleton. This is not restricted to the plasma membrane but is relevant also for internal membrane-enveloped vesicles. Thus, specific protein factors regulate the actin cytoskeleton in shape changes and vesicular trafficking.

The next three chapters reveal the interactions between pathogenic prokaryotes and the actin cytoskeleton of their hosts: to optimize bacterial multiplication, many different bacterial toxins can covalently modify the actin molecule itself, but also regulators of actin polymerization and organization (Aktories, Schwan and Lang). The articles on pathogenic bacteria (Tran Van Nhieu and Romero) and viruses (Marzook and Newsome) describe the crosstalk of these pathogens with their host cells. Adhesion, infection and intracellular multiplication depend on highly sophisticated ways invented by the pathogens to abuse the actin cytoskeleton—with disastrous consequences on cellular and tissue integrity.

The task of the actin cytoskeleton in organizing cells into functional tissues and organs is the topic of the next chapter which relates cell and tissue polarity to the intrinsic polarity of actin filaments and their associated adhesive structures (Luxenburg and Geiger). Notably, the vital role of actin polarity and dynamics in providing mechanical support for tissue development during morphogenesis is not confined to vertebrates, but also relevant for insects, as shown in the article on *Drosophila* development (Brüser and Bogdan).

Another complex of actin functions is presented in the chapter on nuclear actin (Viita and Vartiainen). This activity of actin has led a rather cryptic life for several decades, since the existence of nuclear actin had been doubted. Today, there is a wealth of solid and well-accepted evidence that actin operates also in the nucleus; however, the exact details of its function and molecular organization are still a matter of debate.

Finally, there remains a catalogue of unanswered questions. Many mechanistic details on actin filament generation, life span and regulation by the different binding partners are still left for future investigations (Pollard).

The progress in understanding the structure, function and regulation of the actin cytoskeleton made over the past seven decades reflects the enormous advance in techniques. Molecular genetics, refined biochemistry and structural biology, and live microscopy on cells expressing fluorescently tagged proteins have been employed in the studies reported here. Notably, pharmacology has contributed to many fundamental studies on actin dynamics, which is sensitive not only to bacterial toxins but also to a large and still growing number of toxins from sponges and fungi. The synthesis of specific inhibitors will be a platform for future pharmacological research in diagnosis and therapy of the numerous human diseases based on mutations in actin isoforms, actin regulators and myosin.

Braunschweig, Germany Brigitte M. Jockusch

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Mammalian Actins: Isoform-Specific Functions and Diseases

Christophe Ampe and Marleen Van Troys

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Abstract

Actin is the central building block of the actin cytoskeleton, a highly regulated filamentous network enabling dynamic processes of cells and simultaneously providing structure. Mammals have six actin isoforms that are very conserved and thus share common functions. Tissue-specific expression in part underlies their differential roles, but actin isoforms also coexist in various cell types and tissues, suggesting specific functions and preferential interaction partners. Gene deletion models, antibody-based staining patterns, gene silencing effects, and the occurrence of isoform-specific mutations in certain diseases have provided clues for specificity on the subcellular level and its consequences on the organism level. Yet, the differential actin isoform functions are still far from understood in detail. Biochemical studies on the different isoforms in pure form are

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just emerging, and investigations in cells have to deal with a complex and regulated system, including compensatory actin isoform expression.

Keywords
Actin disease mutation • Actin genes • Baraitser-Winter syndrome • Cytoskeleton • Deafness • Isoform switching • Myopathy • Thoracic aortic aneurysms and dissection

1 Introduction

Eukaryotes have three types of cytoskeletal elements in their cytosol: tubulins, intermediate filaments, and actin filaments (Zampieri et al. 2014). The latter are the main component of the actin cytoskeleton that contain, next to actin molecules, actin-binding proteins and regulatory proteins. The actin cytoskeleton differs from cell type to cell type thereby gaining differential functions. For instance, in muscle cells, actin and myosin filaments form the contractile apparatus together with specific actin-binding proteins. Especially in striated muscle cells, this is organized in a highly regular manner (Sanger et al. 2016; Gautel and Djinovic-Carugo 2016). In (cultured) non-muscle cells, the actin cytoskeleton is more versatile and can be highly dynamic depending on the subcellular localization (Lehtimäki et al. 2016). Indeed, as we will describe below, actin filaments are formed via a complex polymerization cycle. The formation of higher-order cellular structures (i.e., actin-rich networks or bundles) is assisted by a broad range of actin-binding proteins with distinct and sometimes overlapping activities (Pollard 2016). These dynamic structures support the formation of membrane protrusions such as lamellipodia, filopodia, (micro)spikes, microvilli, podosomes, or invadopodia (Alblazi and Siar 2015; Blanchoin et al. 2014). With the exception of microvilli, these actin-rich structures are connected to migration or invasion of cells. In addition, in cells actin bundles may form various types of stress fibers in some cases emanating from focal adhesions that attach cells to the extracellular matrix (Vallenius 2013). In addition, in most cells cortical actin structures or networks exist (Gutierrez 2012; Eghiaian et al. 2015).

All eukaryotes express at least one actin, but it is not uncommon that species express more isoforms and this is the rule in vertebrates. What is known on how these isoforms differentially contribute to the functions of the mammalian actin cytoskeleton is reviewed here. We will mainly focus on actin isoforms in the cytoplasm, since in the nucleus their specific activities have hardly been investigated, although circumstantial evidence indicates there must also be nuclear actin isoform specificity (Tondeleir et al. 2012; Bunnell et al. 2011; Lechuga et al. 2014; Almuzzaini et al. 2016) (see below). For a general discussion of actin function in the nuclear compartment, we refer to another chapter (Viita and Vartiainen 2016).

