Chapter 6

Expression of CHAP in adult mouse tissues is correlated with filamentous actin expression

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Abstract

Previously, we have shown that CHAP is expressed in striated muscles during developmental stages and in adult tissues. Here, we analyzed CHAP expression in multiple mouse adult tissues and show that besides expression in striated and smooth muscle, CHAP is expressed in small intestine, kidney and brain in an isoform specific manner. Expression of CHAP in small intestine and kidney was co-localized with expression of filamentous actin. These results suggest that CHAP may be involved in actin signaling with a broader function as previously expected.

Introduction

Actin is the major component of the cytoskeleton of eukaryotic cells. Many cellular processes, such as division, cell migration, vesicle transport, contractile force generation, cell polarity and cell shape changes are regulated by changes in actin dynamics[1-3]. In mammals and birds there are six different actin isoforms, which are encoded by six different genes. Four of these actin isoforms are muscle specific: α-skeletal actin, α-cardiac actin, α-smooth actin and γ2-smooth actin. The γ1-actin and β-actin are non-muscle isoforms and function in the assembly of the cytoskeleton of non-muscle cells[1, 3]. Monomeric actin (globular or G-actin) can assemble in filamentous actin (F-actin), and this balance is regulated by several actin binding proteins. Cofilin enhances actin filament turnover by severing actin filaments and promoting dissociation of actin monomers from the pointed ends of actin filaments. Profilin on the other hand, promotes assembly of F-actin fibers. Myosin binding to F-actin is essential for contraction in muscle cells. The myosin-actin interaction is also in important for other cellular processes, such as cytokinesis and cell migration[1, 3].

In the previous chapters we have described a novel Z-disc protein, which we named Cytoskeletal Heart-enriched Actin-associated Protein (CHAP)[7]. Two isoforms of CHAP exist: a longer isoform CHAPa and a shorter isoform CHAPb. Whereas CHAPb is expressed in the heart and somites during embryonic development CHAPa is expressed in adult heart and skeletal muscle[8]. We have previously shown that overexpression of CHAP resulted in actin stress fiber formation *in vivo* and *in vitro* (see chapter 4 and 5), suggesting a possible role in actin signaling. This is further corroborated by the fact that CHAP belongs to synaptopodin protein family, of which its other members have also been shown to be involved in actin bundling[4, 5]. Synaptopodin, the first described protein of this family, regulates formation of actin stress fibers, via RhoA signaling[6] and is expressed in brain and kidney[4]. Furthermore, myopodin, a second member of this family, is expressed in muscle cells and is also involved in actin signaling as well. It contains an actin binding site, binds directly to actin and has actin bundling activity[5].

In this chapter we analyzed the expression of CHAP in multiple adult tissues. We confirmed predominant expression of CHAPa and CHAPb isoforms in skeletal muscle by western blot analysis. However, low levels of CHAPb could also be detected in adult kidney, brain, small intestine and large intestine. This was confirmed by specific CHAP immunofluorescent staining on adult mouse tissue sections of kidney and intestine, which strongly co-localized with filamentous actin.

Material and methods

Animals
Adult Swiss mice were sacrificed for the collection of organs. For RNA and protein isolation organs were rinsed in PBS, snap frozen in liquid nitrogen and stored at -80 °C until further use.
Organs for cryosections were perfused, collected and rinsed in PBS. Subsequently, organs were processed as described in Bajanca et al[9]. Briefly, organs were fixed in 0.2% PFA solution containing 4% sucrose, 0.12mM $CaCl_2.2H_2O$, 0.2M $Na_2HPO_4.2H_2O$, 0.2M $NaH_2PO_4.H_2O$ over night at 4 °C. Then, organs were washed in the same solution without PFA during the day at 4 °C, followed by 0.24M phosphate buffer and 30% sucrose over night at 4 °C. The next day embryos were embedded in Tissue-Tek (Sakura Finetek) on dry ice and stored at -20 °C until sectioning.

RNA isolation and cDNA synthesis
Organs were homogenized in Trizol (Invitrogen) using the Ultra Turrax tissue separator (IKA, Germany). Then RNA was isolated according to the manufacturers protocol. RNA was treated with DNase (DNA-free, Ambion) and cDNA was synthesized (iScript BioRad). qPCR was performed using the CFX96 Real-Time PCR detection system (Bio-Rad). The following primers were used: *ChapA* (sense: 5'-GAGGAGGTGCAGGTCACATT-3'; antisense: 5'-CTGAAGAGCCTGGGAAACAG-3'), *ChapB* (sense: 5'-CCGCCGCTTCTTAAACATAA-3 antisense: 5'-GGCTTTAAAGGGCCTTGG-3') and as reference gene *Gapdh* (sense: 5'-GTTTGTGATGGGTGTGAACCAC-3', antisense: 5'-CTGGTCCTCAGTGTAGCCCAA-3'). Data were analyzed with Bio-Rad CFX Manager and presented as mean +/- standard error.

