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Smooth muscle specific expression of calponin

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Calponin is an actin-, calmodulin-, and tropomyosin-binding protein that has been isolated from smooth muscle tissue. Using a monoclonal antibody specific for avian calponin, we demonstrate a differentiation-linked increase in calponin expression in embryonic chick gizzard. Cultivation of gizzard smooth muscle cells in vitro resulted in a down-regulation of calponin expression after the first 48 h that was paralleled by a loss of synthesis of metavinculin and the high molecular weight isoform of caldesmon. In early cultures of smooth muscle cells calponin was localised in the actin-containing stress fibres but labelling was restricted to the central parts of the actin cytoskeleton. Calponin expression is suggested as a potentially useful index of smooth muscle differentiation.

Calponin; Smooth muscle; Expression; Localization

1. INTRODUCTION

Calponin is a 34 kDa actin- and calmodulin-binding protein originally purified from vertebrate smooth muscle tissue by Takahashi and colleagues [1]. From the demonstrated binding of calponin also to tropomyosin [2,3], its apparent presence in native thin filaments isolated from smooth muscle [4] and its in vitro inhibition of smooth muscle actomyosin ATPase [4,5] this protein has been attributed with a regulatory role in the contractile machinery. While it has been shown that calponin is absent from certain non-muscle tissues [6], it has not been demonstrated how the expression of calponin correlates with the differentiation state of smooth muscle cells. In this report we show that calponin accumulates during smooth muscle differentiation and disappears concomittantly with two already established smooth muscle markers, metavinculin and the heavy caldesmon isoform, during proliferation of smooth muscle cells in vitro.

2. MATERIALS AND METHODS

2.1. *Electrophoresis*

Analytical gel electrophoresis was carried out on 8-22% acrylamide minislab gels according to the procedure of Matsudaira and Burgess [7] in the buffer system of Laemmli [8]. Protein samples were supplemented with SDS sample buffer (final concentrations: 2.5% SDS, 1% 2-mercaptoethanol, 7% glycerol, 0.001% Bromophenol blue, 62.2 mM Tris, pH 6.8) and boiled for 1 min at 100°C. The gels were stained for 30 min at 60°C in 0.2% Coomassie brilliant blue G250 (Sigma) dissolved in 50% methanol and 10% acetic acid and destained in 10% acetic acid at 70°C.

Whole muscle samples from embryos and adult gizzard were weighed and dissolved at a fixed concentration in the sample buffer and equal amounts applied to the gels.

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2.2. Immunological techniques

Blotting of minislab gels onto nitrocellulose sheets (Schleicher and Schuell, FRG) was performed according to Towbin et al. [9] and silver-enhanced immunogold-staining carried out as described by Moeremans et al. [10] using a secondary antibody with a 20-nm gold tag (Janssen Pharmaceutica, Belgium). Monoclonal antibodies to vinculin, calponin and caldesmon, used for immunoblotting, were from Sigma (vin 11-5, CP-93 and Clone C 0297, respectively).

Monoclonal antibody to calponin clone CP-93 was derived from a fusion between a mouse myeloma cell line P3-NS1-Ag 41 and splenocytes from BALB/c mice immunized with turkey gizzard calponin following standard procedures of hybridoma technology. Positive wells were selected using multiple-slot immunoblotting and immunofluorescence labeling. Clone CP-93 was found to be of IgG1 subclass determined by Sigma Immunotype kit (Sigma, St. Louis).

2.3. *Primary cultures*

Cultured cells were prepared from gizzards taken from 16-day-old chick embryos using collagenase (3 mg/ml) for cell dispersion. Cultures were maintained in DMEM containing 10% fetal calf serum at 37°C in the presence of 5% CO_2.

2.4. *Fluorescence microscopy*

Cultured chick gizzard cells were fixed in a mixture of Triton X-100 (0.2%) and paraformaldehyde (3%) in cytoskeleton buffer [11] for 30 min at room temperature. They were then processed for immunofluorescence microscopy essentially as described elsewhere [12]. The polyclonal antibody against chicken vinculin was kindly provided by Prof. B. Geiger (Weizmann Institut, Israel) and the rhodamine- and coumarin-conjugated phalloidins by Prof. H. Faulstich (Max-Planck-Institute, Heidelberg).