2 Actin Isoforms: A Highly Conserved Family of Proteins

The number of actin-expressing genes varies between vertebrate species, but it appears that most mammals express six isoforms of actin and do so in a tissue-specific manner. In mouse and man, one distinguishes four muscle forms, namely, alpha-skeletal muscle actin, alpha-cardiac actin, alpha-smooth muscle actin, and gamma-smooth muscle actin, and two non-muscle actins referred to as beta- and gamma-cytoplasmic actin (Table 1 and Fig. 1). The designation of alpha, beta, or gamma comes from the electrophoretic mobility on 2D PAGE (Garrels and Gibson 1976) owing to a difference in isoelectric point. This difference is due to the number and type of acidic residues at the N terminus (Fig. 1) (Vandekerckhove and Weber 1978).

As can be appreciated from the aligned sequences (Fig. 1), actin is a very conserved protein. The six mammalian actins are extremely similar with highest

Table 1 Actin isoforms and associated mouse knockout models

Protein	Gene (mouse/ human)	KO phenotype (compensatory actin expression)	Rescue (*gene*) in KO context	Reference
Alpha-skeletal muscle actin	*Acta1* *ACTA1*	Neonatal lethal (Actc, Acta2)	Viable if high expression with lower myofiber force production (*Actc*)	Crawford et al. (2002) Nowak et al. (2009), and Ochala et al. (2013)
Alpha-cardiac actin	*Actc* *ACTC*	Perinatal lethal (Acta1, Acta2)	Hypodynamic and enlarged heart (*Actg2*)	Kumar et al. (1997)
Alpha-smooth muscle actin	*Acta2* *ACTA2*	Impaired vascular contractility (Acta1)	n.a.	Schildmeyer et al. (2000)
Gamma-smooth muscle actin	*Actg2* *ACTG2*	n.a.	n.a.	
			Conditional KO: (*organ*) *phenotype*	
Beta-cytoplasmic actin	*Actb* *ACTB*	E7 E10,5 (Actg1, Acta2)	(Motor neurons) no phenotype (CNS) hyperactivity (Skeletal muscle) quadriceps myopathy (AHC) impaired stereocilia maintenance[a]	Shmerling et al. (2005), Bunnell et al. (2011), Tondeleir et al. (2012), Cheever et al. (2011), Cheever et al. (2012), Prins et al. (2011), and Perrin et al. (2010)
Gamma-cytoplasmic actin	*Actg1* *ACTG1*	Progressive hearing loss (Actb)	Progressive myopathy Impaired stereocilia maintenance[a] (AHC)	Sonnemann et al. (2006) and Perrin et al. (2010)

AHC auditory hair cell, *CNS* central nervous system, *KO* knockout, *n.a.* not available
[a]Distinct patterns with distinct onset of hearing loss