Protein isolation and western blot
For protein isolation organs were crushed on liquid nitrogen and dissolved in T-PER tissue protein extraction reagent (Pierce) with extra added protein inhibitors (protease inhibitor cocktail tablets (10 µg/ml; Roche, Germany), 0.1 mmol/L dithiothreitol (DTT; Invitrogen) and 1 mmol/L phenylmethanesulfonylfluoride (PMSF; Sigma Aldrich), 5 mmol/L NaF and 1 mmol/L Na_3VO_4). Samples were incubated on ice for 15 minutes and centrifuged at 10.000 RPM at 4 °C for 10 minutes and supernatants were transferred to new tubes. Protein concentration was measured with the Bradford assay (Bio-rad) using bovine serum albumin (BSA) for a standard curve. Then 5x sample buffer (100 mM Tris-HCl pH 6.8, 10% SDS, 50% glycerol, 25% β-mercaptoethanol and bromphenol blue) was added and samples were boiled for 5 minutes at 95 °C. Protein gels were loaded with 50 µg protein. Gels were blotted (Hybond-P, GE healthcare) for 3 hours at room temperature and blocked for one hour with 5% milk/Tris Buffered Saline-tween (TBS-T: 50mM Tris-HCl pH 7.5, 125 mM NaCl, 0.02% Tween-20). First antibody diluted in 5% milk/TBS-T was applied over night at 4 °C. Antibodies used were CHAP (1:200, custom made by Eurogentec) and GAPDH (1:10000, Millipore). Secondary antibody was applied for 1 hour at room temperature. Secondary antibodies used were anti-mouse IgG HRP (1:1000, Cell Signaling Technology) or anti-rabbit IgG HRP (1:2000, Cell Signaling Technology) dissolved in 5% milk/TBS-T. Blots were visualized by using SuperSignal West Pico Chemiluminescent Substrate (Pierce).

Immunofluorescence

Organs were sectioned (5 μM) and mounted on starfrost slides (Knittel). Antibody stainings were performed as described by van Laake et al[10]. Antibodies used were CHAP (1:50, custom made by Eurogentec), α-smooth muscle actin (1:500, 1A4, Sigma-Aldrich Chemie), RhoA (1:100, Santa Cruz). Secondary antibodies were as follows: Cy-3 conjugated anti-rabbit (1:250, Jackson Immunoresearch Laboratories) and Alexa488 conjugated anti-mouse (1:200, Invitrogen). F-actin fibers were stained with phalloidin conjugated Alexa488 (1:100, Invitogen) in PBS for 20 minutes. Cell nuclei were stained with TO-PRO (Invitrogen) and slides were enclosed with Prolong Gold (Invitrogen). Stainings were analyzed with SL confocal microscope.

Results

We have previously demonstrated that CHAP is expressed embryonic and adult heart and skeletal muscle and also in smooth muscle cells. To analyze CHAP expression in other adult organs we first isolated RNA from different mouse organs, followed by quantitative analysis of *ChapA* and *ChapB* expression levels (Figure 1A). Expression levels for *ChapA* and *ChapB* were, as expected, highest in skeletal muscle. Nevertheless, expression levels for particularly *ChapB* could also be observed in the other tissues: kidney, brain, stomach, intestine, spleen and lung. *ChapA* was expressed at lower levels but still detectable in kidney small intestine and lung. In order to determine whether CHAPa and b proteins were expressed in these tissues we performed western blot analysis (Figure 1B). Whilst in skeletal muscle both CHAPa (140 kDa) and CHAPb (110 kDa) could be clearly detected, in the other tissues examined we only could detect expression at the height, which corresponds to the size of CHAPb, in kidney, brain, small intestine and large intestine, confirming qPCR results the mRNA expression levels. Although there was a slight difference in the height of the bands between the organs, this may be explained by posttranslational modifications.

