Protoplasma (2014) 251:985–988
DOI 10.1007/s00709-013-0608-6

SHORT COMMUNICATION

Unusual ultrastructures of the *Branchiostoma* IF protein C2 containing heptads in the tail

Anton Karabinos · Jürgen Schünemann

Received: 7 November 2013 / Accepted: 21 December 2013 / Published online: 12 January 2014

Abstract *Branchiostoma* intermediate filament (IF) protein C2 contains a long tail domain consisting of several degenerate repeats which display a heptad repeat pattern. This unique tail sequence is predicted to constitute a long coiled coil domain in C2, which is separated from the rod by a glycine-rich linker L3. The recombinant IF protein C2 shows, in electron microscopy (EM), parallel rodlike dimers of 66.7 nm decorated by a larger globule on one side and a smaller globule on the other side. In contrast, the length of the tailless C2 dimers, decorated by only one small globule, is about 26 nm shorter. These results indicate that both the rod domain and the newly predicted coiled coil segment 3 participate in the formation of a double-stranded coiled coil dimer. Moreover, the two to four C2 dimers are able to associate via their globular tail domain into multiarm oligomers, an ability not seen by the tailless C2 mutant or the other currently known protostomic and vertebrate IFs.

Keywords *Branchiostoma* · Intermediate filament · Tail domain · Coiled coil · Dimer

Introduction

About 70 different members of the intermediate filament (IF) family of proteins in vertebrates (Hesse et al. 2004) are separated to the nuclear lamins (type V) and the four cytoplasmic IF types. All IF proteins possess a central rod domain with segments 1A, 1B, 2A and 2B, characterised by heptad repeats. The rod is flanked by head and tail domains that differ significantly in sequence both between chains and between chain types (for reviews, see Fuchs and Weber 1994; Parry and Steinert 1995; Herrmann et al. 2009). The primary function of the cytoplasmic IF cytoskeleton seems to be resistance against mechanical stress (i.e. McLean and Lane 1995; Hesse et al. 2000; Karabinos et al. 2001a; Vijayaraj et al. 2009).

Previously, we cloned and characterised 13 cytoplasmic IFs from cephalochordate *Branchiostoma*. Five *Branchiostoma* IF proteins, identified as *bona fide* type I (k1, Y1, E1) and type II (D1, E2) keratins, form in vitro typical heteropolymeric filaments (Karabinos et al. 2000), are developmentally regulated and represent the major IF proteins in the *Branchiostoma* nerve cord (Karabinos et al. 2001b). Another five *Branchiostoma* keratin-like IFs, A1, A2, A3, B1 and B2, were proposed to be lancelet specific (Karabinos et al. 2002). The B1 protein is mesodermally expressed and forms homopolymeric IF in vitro. In contrast, its closest relative B2 is co-expressed with the three homologous proteins A1–A3 in the intestinal epithelia and forms heteropolymeric IF with A3, triggered by a short sequence in the segment 1B (Karabinos et al. 2012). Finally, the three remaining currently known *Branchiostoma* IF proteins X1, C1 and C2 possess some keratin-like sequences (Karabinos et al. 2000, 2002) and are integrated into the epidermal and neuronal keratin meshwork (Karabinos et al. 2001b), but their precise role in the latter structures remains unknown.

In this work we described a more detailed characterisation of the *Branchiostoma* IF C2 sequence and analysed its structure by electron microscopy (EM).

Materials and methods

Nucleic acid techniques

Cloning of the *Branchiostoma lanceolatum* C2 is described elsewhere (Riemer et al. 1998). The tailless deletion mutant of

Handling Editor: Pavel Dráber

A. Karabinos (✉)
SEMBID, s.r.o.—Research Center of Applied Biomedical Diagnostics, Masarykova 16, 08001 Prešov, Slovakia
e-mail: sembid.pp@gmail.com

J. Schünemann
Department of Cellular Logistics, Max Planck Institute for Biophysical Chemistry, Am Fassberg 11, 37077 Goettingen, Germany

the *B. lanceolatum* protein C2 was prepared by cloning of the corresponding PCR fragments amplified from the C2 complementary DNA (cDNA) (Riemer et al. 1998) into the pET23a expression vector. The tailless C2 fragment contains the entire head and rod domains and ends with the first three residues "MAG" of the linker L3 (for details, see Fig. 1a, c). The primers used for preparation of the deletion mutant, described above, are available upon request. The tailless C2 DNA construct was sequenced to ensure that the correct deletion was generated.