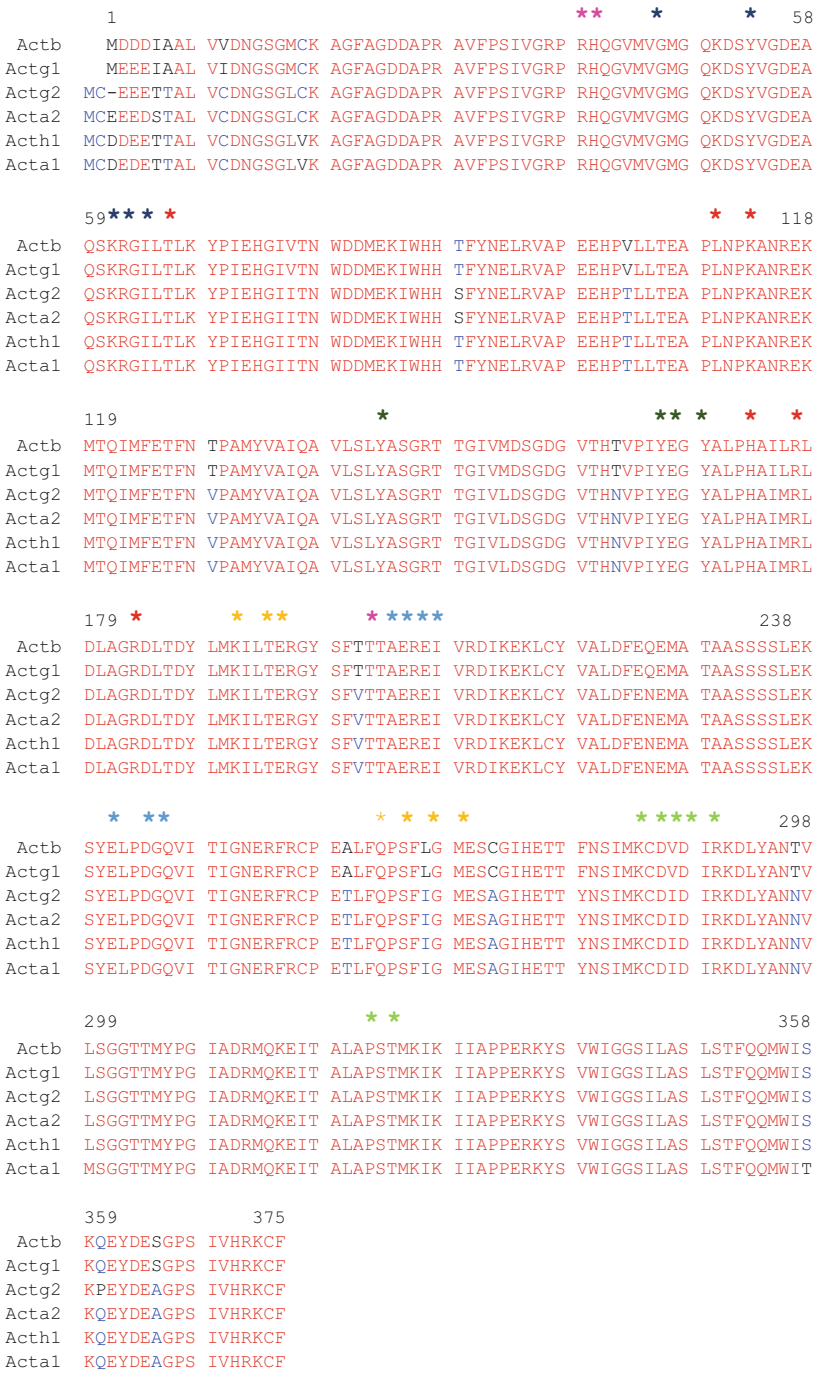

Fig. 1 Alignment of human actin sequences showing high sequence conservation: *red* indicates identical residues, and *blue* and *black* indicate that at that position at least one isoform is different,

divergence between cytoplasmic and muscle actins. It should be noted that the initiator methionine and the cysteine in muscle actins are posttranslationally removed and the first acidic residue is subsequently acetylated (the numbering used in the general section takes this into account and numbering starts with Asp1 of alpha-skeletal muscle actin; the numbering of mutated residues in disease variants is isoform specific). The non-muscle actins lack this cysteine and only the initiator methionine is removed prior to acetylation (references in Cook et al. 1991). Next to the well-established acetylation at the N terminus and the methylation of His73, mammalian actins may undergo a number of different posttranslational modifications. In general the role of these modifications is poorly understood, let alone isoform-specific effects. We refer the interested reader to Terman and Kashina (2013).

The conservation of the primary structures implies that the actin isoforms have the same fold and present similar surfaces. The actin molecule, also referred to as actin monomer or globular (G)-actin, adopts the hexokinase superfold (Bork et al. 1992; Kabsch and Holmes 1995). Traditionally it is divided in a large and small domain in between which the nucleotide (ATP or ADP) and the divalent metal ion (Mg^{2+} or Ca^{2+}) bind. Both domains are each subdivided into two structural subdomains (Fig. 2a) (Kabsch et al. 1990). Subdomain 1 contains the N and C terminus. In Fig. 2c the differential residues between the human actin isoforms have been mapped on the three-dimensional structure. This demonstrates no real hot spot domain of divergence exists with the exception of the N terminus.

Taking into account the ancient nature of the actin family, the combination of strong general conservation within all isoforms and the additional conservation within the cytoplasmic and muscle subgroups (e.g., at the N termini) is indicative of a high selective pressure both on the similarities and on the differences. This on the one hand reflects the shared properties of the actin isoforms, but on the other hand also underscores the importance of their uniqueness and functional specificity. As suggested by Gunning et al. (2015), the very limited sequence divergence in actin itself implies only subtle differences between the isoforms that may however be magnified by a more extensive divergence of the actin-associated proteins (Gunning et al. 2015). Together, this enables the diversity in dynamic actin-rich structures in specific cells or specific locations in cells, as mentioned above and further detailed below.

Fig. 1 (continued) but note that in these cases, it usually is a conserved substitution. Reference sequences were retrieved from the NCBI protein database: http://www.ncbi.nlm.nih.gov/protein/ and aligned with multalin: http://multalin.toulouse.inra.fr/multalin/multalin.html using the blosum62-12-2 and default settings. These sequences were also used for generating Fig. 2c. Residues with "*" are important in F-actin formation and are also displayed in space filling in Fig. 2b (same color code). Note that all residues marked with * are identical with the exception of the conserved substitution of valine or isoleucine at position 287. All actins are therefore assumed to form very similar polymer structures