3. RESULTS AND DISCUSSION

The differentiation of gizzard smooth muscle in situ has formerly been shown to be accompanied by characteristic changes in the expression of cytoskeletal and contractile proteins. These include increases in the expression of desmin [13,14], caldesmon [15], metavinculin [16], the smooth muscle isoforms of tropomyosin [17], and changes in the myosin light chain pattern [18,19]. Using a monoclonal antibody that labels only calponin in total extracts of gizzard muscle (Fig. 1), we

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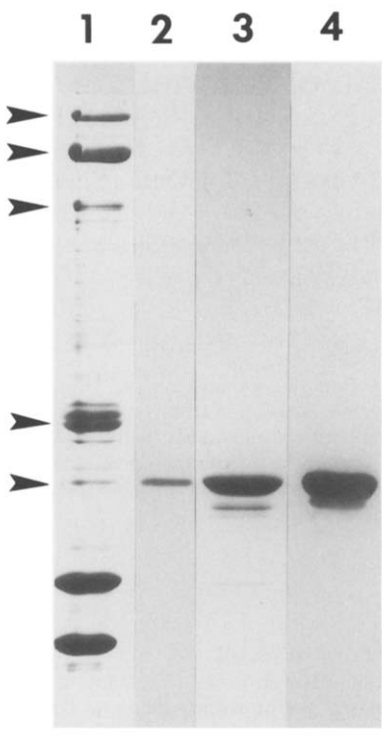

Fig. 1. Immunoblot with monoclonal CALP 93 antibody. (1 and 3) Coomassie-stained gels of total turkey gizzard smooth muscle extract (1) and purified calponin (3). (2 and 4) Corresponding immunoblots. In addition to intact calponin, the antibody stains the 31 kDa proteolytic fragment: a single band is labelled in gels of the whole muscle extract. Arrows indicate filamin, myosin heavy chain, caldesmon, actin and calponin (from top to bottom).

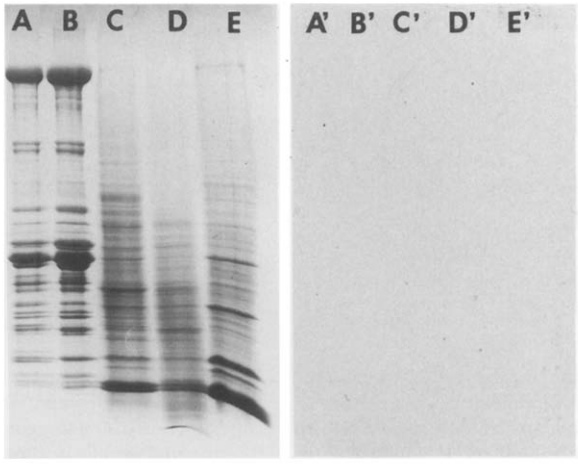

Fig. 3. Absence of calponin from skeletal muscle and non-muscle tissues. Samples shown in gel (left) and corresponding immunoblot with the calponin antibody (right) are of the following chicken tissues: (A,B) skeletal muscles, respectively scapulohumeralis posterior and biceps propatagialis; (C) kidney; (D) liver; (E) spleen.

have shown that there is also a differentiation-linked increase in calponin expression in embryonic chick gizzard (Fig. 2).

In agreement with the data from Takahashi et al. [6] non-muscle tissues, kidney, liver and spleen, were negative for calponin (Fig. 3C–E). We were also unable, with out monoclonal antibody, to detect any immunoreaction in extracts of both skeletal muscle