Protein techniques

Expression and purification of the recombinant *Branchiostoma* C2 proteins were as described (Karabinos et al. 2000). Aliquots of the purified recombinant C2 and C2-tailless proteins in 8 M urea buffer at 0.2 mg/ml were dialysed against 50 mM NaCl and 20 mM Tris-HCl (pH 7.4) and processed for glycerol spraying/rotary metal shadowing and EM essentially as described (Karabinos et al. 2003). Other tested assembly buffers were 50 mM NaCl, 10 mM Tris-HCl (pH 7.4), 20 mM Tris-HCl (pH 7.2), 20 mM Tris-HCl (pH 7.4), 20 mM Tris-HCl (pH 7.6) and 2 mM Tris-HCl (pH 9.0). All buffers were 1 mM in DTT and were made in Milli-Q water (Millipore, Escheborn, Germany).

Results and discussion

The tail domain of C2 contains degenerate repeats displaying a coiled coil-forming ability

During preparation of the recombinant C2 proteins in this study (see "Materials and Methods"), we found

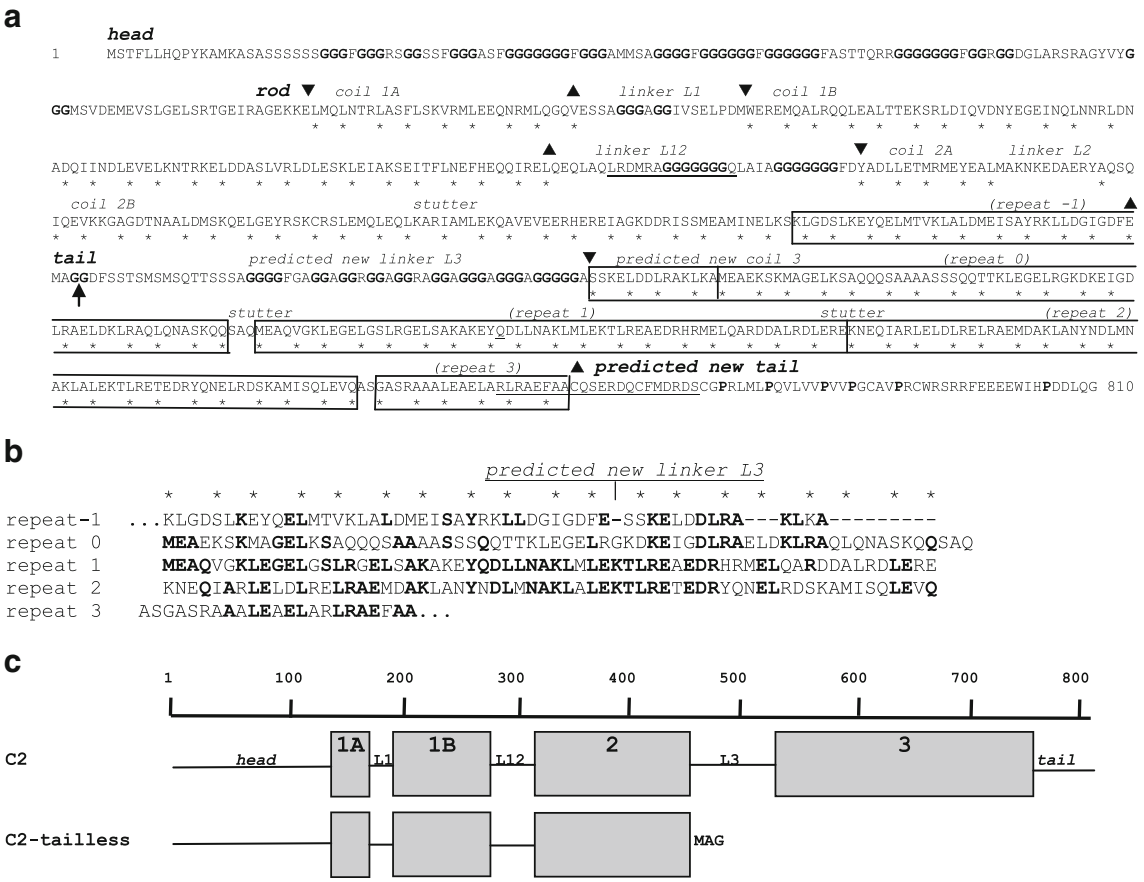

Fig. 1 **a** Amino acid sequence of the *B. lanceolatum* IF protein C2. The head, coils 1A, 1B, 2A, 2B and 3 and the linkers L1, L12, L2 and L3 are indicated. Also indicated are positions of the originally and newly predicted tail domains. *Arrows* pointing downwards and upwards respectively mark the beginning and the end of the rod segments 1A, 1B and 2 as well as of the newly predicted coiled coil 3. *Asterisks* below the rod and coiled coil 3 segments mark the *a* and *d* positions of the heptad repeat pattern. The position of stutters in coils 2 and 3 is indicated. The degenerate repeats −1 to 3 are boxed. The *underlined* sequences in the linker L12 and in the tail domain are new due to a revision of the original C2 sequence (see text for details). Glycine-rich regions in the head domain and in the linkers L1, L12 and L3 are indicated in *bold* type. The *arrows* mark the end of the tailless C2 fragment. **b** The C-terminal part of the rod domain and the attached part of the tail domain display five degenerate repeats (repeats 0, 1 and 2) or their fragments (repeats −1 and 3). *Bold letters* mark residues shared by two, three, four or five repeats. The position of the predicted new linker L3 as well as the new predicted tail is indicated. **c** Schematic representation of the *Branchiostoma* protein C2 and its tailless fragment. The tailless C2 fragment ends with the three residues "MAG" which are derived from the predicted new linker L3