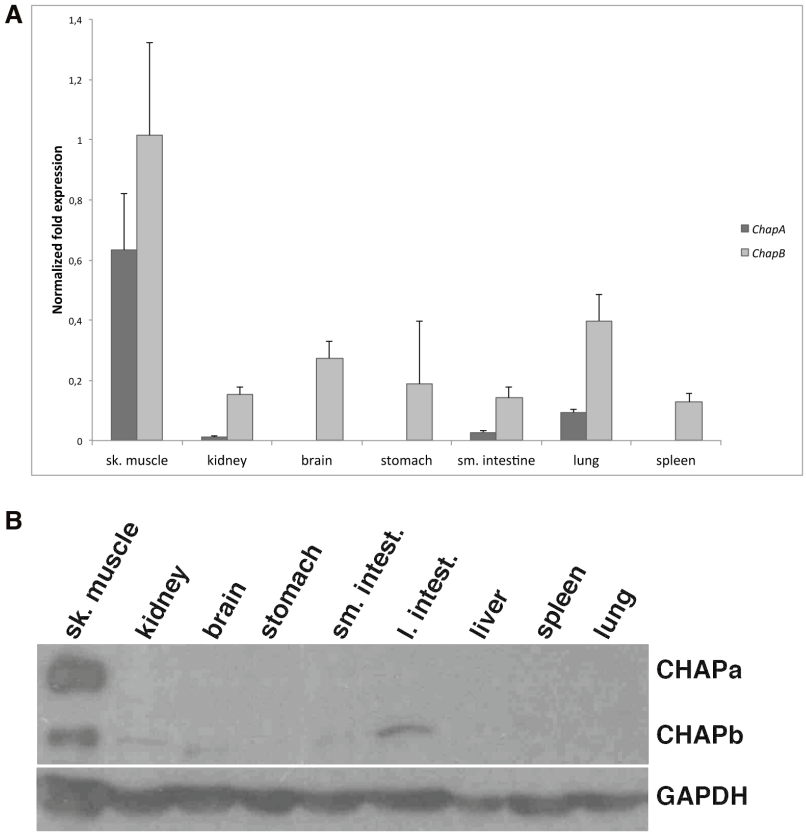

Figure 1: Expression analysis of CHAP in adult tissues . A) qPCR expression analysis of *ChapA* (dark gray bars) and *ChapB* (light gray bars) in skeletal muscle, kidney, brain, stomach, small intestine, lung and spleen. *Gapdh* was used as internal control. B) Western blot showing expression of CHAP in kidney, brain, small intestine and large intestine. GAPDH was used as loading control.

Next we analyzed expression of CHAP by immunohistochemistry on cryosections of these organs. Since we have recently described that CHAP is expressed in smooth muscle cells we co-stained sections of the intestine with α-smooth muscle actin (ASMA). However, no significant CHAP staining was observed in the smooth muscle layer of the small intestine (Figure 2A). Instead, we found CHAP to be expressed in the villus of the small intestine. CHAP expression was not detected in the bottom of the crypt, but was found in differentiated endothelial cells in a dotted pattern and expression was lost at the tip of the villus. Thus expression of CHAP was restricted to the central part of the villus, which contains differentiated endothelial cells. Besides this expression of CHAP, expression was also found in the center of the villus (Figure 2B). The homolog of CHAP, Synaptopodin, is involved in actin polymerization via RhoA and found that RhoA was upregulated and ectopically expressed in CHAPb Tg mice (see chapter 4). Therefore, we co-stained the small intestine for RhoA. Similar to the localization of CHAP, RhoA was expressed in the center of the villus. However, no co-immunostaining of CHAP and RhoA was found (Figure 2C), indicating that CHAP and RhoA were not expressed in the similar cells.

