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A rod domain sequence in segment 1B triggers dimerisation of the two small *Branchiostoma* IF proteins B2 and A3

Anton Karabinos[a,*], Jürgen Schünemann[b], David A.D. Parry[c]

[a] SEMBID, s.r.o.-Research Center of Applied Biomedical Diagnostics, Hviezdoslavova 22, 08001 Prešov, Slovakia
[b] Max Planck Institute for Biophysical Chemistry, Department of Cellular Logistics, Am Fassberg 11, 37077 Goettingen, Germany
[c] Institute of Fundamental Sciences, Massey University, Palmerston North 4442, New Zealand

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ABSTRACT

Previously, we cloned two *Branchiostoma* IF proteins A3 and B2 and demonstrated that both can form heteropolymeric IF based on a coiled coil dimer consisting of one B2 and one A3 polypeptide. In this study we continued in the characterisation of the B2/A3 heterodimer by searching for the sequences that play an important role in the triggering of the B2/A3 heterodimer. Using a series of deletion and chimeric B2, A3 and B1 constructs and the overlay assay as a tool, we were able to identify a part of the B2 sequence (segment 1A, linker L1 and the N-terminal part of segment 1B) which retains the ability of the full length protein B2 to specifically recognize A3 in blot overlays. Moreover, inspection of this A3-competent B2 fragment identified a short sequence in segment 1B which shares with the currently known trigger-like motif of cortexillin and other coiled coil proteins potential to form multiple inter-chain ionic interactions. Thus, a common and essential feature of trigger sequences with different primary structures found so far in IF and other coiled coil proteins seems to be their ability to form multiple inter-chain ionic interactions which brings the chains close to one another and allows coiled coil formation to propagate accordingly.

Introduction

There are about 70 different members of the IF family of proteins in man (Hesse et al., 2000, 2004) and, in vertebrates, these are subdivided into six types based on sequence similarities, gene structures, expression profiles and polymerisation properties (for reviews see Fuchs and Weber, 1994; Parry and Steinert, 1995; Herrmann et al., 2003, 2009). Keratins, which form epithelial filaments, are based on obligatory heteropolymeric double-stranded coiled coils and each molecule contains one type I and one type II chain. The type III chains include the four mesenchymally expressed proteins – desmin, vimentin, GFAP and peripherin – and these generally form homopolymeric IFs. The type IV classification includes the three neurofilament proteins and α-internexin, the type V group comprises the nuclear lamin proteins, and the two eye lens IF proteins – filensin and phakinin – represent the type VI chains. Orthologs of the vertebrate cytoplasmic type I to III IF proteins have also been found in early chordates (Karabinos et al., 1998, 2000; Luke and Holland, 1999; Wang et al., 2000, 2002).

The primary function of the cytoplasmic IF cytoskeleton seems to be resistance against mechanical stress, provided in large part in metazoan cells by the IF filamentous network. This view is supported by a variety of epidermal fragility syndromes induced by mutated human keratin genes (McLean and Lane, 1995), by knockout technology in mice (*i.e.* Hesse et al., 2000; Vijayaraj et al., 2009) as well as by reverse genetics in the nematode *C. elegans* (Karabinos et al., 2001b; Hapiak et al., 2003; Woo et al., 2004; Hüsken et al., 2008; Zhang et al., 2011).

Structurally, all IF proteins are similar. Each possesses a central rod domain characterized by α-helix-favouring heptad repeats. At its N- and C-terminal ends lie head and tail domains, respectively, that differ significantly in sequence both between chains and between chain types. The central rod domain is subdivided into segments 1A, 1B, 2A and 2B and these are connected to one another by the linkers L1, L12 and L2 respectively. However, the nuclear lamins and the cytoplasmic IFs from protostomia differ from the cytoplasmic IFs of vertebrates, cephalochordates and urochordates as each contains a longer rod domain due to the insertion of an additional 42 residues (six heptads) in segment 1B. In addition, the nuclear lamins have a unique tail containing an Ig-like segment, a nuclear localisation signal and, in most cases, a CaaX box (Erber et al., 1999). It seems reasonable to assume that lamins represent an ancestor sequence of cytoplasmic IFs (Fuchs and Weber, 1994; Parry and Steinert, 1995; Erber et al., 1998; Herrmann et al., 2003).