several C2 cDNA clones containing a 14-residue-long glycine-rich insertion in the linker L12 (underlined in Fig. 1a), not observed in our original cDNA analysis (Riemer et al. 1998). Moreover, we corrected an open reading frame of the C2 tail sequence by exchanging the 22 residues "ACVPSLLPASPSATSASWTATR" by another 22 residues "RLRAEFAACQSERDQCFMDRDS" (underlined in Fig. 1a), previously overlooked due to a sequence error. The latter correction helped us identify three more degenerate repeats (or their fragments) in the C2 tail domain (numbered as −1, 0 and 3 in Fig. 1a, b), related to the two originally reported degenerate repeats 1 and 2 (Riemer et al. 1998). Interestingly, all five latter sequences display a heptad repeat pattern predicted to form a new 232-residue-long coiled coil segment 3 in C2. The latter segment contains two stutter-like discontinuities of the coiled coil phasing and is separated from the rod by a 58-residue-long se quence named here as a linker L3 (Fig. 1a). Thus, based on the sequence analysis, described above, the revised C2 protein sequence is predicted now to contain a 139-residue-long head, the coiled coil segments 1A, 1B, 2A, 2B and 3 as well as the 57-residue-long tail domain (Fig. 1a, c). Interestingly, the latter domain is rich in proline and cysteine residues, while the head and the L1, L12 and L3 linkers are rich in glycine residues (Fig. 1a).

Electron microscopy reveals C2 dimers and multiarm oligomers

The first step in the assembly process of all IF proteins is the formation of the parallel double-stranded IF coiled coil (for reviews, see Fuchs and Weber 1994; Parry and Steinert 1995; Herrmann et al. 2009). The conserved assembly mode of the IF proteins and the domain structure prediction for C2, described above, suggest that C2 might form a long double-stranded dimer based on the coiled coil segments 1A, 1B, 2 and 3 (Fig. 1). In order to experimentally prove this suggestion, we have analysed the purified recombinant C2 protein by EM. Figure 2 shows electron micrograph containing a rodlike structure with a larger globule on one side and a smaller globule on the other side (Fig. 2a, b, e). This structure most probably represents a parallel double-stranded dimer. Moreover, multiarm oligomers composed of larger globules creating a prominent globular core and with two to four rodlike arms bending to varying degrees were also revealed (Fig. 2c–e). The latter structures are thought to represent dimers oligomerising via their C-terminal globules because this ability was not seen by the tailless C2 protein (see below). The length of the whole dimer, obtained from 216 measurements, was 66.7 nm. This value was about 40 % higher than 40.5 nm, measured for the 391 C2-tailless dimers prepared and examined under similar experimental conditions (for the domain structure of the tailless C2, see Fig. 1c). The

