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Calponin

Developmental isoforms and a low molecular weight variant

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Two-dimensional gel analysis of basic proteins in developing human smooth muscle identifies calponin as a prominent marker of the differentiated phenotype. Adult tissue (human and mouse) typically expresses up to four calponin isoforms, three of which appear sequentially during fetal development: adult myometrial cells express the same three isoforms in primary culture in vitro and these are down-regulated, in reverse order, during the subsequent modulation of phenotype. Monospecific, polyclonal antibodies against calponin identify a lower molecular weight variant of calponin (L-calponin) that is strongly and specifically expressed in adult smooth muscles of the human urogenital tract. L-Calponin is down-regulated in benign smooth muscle derived tumors (leiomyoma) and is not expressed in primary cultures of normal uterine tissue.

Smooth muscle; Protein isoforms; Development; Calponin

1. INTRODUCTION

During smooth muscle development the major contractile proteins actin, myosin and tropomyosin show complex patterns of switching, from non-muscle protein isoforms to two or more muscle isoforms in the differentiated cell (see e.g. [1–5]). Antibodies against one or another actin or myosin isoform are already proving useful as markers of smooth muscle cells [3,4,6], but since these isoforms may be differentially expressed in different smooth muscle populations [1,4] and in cells other than smooth muscle [3,7,8] more markers of the smooth muscle phenotype are called for. Studies along the latter lines have already pinpointed metavinculin [9] and the heavy isoform of caldesmon [10] as alternative and complementary markers of the smooth muscle phenotype. The present study was undertaken to establish the possible existence of other smooth muscle specific proteins, emphasis being placed on analysis of the pattern of human basic proteins separated by two-dimensional gel electrophoresis under non-equilibrium conditions. The results confirm and extend more recent studies on chick that have indicated the smooth muscle specific expression of the protein calponin [11]. In addition to showing the differential expression of calponin isoforms in human smooth muscle, during differentiation in vivo and phenotype modulation in vitro we describe a new low molecular weight variant of calponin that is strongly expressed in smooth muscles of the human urogenital tract and that appears to be a late differentiation marker.

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2. MATERIALS AND METHODS

2.1. *Sample preparation*

Human smooth muscle was obtained during routine post mortem examination of patients within 10 hours after death from the following organs. Gastrointestinal: stomach, gallbladder, common bile duct, duodenum, jejunum, ileum, colon. Urogenital: urinary bladder, ureter, uterus, ductus deferens. Cardiovascular: aorta, coronary arteries, carotid artery, mesenteric arteries, inferior vena cava, portal vein. Pulmonary: tracheal muscle. Human fetal tissue was obtained during dissection of fetuses after induced interruption of pregnancy. The gestational ages were 12, 16, 23, 24, 26, 28 and 33 weeks as judged by attending gynaecological staff.

For fetal mouse samples, pregnant Balb/c mice were sacrificed by cervical dislocation at day 15 of gestation and adult intestines and myometrium as well as the fetal intestines were dissected. Intestines of newborn mice were also obtained in the same way. All samples were snap-frozen immediately in liquid N_2 and stored at −70°C.

Samples for two-dimensional gel electrophoresis were produced from 6 μm cryostat sections of the tissue blocks, to ensure fine tissue dispersion; the sections were directly dissolved in lysis buffer [12] on the collecting slide.

2.2. *Electrophoresis and immunoblotting*

Non-equilibrium pH gradient electrophoresis (NEPHGE) was carried out according to Bravo et al. [12]. Carrier ampholines, 2.5% pH range 7–9; 2.5% pH range 8–10, were from Bio-Rad. 200–300 μg of protein were applied to the first dimension and the gels run at 400 V for 4 h 30 min at room temperature. The first dimension gels were applied to 15% slab gels lacking stacking gel and run overnight at 12 mA. Visualisation of proteins was performed using the silver stain method according to Heukeshoven and Dernick [13].

Blotting of gels onto nitrocellulose sheets (Schleicher and Schuell, Germany) was carried out according to Towbin et al. [14] and immu-