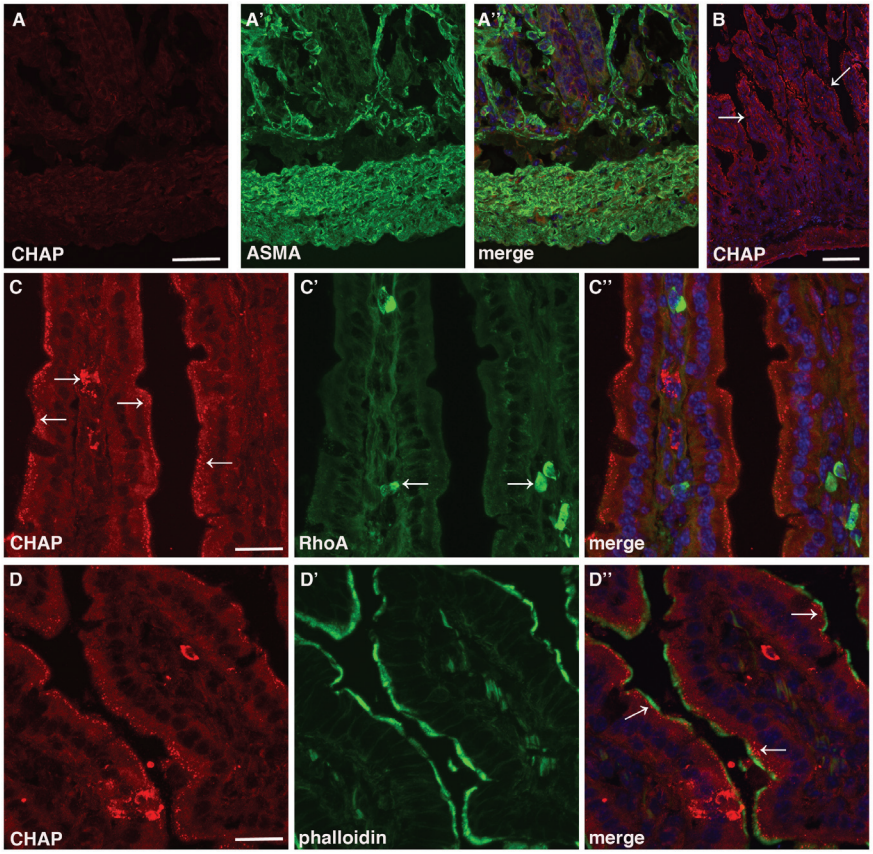

Figure 2: CHAP expression in the small intestine. A) Staining of the smooth muscle layer for CHAP (red; A) and α-smooth muscle actin (green, A'), merge images are shown (A"). B) CHAP is expressed in the villi of the small intestine in a dotted pattern (arrows). C) Staining of the villus for CHAP (red; C) and RhoA (green; C'), merge images are shown (C"). D) Staining of the villus for CHAP (red; D) and phalloidin (green, D'), merge images are shown (D"). Scale bars 20 µm in A, C and D and 100 µm in B.

In the hearts of CHAPb Tg mice we observed actin stress fibers and upregulation of the actin pathway (see chapter 4). To investigate if CHAP localization correlated with filamentous actin (F-actin) localization, we co-stained with phalloidin. F-actin stained the microvilli of a villus. No co-localization of CHAP with F-actin was observed. Instead, CHAP was expressed adjacent to the F-actin stained microvilli (Figure 2D), showing that expression of CHAP was restricted to the roots of the microvilli.
Next we analyzed expression of CHAP in the kidney. Synaptopodin is expressed in kidney podocytes of the glomerulus. Staining of the kidney for CHAP showed that CHAP was expressed in the kidney tubules but not in the glomerulus (Figure 3A and B). Similar as in the small intestine, no co-staining was found of CHAP with RhoA, which was expressed in the glomerulus (Figure 3A). However, co-staining of CHAP with F-actin, which stains the tubule microvilli, showed that as in the small intestine, CHAP localization was adjacent to that of F-actin in the kidney tubules (Figure 3B and C). Thus, CHAP expression was restricted to the roots of the kidney tubule microvilli, suggesting a similar staining pattern as observed in the small intestine.
We also analyzed the expression of CHAP in the brain, however no specific CHAP immunostaining could be detected, although we cannot exclude CHAP expression in specific locations in the brain, that we may have missed in the current analysis.