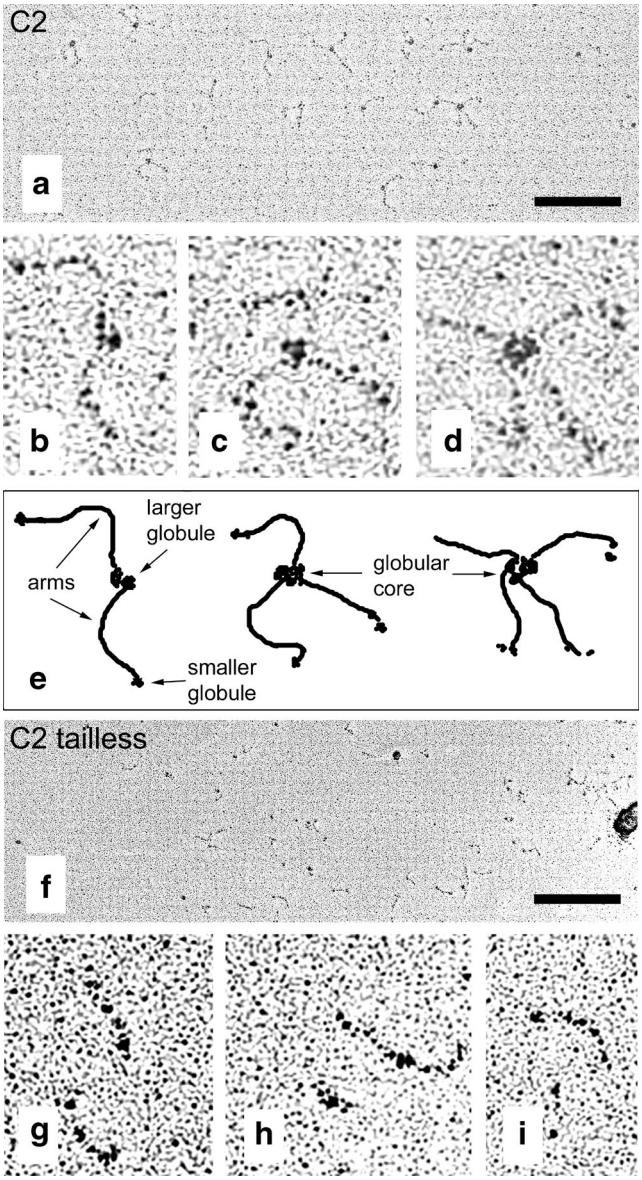

Fig. 2 EM analysis of recombinant *Branchiostoma* protein C2 and its tailless mutant. **a** Electron micrograph containing C2 rodlike dimers, decorated with a larger globule on one side and a smaller globule on the other side as well as multiarm oligomers composed of a prominent globular core and two to four rodlike arms with several flexible linkers bending the rod to varying degrees. Each arm is thought to represent a C2 dimer (see text for details). A detailed micrograph of the two- (**b**), three- (**c**) and four-arm (**d**) oligomers with the corresponding sketches (**e**) to show the arrangement of the individual structures. **f** Electron micrograph containing individual C2-tailless rodlike dimers containing a small globule on one end. Note the absence of oligomerisation on this micrograph. **g–i** A detailed micrograph of individual rodlike dimers of the tailless C2 protein. *Bars* represent 200 nm

C2-tailless dimer shows only single rods with small globules on one side which probably correspond to the head domain of the C2 protein. Moreover, no oligomerisation was seen in the tailless C2 preparation (Fig. 2f, g, i), except a very rare finding of two dimers that appear to be connected (Fig. 2h).

The heptad repeat structures in the rod domain of the cytoplasmic IFs, as mentioned above, are responsible for the formation of homo- or hetero-coiled coil dimers as a first step in the IF assembly. In line with this conserved IF assembly mode, we found here a double-stranded C2 coiled coil (Fig. 2); however, several unexpected features were also found. First, based on a direct comparison of the C2 and tailless C2 molecules in EM (see Fig. 2 and text above), it seems likely that at least a part of the coiled coil segment 3 also participates in the formation of the C2 dimer. Second, the C2 dimers associate via their larger C-terminal globular domains and do not form typical IF tetramers or higher order structures in buffers, tested in this study (see "Material and Methods"). There are several possible explanations for this unusual C2 assembly behaviour. First, it could be that the long *B. lanceolatum* C2 dimer is for some structural reason unable to form tetramers or higher order structures of the typical cytoplasmic IF protein. Such interpretation would be in agreement with the unique tail heptad repeats, found so far only by the two *Branchiostoma* protein C (Riemer et al. 1998). Moreover, the unusual oligomers might indicate the potential of C2 to bundle and/or stabilise the *Branchiostoma* epidermal and neuronal keratins where C2 is a minor component (Karabinos et al. 1998, 2001b). Alternatively, the long C2 dimers and their unusual oligomers are a non-specific product of our in vitro assembly experiments, and a usual IF polymerisation of this protein is triggered by some yet unknown co-polymerisation IF partner. In this respect, we want to note that the overlay experiments with several *Branchiostoma* (D1, E1, k1) or human (K8, K18) type I and type II keratins did not indicate any heterotypic C2 interactions (our unpublished results). Thus, further molecular analyses, for example, using an immunoprecipitation assay with the C2-specific antibody (Riemer et al. 1998), are needed to provide clues to the role which this unique IF protein plays in the *Branchiostoma* epidermal and neuronal keratins.