The structure of the rod domain in IF molecules enables them to assemble both *in vitro* and *in vivo* into one of four closely related 10 nm-like filaments (Fuchs and Weber, 1994; Parry and Steinert, 1995; Herrmann et al., 2003, 2009). Formation of the

* Corresponding author. Tel.: +421 905 402 683.
E-mail address: sembid.pp@gmail.com (A. Karabinos).

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double-stranded coiled coil is driven by internalisation of the apolar residues in positions *a* and *d* of the heptad repeats. The last ten years has revealed significant progress in the elucidation of the atomic structure of the vimentin coiled coil (Strelkov et al., 2002; Meier et al., 2009; Nicolet et al., 2010). However, the elementary question, regarding the trigger mechanism through which coiled coil propagation is initiated in the IF coiled coil remains an open one. In order to provide insight into this process Wu et al. (2000) searched for trigger motifs within keratin K5/K14. These authors prepared a series of point substitutions in both chains and tested their ability to form filaments both *in vitro* and *in vivo*. In addition, they tested the stability of particular dimers and tetramers using a urea disassembly assay. This resulted in the identification of two trigger-like motifs. The first was located on the largely conserved C-terminal end of 2B and this was thought likely to be a common feature in all IF chains. The second motif identified in this study was located in the C-terminal half of coil 1, specifically between residues 79 and 91 of segment 1B. Interestingly, no trigger-like motif was found in segment 1A (Wu et al., 2000).

Previously, we cloned and characterized 13 cytoplasmic IFs from cephalochordate *Branchiostoma.* Five proteins were identified as bona fide keratins. This assignment was confirmed by the obligatory heteropolymeric filament formation of the recombinant proteins. Any stoichiometric mixture of type I (k1, Y1, E1) and type II (D1, E2) proteins provided IF. In addition, two of the *Branchiostoma* type I keratins formed chimeric IF when mixed with human type II keratin 8 (Karabinos et al., 2000). Three keratins (k1, Y1, D1) and protein X1 are the only IF proteins expressed in the gastrula. The number of lancelet IF proteins increases at the neurula and early larval stages to 7 and 11 respectively, and in the adult 13 different proteins have been found. The keratins are the major IF proteins in the *Branchiostoma* nerve cord. Proteins X1, C1 and C2 possess some keratin-like characters and were shown to be integrated into the epidermal and neuronal keratin meshwork (Karabinos et al., 2001a). Finally, the last currently known *Branchiostoma* IF proteins A1, A2, A3, B1 and B2 formed a separated A/B branch in the evolutionary trees and were proposed to be lancelet-specific (Karabinos et al., 2002). The B1 protein is expressed in mesodermally derived muscle tails and in coelomic epithelia, and is also able to form homopolymeric IF *in vitro*. In contrast, its closest relative B2 is co-expressed with the three homologous proteins A1-A3 in the intestinal epithelia. It can form heteropolymeric IF with A3 essential in the formation of filaments, based on a coiled coil dimer consisting of one B2 and one A3 polypeptide. Interestingly, both IF proteins A3 and B2, which have been previously cloned from the *Branchiostoma floridae*, essentially lack a tail domain and are designated therefore as "small" (Karabinos et al., 2002).