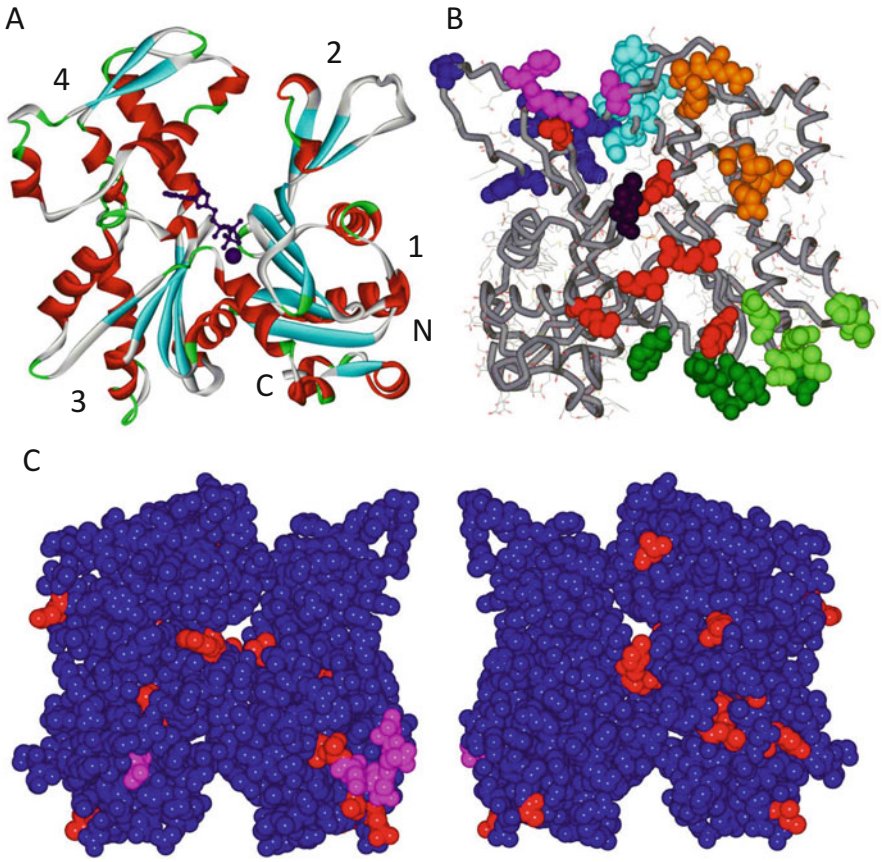

Fig. 2 Structural views of the actin molecule. (**a**) Ribbon presentation of monomeric actin with the alpha-helices in *red*, beta-strands in *blue*, beta-turns in *green* and loops in *gray*. This view is generally considered as depicting the front of the molecule. The molecule can be divided in subdomains 1–4. Subdomain 1 contains both the N and C termini. Subdomains 2 and 4 are at the pointed end; subdomains 3 and 4 are at the barbed end (based on their location at actin filament ends). ATP and the divalent ion are in *purple*. Taken from PDB entry 2BTF (Schutt et al. 1993) with omission of profilin. (**b**) The actin protomer in an orientation rotated 180° along a vertical axis in the plane relative to the molecule in 1A (thus viewing the back of the molecule). The main chain is in *gray*. The residues in space filing form contacts with neighboring protomers in the filament (as derived from Table 2). *Blue* and *green* indicate longitudinal contacts (light makes contact with light, and dark with dark); *red* and *orange* indicate contacts across the filament axis (residue making contacts via main chain atoms are not indicated; see Table 2). The residues in *magenta* (R39, H40, R183) make both types of contacts. The template structure was taken from PDB entry 3MFP (Fujii et al. 2010). (**c**) Scop3 visualization (Vermeire et al. 2015) of the variation of human actin isoactins plotted on the 3D structure: *blue*, identical residues; *red* and *magenta*, positions where mutations occur. Sites in magenta have a higher mutational tolerance. Two orientations are seen: on the left the actin molecule is in the same orientation as in Fig. 2a with the N-terminal residues (in *pink*) at the lower right. On the right is the same actin molecule in an orientation similar to 2b. The template structure of the actin molecule was taken from PDB entry 2BTF

Despite the identification of the actin isoforms nearly half a century ago, many questions still remain on how they actually differ. To what extent do we understand the isotype redundant and nonredundant functions? What is the role of isotype-specific expression and how is this regulated? Do isotype levels affect the cellular proteome? To what level do actin isoforms segregate in cells? What did the community learn from KO mouse models and isoform-specific mutations in disease? We will try to illustrate where this journey has led us thus far. To start with we briefly state common functions between the isoforms.

3 Actin Isoforms: General Properties

With general properties, we mean that all mammalian actin isoforms share them, although it does not exclude subtle differences.

All isoforms are assumed to require the chaperonin CCT to reach the native state. This has been experimentally shown for beta-cytoplasmic actin, alpha-skeletal actin, and alpha-cardiac actin (Vang et al. 2005; Rommelaere et al. 1993; Neirynck et al. 2006; Costa et al. 2004; Grantham et al. 2000). Because there is an absolute requirement for CCT to fold actin and because multiple sites of actin interact with CCT (Neirynck et al. 2006), the high sequence conservation between the isoforms allows assuming that also gamma-cytoplasmic actin and alpha- and gamma-smooth muscle actins require chaperonin-assisted folding.

The next shared property obviously is the core business of the actin molecule: the capacity to form polymers. Actin monomers self-associate in a head to tail fashion and thus form polarized filamentous structures, termed F-actin. Polymerization dynamics have initially been deciphered in vitro, mostly using alpha-skeletal muscle actin, and corroborated in vivo. We refer to Carlier et al. (2015) for a detailed description. When inducing polymerization of purified Ca^{2+}-ATP-actin monomers (by adding KCl and $MgCl_2$ to relatively low concentrations), one observes a lag phase, termed nucleation phase. Interestingly, when starting from Mg^{2+}-actin monomers, this phase is shortened (Carlier et al. 1986). The lag phase is followed by a phase in which both ends elongate, albeit at different rates. Polymerization is accompanied by hydrolysis of ATP to ADP-P_i, but subsequent P_i release lags behind. Together, this finally leads to a steady state in which treadmilling occurs; this is the cycling of actin protomers (subunits within the polymer) through the filament. ATP-actin monomers preferentially add to the ATP side of the actin filament (also termed the barbed or fast polymerizing end), whereas ADP-actin protomers preferentially dissociate from the ADP filament end (also termed the pointed or slow depolymerizing end). Obviously, ADP needs to be exchanged by ATP if an actin molecule reenters the polymerization cycle (Tondeleir et al. 2011).