Acknowledgments We thank D. Riedel and G. Heim (Laboratory for Electron Microscopy, Max Planck Institute for Biophysical Chemistry, Goettingen) for the help with measurement of the EM molecules. This work was funded by the European Regional Development OPVaV-2009/2.2/05-SORO (ITMS code:26220220143).

Conflict of interest The authors declare that they have no conflict of interest.

References

Fuchs E, Weber K (1994) Intermediate filaments: structure, dynamics, function and disease. Annu Rev Biochem 63:345–382

Herrmann H, Strelkov SV, Burkhard P, Aebi U (2009) Intermediate filament: primary determinants of cell architecture and plasticity. J Clin Invest 119:1772–1783

Hesse M, Franz T, Tamai Y, Taketo MM, Magin TM (2000) Targeted deletion of keratin 18 and 19 leads to trophoblast fragility and early embryonic lethality. EMBO J 19:5060–5070

Hesse M, Zimek A, Weber K, Magin TM (2004) Comprehensive analysis of keratin gene clusters in humans and rodents. Eur J Cell Biol 83:19–26

Karabinos A, Riemer D, Erber A, Weber K (1998) Homologues of vertebrate type I, II and III intermediate filament (IF) proteins in an invertebrate: the IF multigene family of the cephalochordate *Branchiostoma*. FEBS Lett 437:15–18

Karabinos A, Riemer D, Panopoulou G, Lehrach H, Weber K (2000) Characterisation and tissue-specific expression of the two keratin subfamilies of intermediate filament proteins in the cephalochordate *Branchiostoma*. Eur J Cell Biol 79:1–10

Karabinos A, Schmidt H, Harborth J, Schnabel R, Weber K (2001a) An essential role for four intermediate filament proteins in *Caenorhabditis elegans* development. Proc Natl Acad Sci U S A 98:7863–7868

Karabinos A, Wang J, Wenzel D, Panopoulou G, Lehrach H, Weber K (2001b) Developmentally controlled expression pattern of intermediate filament proteins in the cephalochordate *Branchiostoma*. Mech Dev 101:283–288

Karabinos A, Schunemann J, Parry DAD, Weber K (2002) Tissue-specific co-expression and in vitro heteropolymer formation of the two small *Branchiostoma* intermediate filament proteins A3 and B2. J Mol Biol 316:127–137

Karabinos A, Schunemann J, Meyer M, Aebi U, Weber K (2003) The single nuclear lamin of *Caenorhabditis elegans* forms in vitro stable intermediate filaments and paracrystals with a reduced axial periodicity. J Mol Biol 325:241–247

Karabinos A, Schunemann J, Parry DAD (2012) A rod domain sequence in segment 1B triggers dimerisation of the two small *Branchiostoma* IF proteins B2 and A3. Eur J Cell Biol 91:800–808

McLean WH, Lane EB (1995) Intermediate filaments in disease. Curr Opin Cell Biol 7:118–125

Parry DAD, Steinert PM (1995) Intermediate filament structure. Springer, New York

Riemer D, Karabinos A, Weber K (1998) Analysis of eight cDNAs and six genes of intermediate filament (IF) proteins in the cephalochordate *Branchiostoma* reveals differences in the IF multigene families of lower chordates and the vertebrates. Gene 211:361–373

Vijayaraj P, Kröger K, Reuter U, Windoffer R, Leube RE, Magin TM (2009) Keratins regulate protein biosynthesis through localization of GLUT1 and -3 upstream of AMP kinase and Raptor. J Cell Biol 187:175–184