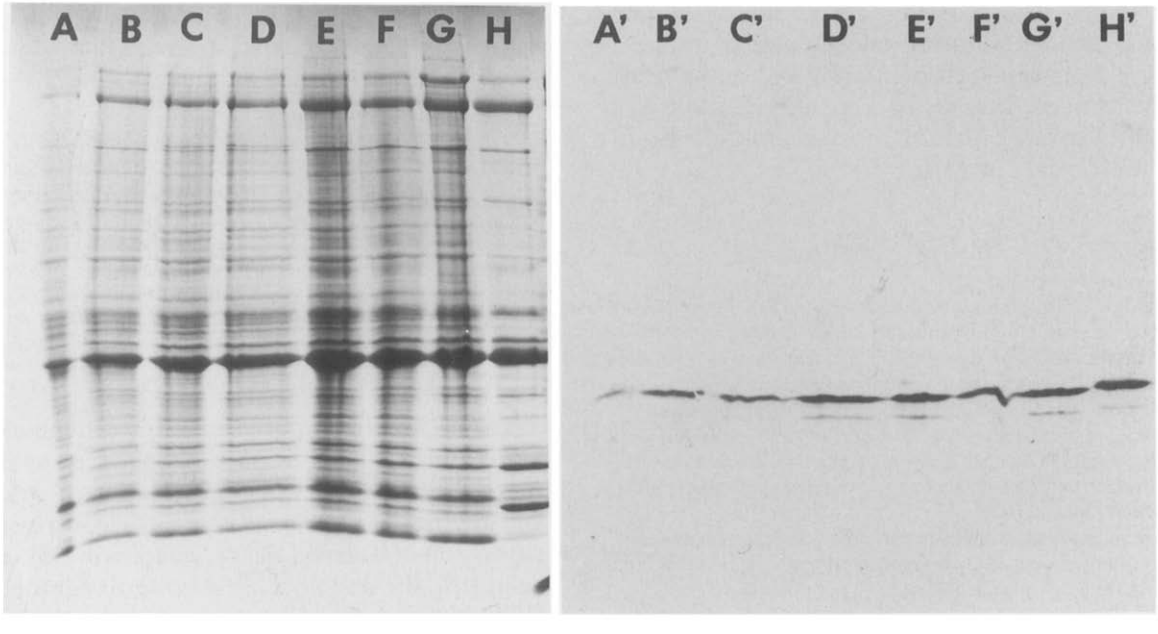

Fig. 2. Expression of calponin in embryonic chick gizzard during development. Samples shown in gel (left) and corresponding immunoblot with the calponin antibody (right) are: (A–G) embryonic gizzards taken at, respectively, 12, 13, 14, 15, 16, 17 and 19 days of incubation and H, adult chicken gizzard.

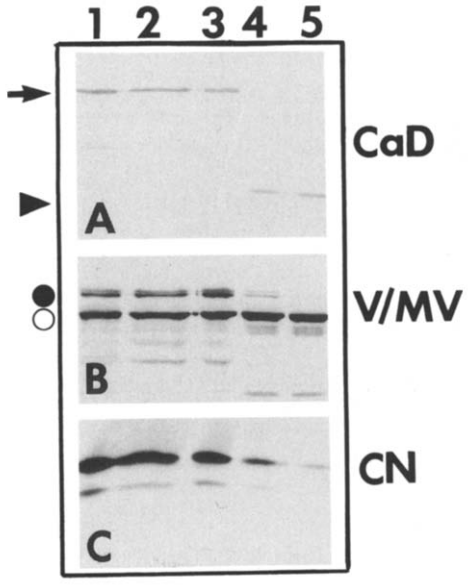

Fig. 4. Down-regulation of calponin expression in gizzard smooth muscle cells cultured in vitro; correlation with expression of caldesmon and metavinculin. Immunoblot shows samples taken from: (1) 16-day embryo gizzard; (2–4) primary culture of 16-day gizzard cells sampled at 3, 24 and 48 h, respectively; (5) same cells as in 2–4 but at 72 h, 24 h after the first replating. The same samples were blotted with antibodies to caldesmon (A), vinculin (B) and calponin (C). Abbreviations: CaD, caldesmon; arrow and arrowhead indicate heavy and light isoforms of caldesmon, respectively; V, vinculin (open circle); MV, metavinculin (filled circle); CN, calponin.

(Fig. 3A,B) and cardiac muscle (not shown). The absence of a cross-reaction in skeletal muscle differs from that observed by Takahashi and colleagues [20] who detected a weak labelling of Troponin T; we presume that the polyclonal antibody used by them contained fractions reactive with troponin T-like epitopes on the molecule, not recognised by our antibody.

When cells from 16-day chick embryo gizzards were taken into culture, they continued to express substantial amounts of calponin during the first 24 h (Fig. 4C, lanes 2 and 3). However, there was a subsequent, dramatic drop in calponin synthesis that was unaffected by the cells reaching confluency (Fig. 4C, lane 4; 48 h). Following the first passage practically no calponin could be detected by immunoblotting (Fig. 4C, lane 5; 72 h). These changes in calponin synthesis in cultured cells in vitro paralleled the loss of metavinculin (Fig. 4B) and the switch from the heavy to the light isoform of caldesmon in the same samples (Fig. 4A). Changes in expression of the latter two proteins on cultivation of smooth muscle cells in vitro have been earlier documented [15,21].