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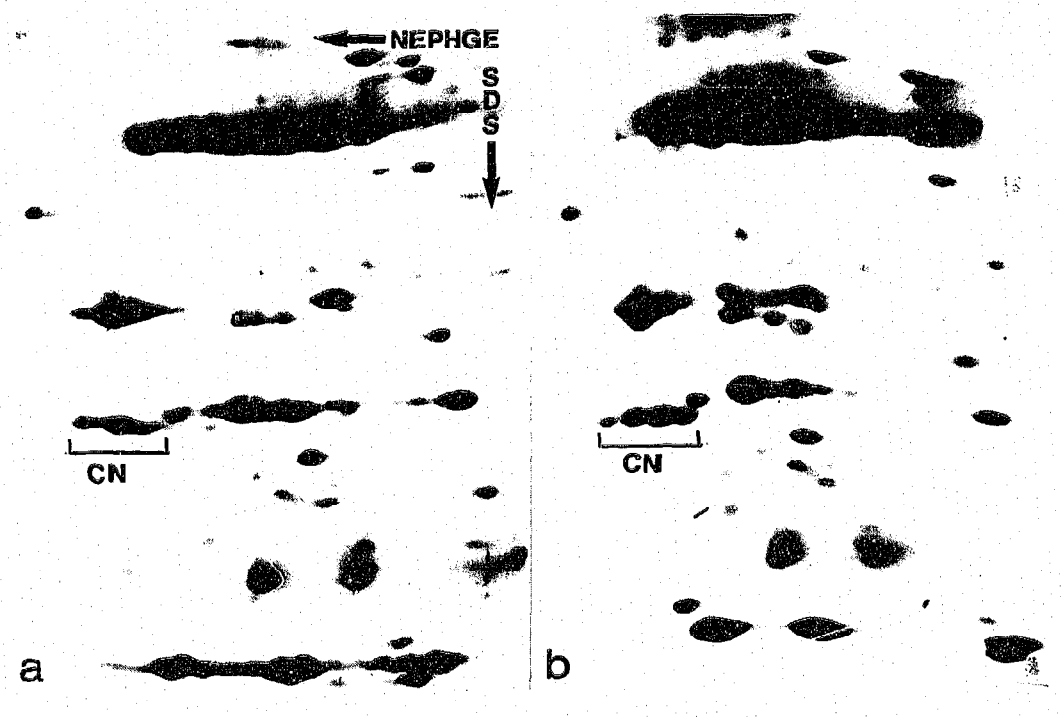

Fig. 1. Non-equilibrium 2D gels (NEPHGE) of smooth muscle samples taken from the human vascular system: four calponin isoforms are expressed in human inferior vena cava (a) and three in the left anterior descending coronary artery (b). CN, calponin

noreactions detected using the silver enhanced immunogold staining method, as described by Moeremans et al. [15] using a secondary antibody with a gold tag (Janssen Pharmaceutica, Belgium). The monoclonal antibody to desmin (U10) was kindly provided by Prof. M. Osborn; antibodies to α-smooth muscle actin, vinculin and caldesmon were from Sigma. The polyclonal antibody to porcine stomach calponin (Gimona et al., in preparation) was raised in rabbits and then purified by affinity chromatography on porcine stomach calponin coupled to CNBr-activated Sepharose 4-B (Pharmacia Fine Chemicals, Sweden).

2.3. *Tissue culture*

Human smooth muscle for tissue cultures was obtained from hysterectomy specimens immediately after surgery. Parallel analysis of normal and tumorous myometrium was carried out by simultaneously processing each specimen for 2D-electrophoresis (see above) and tissue culture. Briefly, tissue pieces of 4–5 g were chopped with a scalpel and vigorously stirred for 3 h at 37°C in 0.125 mg/ml collagenase I (Sigma), 0.13 mg/ml hyaluronidase (Sigma) in MEM medium with Hank's salts (Gibco). After filtration through a gauze layer, the cells were seeded into 35-mm Falcon plastic dishes and cells were cultured in BME medium supplemented with 10% fetal calf serum, 0.1% penicillin/streptomycin and 0.1% glutamine (Gibco). Confluency was reached after 5 days (high seeding density) or after 12 days (moderate seeding density). After reaching confluency they were washed in low salt buffer (imidazole 5 mM, KCl 30 mM, $MgCl_2$, 1 mM, pH 7.4) and dissolved in lysis solution.