The first model of F-actin was based on fiber diffraction of oriented gels of actin filaments and on the original G-actin structure (Kabsch et al. 1990; Holmes et al. 1990; Lorenz et al. 1993); see details in Dominguez and Holmes (2011).

Two high-resolution cryo-EM structures of alpha-skeletal F-actin have been published in 2010 (Fujii et al. 2010; Murakami et al. 2010) and a high-resolution structure with tropomyosin in 2015 (von der Ecken et al. 2015). The paper by Fujii et al. focused on the contact sites between the protomers within the filament and the paper by Murakami et al. on nucleotide binding, inorganic phosphate, and Mg^{2+} interaction. We will use these structures in relation to the differences in the amino acid sequence of isoactins.

In all recent models, the structure of the actin protomer is more flattened than the G-actin structure as derived from various crystallographic models (reviewed in Dominguez and Holmes 2011) indicating that a conformational change is associated with polymer formation. The structure of the filament can be described either as a two-stranded helix with two strands intertwined in a right-handed fashion or as a single left-handed helix (rotation of −166.6° and a translation of 27.6 Å in Fujii et al. 2010; compared to values reviewed in Dominguez and Holmes 2011). Viewing it as a double strand, one should consider contacts along the filament axis and across. We list these contacts in Table 2, show them on the monomer structure (Fig. 2b), and indicate them with color-coded asterisks in the alignment (Fig. 1).

Table 2 Contacts in F-actin based on Fujii et al. (2010), Murakami et al. (2010), and Dominguez and Holmes (2011)

Longitudinal		Across	
Dark blue	*Dark green*	*Orange*	*Red*
V45	Y143	E270	R39[a]
H40[a]	Y169[b]	S265	H40[a]
Y53	E167[b]	G268	H40
K61	E167	267/268 MC[c] ox	H173
R62	D288	K191	L110
I64	Y166	T194	R177
Light blue	*Light green*	E195	K113
Ala 204, Ile208	Ile 287	194 MC ox	110 MC ox (H_2O)
E205	D286, R290	195 MC ox	110 MC ox (H_2O)
R39	D286[b]	Q263/S265 via Mg^{2+}	T66
D244	R290	T202[a]	R183[b] (via P_i at site 1)
E241	T324		
G245	P322		
E207/Q59 via Mg^{2+}	D288[b] D286		
T202[a]	K284		

Color codes refer to Fig. 2b

[a]Magenta in Fig. 2b

[b]The longitudinal contacts at E167, Y169, D286, and D288 are different in the F-actin-tropomyosin structure (ADP) (von der Ecken et al. 2015) and the cryo-EM structures (Fujii et al. 2010) (Murakami et al. 2010) either due to the presence of tropomyosin or because of the presence of the phosphates (probably reflecting ADP + P_i). Most notably in the former there is an intramolecular salt bridge between R183 and Y169, whereas both residues participate in longitudinal contacts in (Murakami et al. 2010)

[c]MC ox: interaction via main chain oxygen (and H_2O)

What has been underappreciated prior to the elucidation of the cryo-EM structure presented by Murakami et al. (2010) is that inorganic phosphate and Mg^{2+} also have a structural role in the filament, on top of their role in regulating ATP hydrolysis and filament turnover (Table 2). Indeed, Glu207 and Gln59 in one protomer coordinate a Mg^{2+}-ion that also interacts with Asp88 along the filament axis. Similarly, it was pointed out that also two inorganic phosphates contribute to F-actin contacts as this ion could stabilize ternary interactions (Table 2). A first such site is near Thr202 and near Arg183 of the actin protomer across the filament and also near Lys284 of the actin molecule along the longitudinal axis. Arg183 also binds a second inorganic phosphate together with Arg206 of a neighboring protomer across the axis. One of these inorganic phosphates could even result from the hydrolysis of ATP to first ADP-P_i (P_i still at the position where it was originally bound, near Ser14) to ADP and P_i which is then transferred to the residues at the first site (Arg183, Thr202, Lys 284). This is consistent with data showing that the methylated His73 (purple in Fig. 2b and near Arg183 in magenta) is a kind of gating residue for the release of the hydrolyzed phosphate (Nyman et al. 2002). The structural model of Murakami may also explain why the hydrolysis rates are different between Mg^{2+} and Ca^{2+}-ATP actin (Carlier et al. 1986) because Ca^{2+} would prevent nucleophilic attack from a water molecule.

Having mapped the filament contacts of skeletal muscle actin on the 3D structure (Table 2 and Fig. 2b), we can now extrapolate this to the information in the primary structures of the other actin isoforms (Fig. 1, asterisks). As can be appreciated from the alignment, all residues suggested as intrafilament contacts are, with exception of residue 287 (Val or Ile), absolutely conserved in the six mammalian isoforms. This is not surprising, as all isoforms are capable of polymerization which has been shown experimentally by in vitro polymerization experiments (Kuroda and Maruyama 1976; Bergeron et al. 2010; Muller et al. 2013). At the same time, it explains that observed isoform-specific differences in polymerization kinetics (see below) are subtle and suggests these are caused indirectly by the differential amino acids. It is also consistent with the notion that actins can form mixed filaments in vitro in these cases that have been studied.

Since the largest divergence between actin isoforms lies in the N terminus, its biophysical properties are potentially important. In the G-actin crystal structure, there is no electron density for the N terminus (Kabsch et al. 1990). In the F-actin models, the N terminus is however well resolved and exposed (Fujii et al. 2010; Murakami et al. 2010) in agreement with the notion that this region is involved in binding myosins as originally proposed for the myosin II-actin structure derived by modeling of the individual structures of actin and the motor domain of myosin II and low-resolution cryo-EM data (Rayment et al. 1993).