Immunofluorescence microscopy showed that calponin was localised in the actin-containing stress fibres of gizzard cells (Fig. 5A,B; 24 h). Interestingly, the entire length of the stress fibres was not labelled. Characteristically, the actin bundles were unstained at

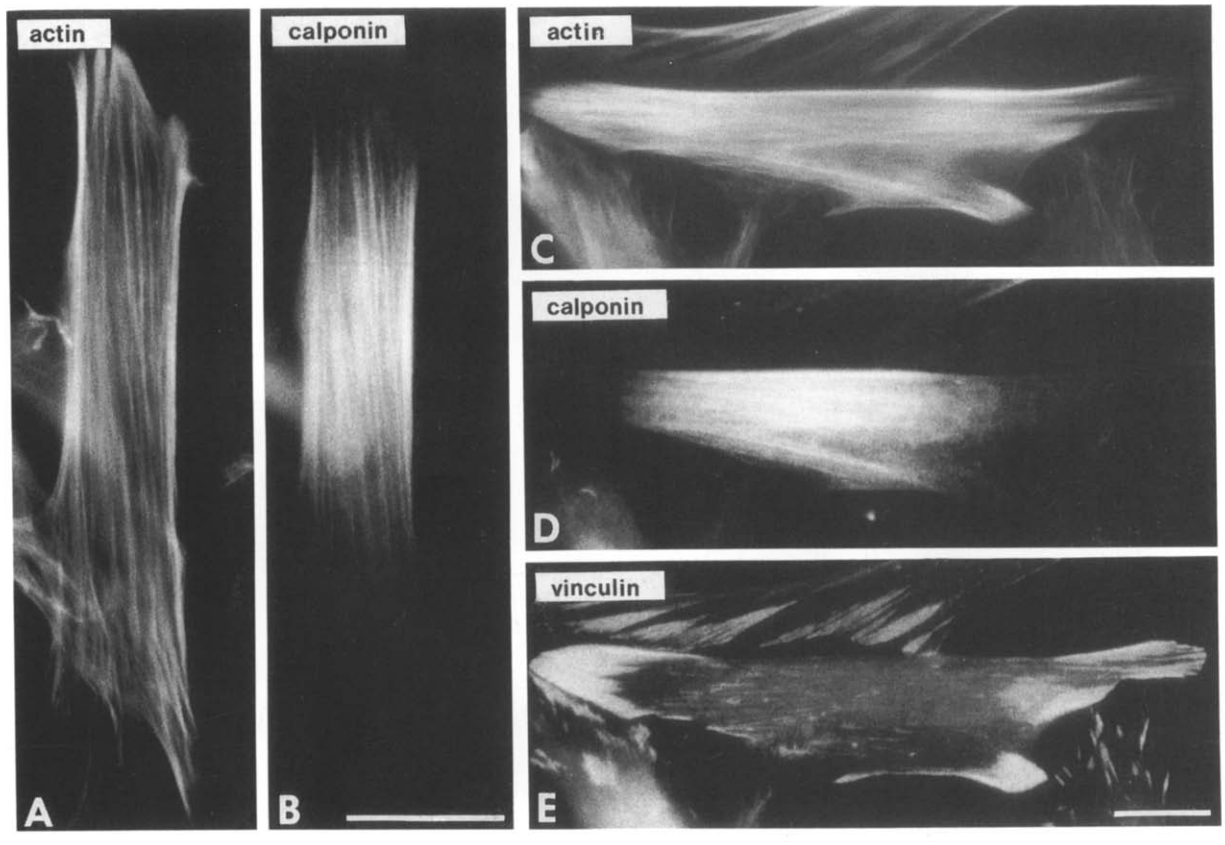

Fig. 5. Colocalization of calponin with a subset of actin filaments in primary cultures of 16-day chick embryo gizzard. (A) actin; (B) calponin; (C–E) triple immunofluorescence microscopy of same cells shows absence of calponin from actin filaments in the region of vinculin rich contacts. (C) actin; (D) calponin; (E) vinculin.

their extreme ends where they approached the substrate-membrane anchorage sites, marked by vinculin antibodies (Fig. 5C–E). In the cell cultures, we typically found cells that were completely unstained for calponin and the proportion of these cells increased with culture time.

The restriction of calponin expression only to smooth muscle points to a tissue-specific function of this protein, the details of which are now under investigation [3–5]. Although isoelectric variants of calponin exist ([20] and our unpublished observations), which may arise from different levels of phosphorylation [5], there is apparently only one molecular weight isoform of this protein, with the apparent molecular weight varying somewhat between species [3,20]. Antibodies to calponin may therefore be of particular value as complementary and alternative markers of smooth muscle differentation.

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