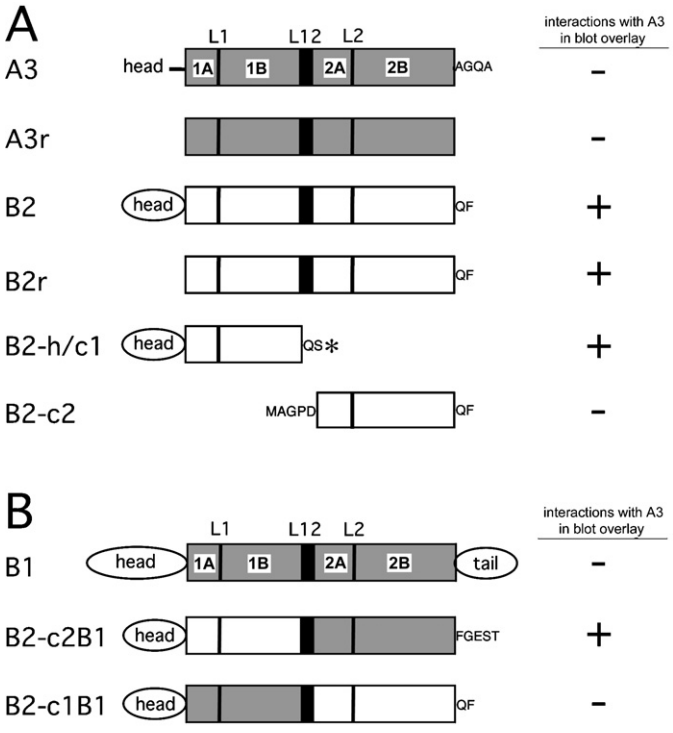

Fig. 1. Schematic representation of the *Branchiostoma* proteins A3, B2, B1 and the corresponding deletion and chimeric mutants used in this study. The structural organisation of an IF protein consists of a head, a rod and a tail domain, as indicated. The helical rod covers segments 1A, 1B, 2A and 2B which are connected by linkers L1, L12 and L2. The plus (+) and minus (−) characters on the right summarize the ability of the corresponding biotin-labelled proteins and mutants to interact with the protein A3 in blot overlays. (A) The fragments A3r and B2r represent the rod domains of the corresponding A3 and B2 proteins. The B2-h/c1 and B2-c2 fragments are deletion mutants of B2 that lack the entire coil 2 and coil 1 segment, respectively. Note the four (AGQA) and two (QF) residue long tail in the full-length A3 and B2 proteins, respectively (Karabinos et al., 2002). The fragment B2-c2 starts with the five residues "MAGPD" which are derived from the linker L12. The asterisk on the B2-h/c1 deletion mutant represents an 24 extra residues (described in Fig. 5A) derived from the expression vector pET. (B) In the chimeric B2-c1B1 and B2-c2B1 mutants the B2 coil 1 and coil 2 segment, respectively, are replaced by the corresponding part of the protein B1. The B2-c2B1 chimeric mutant ends with the four residues FGES derived from the B1 tail and the threonine that is attached to the sequence due to a cloning strategy.

In this study we continued in the characterisation of the B2/A3 heterodimer by searching for the sequences that are responsible for early and highly specific B2/A3 interactions *in vitro*. Such sequences would be expected to play an essential role in the triggering of the B2/A3 heterodimer. Using a series of deletion and chimeric B2, A3 and B1 constructs and the overlay assay as a tool, we were able to identify a part of the B2 sequence (segment 1A, linker L1 and the N-terminal part of segment 1B) which retains the ability of the full length protein B2 to specifically recognize A3 in blot overlays. Moreover, inspection of the A3-competent B2 fragment identified a putative trigger-like sequence in segment 1B that closely resembles the currently defined trigger-motif of cortexillin I and other coiled coil proteins.

Results

Preparation of recombinant proteins and protein mutants

B2 and A3 form a double-stranded coiled coil dimer containing one B2 and one A3 chain. The B2/A3 heterodimerisation represents the first step in the assembly process of these proteins that subsequently ends in the formation of long 10 nm thick IFs *in vitro* (Karabinos et al., 2002). The early and highly specific B2/A3 interactions are best documented in the blot overlay assay (Karabinos et al., 2002). In order to find segments and/or motifs that are responsible for early and highly specific B2/A3-interactions we prepared, expressed and purified a series of deletion and chimeric mutants of B2 and A3 proteins (see Fig. 1). In addition, the full-length protein B1 was expressed and included in this study as a control. The first two deletion mutants B2r and A3r represent the rod domains of the corresponding proteins. The B2-h/c1 deletion mutant contains only the whole head, the coil 1 rod segment and the first two QS residues of linker L12 (Fig. 1A). Moreover, this fragment is terminated by an additional 24 residues derived from the expression vector (depicted by the asterisk in Fig. 1A; see also Fig. 5A). The B2 deletion mutant B2-c2 contains the entire coil 2 segment flanked N-terminally by the linker L12 residues AGPD and C-terminally by the two residue long tail (Fig. 1A). The B2 chimeric mutant B2-c2B1 contains the entire linker L12 but with the coil 2 rod segment replaced by the corresponding B1 sequence terminated by the five residues