A third function shared between actins is that they interact with an extraordinary number of partner proteins (reviewed in Pollard 2016). These partners may bind either the monomer or the polymer (or both) and influence various aspects of the polymerization process or use the filaments for localization or transport purposes. An extensive overview of these proteins is beyond the scope of this review, but some actin-binding proteins will be mentioned below if differences in relation to

actin isoforms have been documented. In addition, mammalian actins bind a number of small molecules from diverse natural sources that have been substantial in studying actin in cells or in vitro. To our knowledge, no actin isoform-specific differences have been observed on the effects of these compounds on actin properties. Actin polymer-interacting agents include the widely used fungus-derived phalloidin and cytochalasin D (Cooper 1987) and jasplakinolide (Bubb et al. 1994). Phalloidin is, in its fluorescent version, used as probe for F-actin in cells and cytochalasin D as an agent for binding the barbed end. Latrunculins (Yarmola et al. 2000) are used to inhibit actin polymerization by monomer sequestration. Apart from being tools for (cellular) functional studies, they have assisted structural studies (Dominguez and Holmes 2011). Given their cytotoxicity, some of these molecules are even considered in antitumor therapies (Kita and Kigoshi 2015). More recently alternative F-actin probes, such as LifeAct, Utrophin, or F-tractin (respectively, containing calponin homology domains of human ubiquitous dystrophin, the actin-binding domain from yeast ABP140 and from rat inositol trisphosphate-3-kinase) fused to a fluorescent protein, are increasingly used in live cell imaging (Burkel et al. 2007; Riedl et al. 2008; Johnson and Schell 2009). Also nanobody-based tools for following cytoplasmic actin dynamics (in plants) have recently been developed (Rocchetti et al. 2014). The capacities of these new tools to aid in elucidating actin dynamics are promising, but this will need to be accompanied by detailed insight in their specificities, since several studies already point out preferential binding to different F-actin-rich structures on the subcellular level or effects on actin dynamics (Courtemanche et al. 2016). Currently, it is not clear whether these tools display actin isoform specificity.

4 Actin Isoforms: The Things that Make Me Different Are the Things that Make Me[1]

4.1 Differential Expression of Actin Isoforms

The expression patterns of actin isoforms are temporally and spatially regulated during development (reviewed in Tondeleir et al. 2009). The names of the actin isoforms are in part derived from the tissue types in which they were first detected (Vandekerckhove and Weber 1978, 1979). From microarray data, it is now evident that most if not all tissues express more than one type (Fig. 3).

Striated muscles in skeletal muscle and the heart each have a different type of alpha-actin. The major form in adult skeletal muscle is alpha-skeletal muscle actin (Vandekerckhove et al. 1986) encoded by the *ACTA1* gene in humans (*Acta1* in mouse, Table 1). Alpha-cardiac actin, encoded by *ACTC*, is the major form in adult heart, but some regions contain considerable amounts of alpha-skeletal muscle actin as well. Figure 3 demonstrates this on the mRNA level. This has also been

[1] A. A Millne (Piglet in Winnie The Pooh).