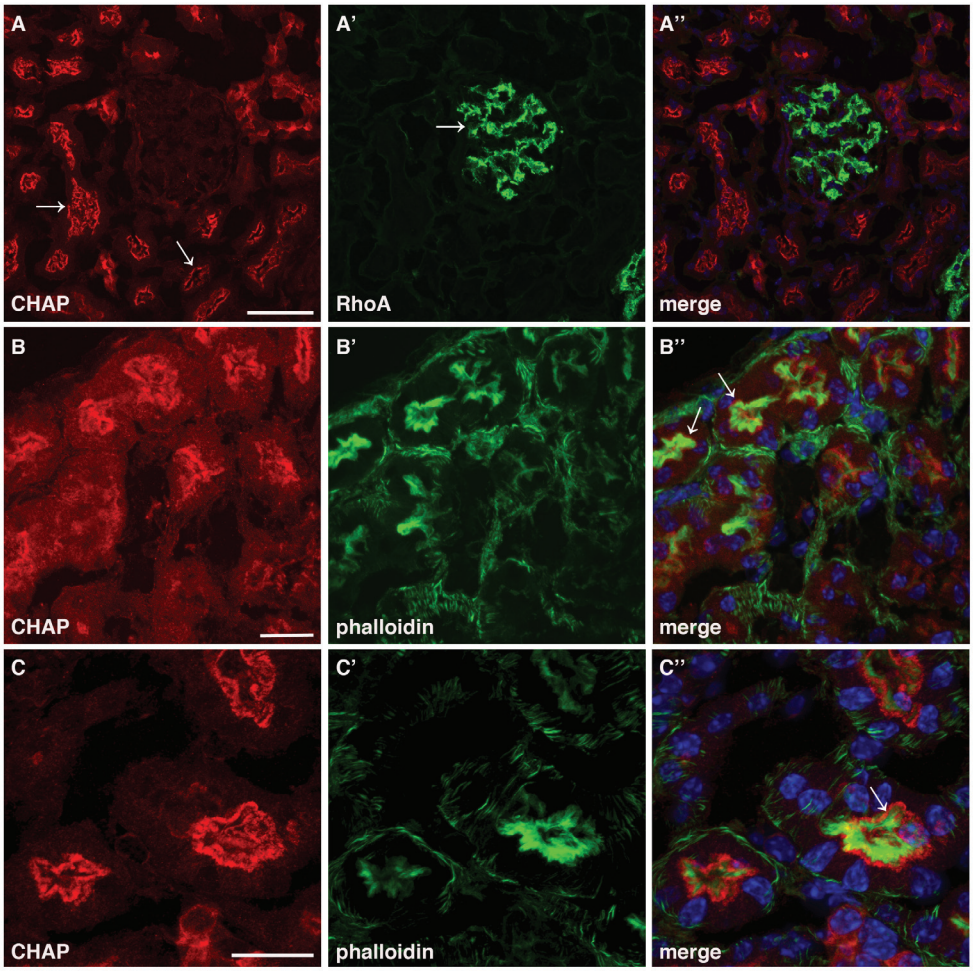

Figure 3: CHAP expression in the kidney. A) CHAP (red) is expressed in the kidney tubules and not in the glomeruli, where RhoA (green) is expressed. Merge images are shown (A"). B and C) Staining of kidney tubules for CHAP (red; B and C) and phalloidin (green; B' and C'). Merge images are shown (B" and C"). Scale bars 50 µm in A, 20 µm in B and C.

Discussion

In this chapter we analyzed the expression of CHAP in a variety of adult organs Previously we have shown that CHAP is expressed in striated muscles (heart and skeletal muscle) and also in smooth muscle cells. Here, we show that CHAP is also expressed in brain, small intestine and kidney. Based on western blot analysis, we conclude that in these organs only CHAPb is expressed. From previous chapters (chapter 4 and 5), we have observed that CHAPb is involved in actin bundling activity (chapter 4 and 5). Immunofluorescence stainings show that CHAP is expressed in the roots of the microvilli of the small intestine and kidney tubules. Microvilli are cylindrical membrane protrusions and have been identified in the small intestine, kidney proximal tubules, and brain. Functions of the villi are increasing the surface area, vesicle releasement, fluid flow sensing and mechano-transduction. Microvilli are composed of actin bundels, which are oriented with the barbed end at the villus tip and the pointed end at the bottom of the bundle. The actin bundles are cross-linked by several cross-linking and membrane-linking proteins, such as myosins. At the bottom of the villus proteins are found that prevent the depolymerization of the actin filaments[11]. For example, tropomyosin is a rootlet protein in microvilli of the small intestine[12] and kidney[13], and has been shown to prevent actin depolymerization by competing with binding to actin with cofilin[13, 14]. Like tropomyosin, CHAP could be involved in preventing actin depolymerization as well.

It would be interesting to investigate expression of CHAP in relation to actin. For example, stereocillia are specialized microvilli on the sensory hair cells of the cochlea and vestibular apparatus[11], where CHAP could be expressed as well. In addition, filopodia are single actin membrane protrusion, involved in cell movement[11], where CHAP expression can be investigated as well. Finally, in cancer cells, actin is necessary for cell movement and in these cells CHAP could function as a tumor suppressor by preventing actin polymerization. Indeed, myopodin has been identified as a tumor suppressor in prostate cancer, as it is (partially) deleted in invasive prostate cancers[15, 16] and inhibits tumor growth *in vitro* and *in vivo*[16].

The results of this chapter show that CHAPb is expressed in a pattern, which is closely related to the presence of filamentous actin and is expressed in different tissue besides heart and skeletal muscle. On the other hand, CHAPa seems to be more restricted to the striated muscles, although we cannot exclude that western blot analysis was not sensitive enough to detect CHAPa protein levels. Furthermore, these results show that CHAPa is muscle-specific expressed, whereas CHAPb is expressed during muscle development and in non-muscle adult tissues.

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