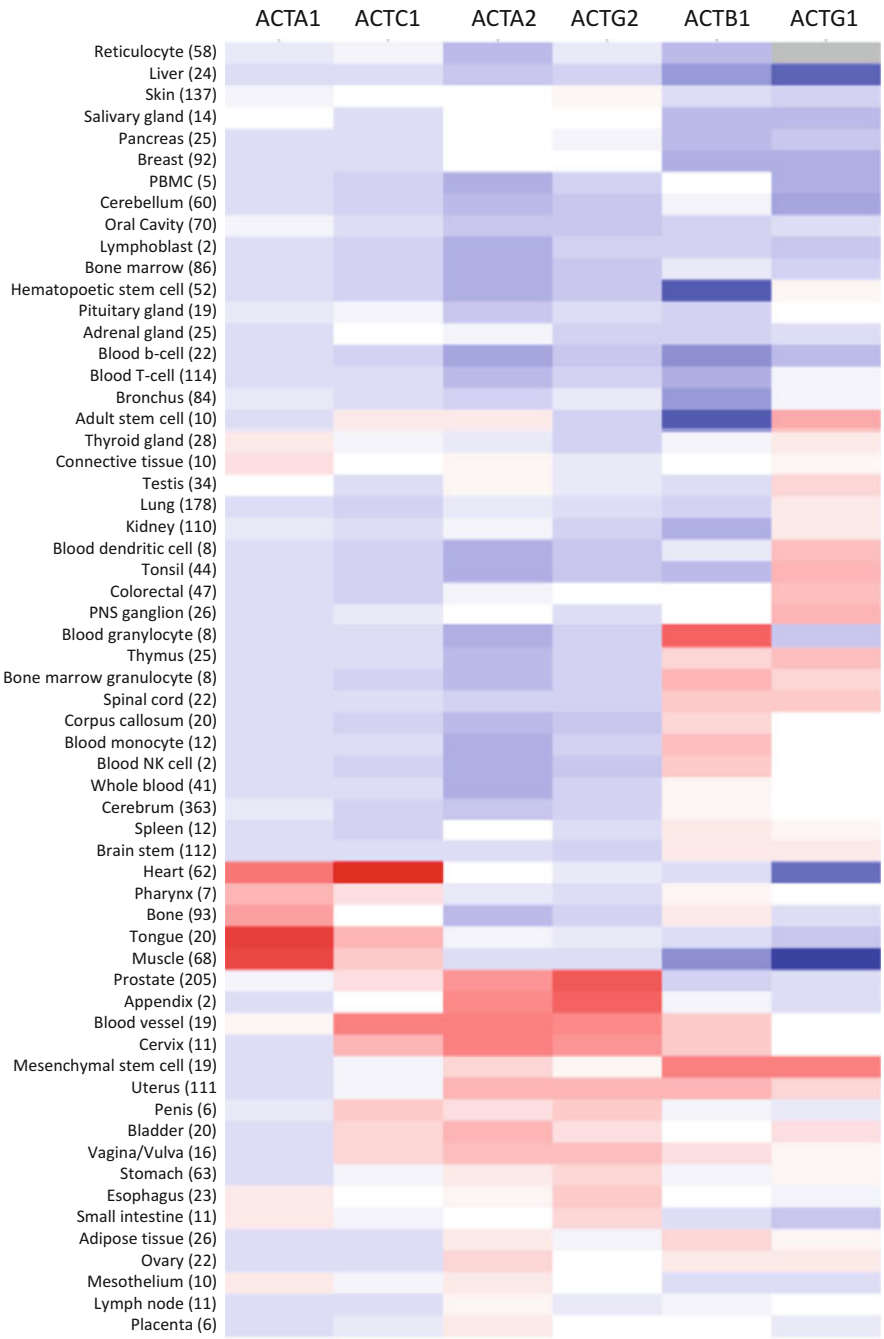

Fig. 3 Tissue-specific differences in actin isoform transcript level. The heat map of actin isoform mRNA levels expressed in the human body was generated in medisapiens in silico transcriptomic

observed at the protein level in healthy hearts (Vandekerckhove et al. 1986; Suurmeijer et al. 2003; Orlandi et al. 2009).

Nonstriated muscle also has typical actin isoforms: *ACTA2* and *ACTG2*, that are often co-expressed as evident from the cluster analysis (Fig. 3), consistent with Fatigati and Murphy (1984). On the protein level, alpha-smooth muscle actin has been shown as the major form in vascular smooth muscle tissues and gamma-smooth muscle actin in enteric tissues (for references, see the introduction of Arnoldi et al. 2013).

For the cytoplasmic actins, the view is variable (Fig. 3). In some tissues or cells, the mRNA for beta-cytoplasmic actin (*ACTB*) is the dominant form (e.g., blood granulocyte, spinal cord corpus callosum), while in others gamma-cytoplasmic actin (*ACTG1*) (e.g., adult stem cell, PNS ganglion, tonsil). Some tissues or cells have nearly similar mRNA levels of the cytoplasmic actins.

These data on tissue- or cell-specific mRNA expression levels evidence tissue-specific regulation of the actin gene promoters. This promoter regulation has mainly been studied in the context of the skeletal and smooth muscle phenotypes and for switches in isoform expression during myogenesis. The important role of the CArG box – serum response factor (SRF) – myocardin (cotranscription factor) controlling axis was established in smooth muscle cells but falls outside the scope of this review (Small 2012; Pipes et al. 2006; Owens et al. 2004; McDonald et al. 2006). However, overall the knowledge of transcriptional control for all six actin isoforms is far from complete.

Obviously the expression data on the mRNA level (Fig. 3) and regulation of the actin promoters are valuable when trying to make correlations of actin expression with diseases such as cancer, but one has to keep in mind that, in general, the correlation between actin mRNA and protein levels has not been made. This is of relevance because in the case of non-muscle actins, various control mechanisms for actin mRNA stability and actin mRNA translation have been described. In mouse two beta-actin transcripts with different lengths of the 3′ untranslated region are expressed in a tissue-specific manner, and translation of the longer transcript is under regulation of a miRNA (Ghosh et al. 2008). Both transcripts contain the so-called zipcode: a 54-nucleotide sequence immediately 3′ of the stop codon (Kislauskis et al. 1997) that targets the transcripts to the periphery of cells where they are locally transcribed upon stimulation (Bassell et al. 1998; Leung et al. 2006; Yao et al. 2006; Huttelmaier et al. 2005). The zipcode is recognized by the zipcode-binding protein 1 (ZBP1) and ZBP2 (Ross et al. 1997; Gu et al. 2002). ZBP2 mainly acts in the nucleus and ZBP1 in the cytoplasm. The translocation through and local activation of translation of beta-actin mRNA in the cytoplasm are dependent on ZBP1 (Huttelmaier et al. 2005; Leung et al. 2006; Yao et al. 2006) and the ZBP1-

Fig. 3 (continued) tool: bodymap (http://ist.medisapiens.com/). Only expression data from healthy tissues were used. Mean expression values were used for clustering tissues and genes. *Blue* indicates low expression levels and *red* high expression levels (*gray* no expression level known)

binding microtubule motor KIF11. Disruption of the interaction of KIF11 with ZBP1 delocalizes β-actin mRNA and affects cell migration (Song et al. 2015). The zipcode is specific to the beta-actin isoform. The RNA-binding protein Sam68 (Src associated in mitosis) may cooperate with ZBP1, regulating localized translation of beta-actin mRNA in dendrites (Klein et al. 2013). The RNA-binding protein HuR stabilizes beta-actin mRNA via a site immediately 3′ of the zipcode sequence and silencing HuR reduces beta-actin mRNA levels (Dormoy-Raclet et al. 2007). Downregulation of β-actin and HuR also affects cell migration of human corneal fibroblasts (Joseph et al. 2014). In addition, downregulation of beta-actin mRNA during myogenesis was attributed to a conserved nucleotide sequence more downstream in the 3′ UTR of the beta-actin mRNA (a sequence located approximately 600 nucleotides 3′ of the stop codon) (DePonti-Zilli et al. 1988). Further details on regulation of beta-actin mRNA transport, stability, and regulation are reviewed in Artman et al. (2014).

In differentiating C2C12 myoblasts, a different type of regulation was proposed to control gamma-cytoplasmic actin expression. During differentiation and fusion, an alternative, noncoding mRNA splice variant is increasingly expressed and its expression correlates with reduction of translation of gamma-cytoplasmic actin protein. This alternative transcript, containing an extra exon that is conserved in Mammalia and is situated between the regular exons 2 and 4, contains an in-frame stop codon, but no corresponding shorter protein was observed using an anti-gamma-cytoplasmic actin antibody. This transcript is muscle specific as it was only present in the skeletal and heart muscle and diaphragm and not in other investigated non-muscle tissues (Drummond and Friderici 2013). In the discussion of this paper, it was suggested that downregulation in the muscle of beta-cytoplasmic actin would be controlled by a similar type of regulated unproductive splicing and translation (RUST) regulation.

Collectively, this indicates that, on top of their differences in primary structure (Fig. 1), the specific functions of actin are also governed by expression regulation and by their ratios in cells when present together.

4.2 Isoform Specificity from Biochemical Experiments

Evidently, obtaining pure actin isoform in sufficient quantities is a prerequisite to study specific biochemical properties of the isoforms. Although this sounds trivial, it has been very cumbersome to recombinantly express actin in the classical expression systems. Because of its requirement for the folding chaperone CCT (Rommelaere et al. 1993), which is not present in prokaryotes, actin cannot be produced in a functional form in bacteria. Beta-cytoplasmic actin can be produced in yeast, but because this organism does not have the appropriate methyltransferase, actin is not methylated at His73 and this affects the polymerization kinetics (Nyman et al. 2002). In addition, in this organism, actin may be incompletely processed N terminally (Cook et al. 1991; Kalhor et al. 1999). Although purification of yeast-produced mammalian actin was valuable for studying mutant actins relative to similarly produced wild type (Nyman et al. 2002; Schuler et al. 1999, 2000a, b), it

cannot be used to fully compare properties of mammalian isoactins because of the potential functional importance of the (differentially) processed N terminus. Along similar lines, tags for easier purification may interfere with proper folding or functioning of actins (Rommelaere et al. 2004; Rutkevich et al. 2006).

People have therefore mostly relied on using alpha-skeletal muscle actin which can be relatively easily purified from skeletal muscle (Spudich and Watt 1971). Beta-cytoplasmic actin has been purified from the profilin-actin complex isolated from calf thymus (Lindberg et al. 1988), but this is difficult to achieve in a truly preparative manner. Given that actins are usually co-expressed, mixed populations are obtained when purifying them from tissue (Gordon et al. 1977; Strzelecka-Golaszewska et al. 1985; Coue et al. 1982), and also commercially available actin protein preparations are consequently isoform mixtures. Thus from a technical point, producing pure actin isoforms in bulk for in-depth biochemical characterization remained challenging for a long time.

The recent progress by producing alpha-cardiac and beta- and gamma-cytoplasmic actin in baculovirus is certainly a breakthrough (Bergeron et al. 2010; Muller et al. 2013; Bookwalter and Trybus 2006; Rutkevich et al. 2006) although in these actin preparations, a measurable amount of insect actin is present for which it is necessary to assume it does not significantly interfere within the experiments. Baculovirus-produced alpha-cardiac actin and classically purified bovine cardiac actin have been compared in relation to their interaction with tropomyosin and troponin, and in this case no difference was observed in contractile and regulatory functions (Bai et al. 2014).

The scientific challenge of biochemical characterization should not be underestimated. Actins have a beautiful but inherently complex biochemistry because of a multistep polymerization process linked to the nucleotide status and because of the multitude of actin-binding proteins. Given the very slight variations in sequence and 3D structure between isoforms, this results in subtle differences in the polymerization process or differential affinities for given actin-binding proteins that require very careful comparative studies, often requiring more than one dedicated assay. This is already evident from the pioneering studies presented below.

Using recombinantly produced beta- and gamma-cytoplasmic actin from insect cells, a number of similarities and differences have been observed. Both cytoplasmic actins are equally thermostable and form similar filaments as judged by electron microscopy. The nucleotide exchange rates of Mg^{2+}-beta- and gamma-cytoplasmic actins were very similar (Bergeron et al. 2010). However polymerization studies showed that gamma-actin (starting from the Mg^{2+}-ATP form) polymerized slower compared to beta-actin. Interestingly, gamma-cytoplasmic actin had a somewhat slower P_i release which was interpreted that treadmilling for beta-actin is slightly faster. It was intriguing that the differences between beta- and gamma-cytoplasmic actin polymerization properties were larger when starting from the Ca^{2+}-ATP actin form. Although in living cells Mg^{2+} is considered the most relevant divalent ion, these authors suggest that, upon activation, local Ca^{2+} increases may be sufficient to affect isoactins differentially. This study using light