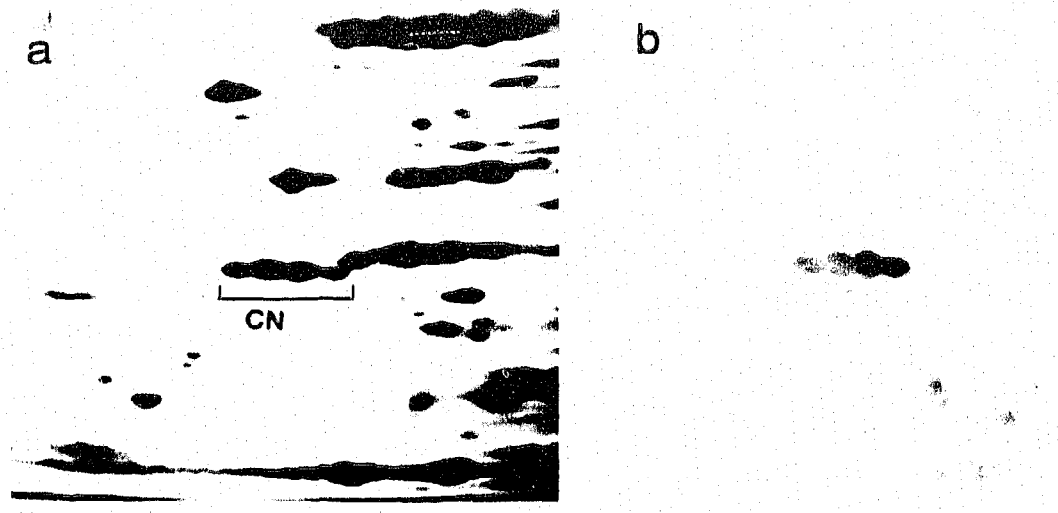

Fig. 2. Human stomach muscle, showing four calponin isoforms. (a) silver stained gel (NEPHGE) and (b) immunoblot with calponin antibody.

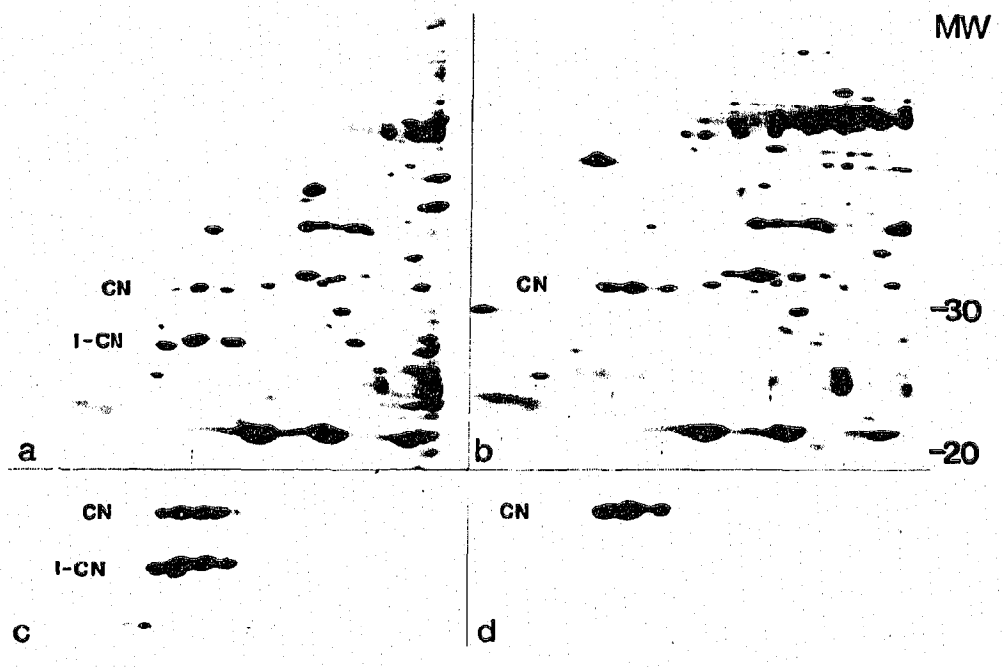

Fig. 3. Calponin expression in normal human myometrium (a,c) and ajdacent tumor tissue (leiomyoma) (b,d). a,b silver staining; c,d corresponding immunoblots with the calponin antibody. Calponin isoforms can be distinguished in both tissues, but l-calponin is only present in normal myometrium. MW, molecular weight × 10^{-3}

3. RESULTS

3.1. *Calponin isoforms in human smooth muscles*

When two-dimensional gel patterns of basic proteins obtained from adult human smooth muscles were compared with a data-base accumulated from human tissues and cultured cells [16] several polypeptides, apparently specific for smooth muscle were detected. Prominent among these was a group of up to 4 spots in the range of 34 kDa (Figs. 1 and 2a) that cross-reacted with an antibody against porcine calponin (Fig. 2b) and could thus be identified as the human equivalent of this protein. As shown in Figs. 1 and 2, three to four isoforms of calponin could be observed in smooth muscles of the vascular system (Fig. 1a,b) and the gastrointestinal tract (Fig. 2).

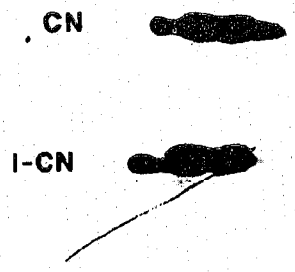

Fig. 4. Immunoblot of human ureter smooth muscle with calponin antibody showing presence of l-calponin.

3.2. *Occurrence of l-calponin; a lower molecular weight calponin variant*

The analysis of uterine likewise revealed multiple isoforms of calponin. A notable finding, however, was the presence of an additional set of polypeptides directly below calponin at a molecular weight of around 29 kDa (Fig. 3a) that cross-reacted with the calponin polyclonal antibody (Fig. 3c). In parallel experiments with porcine stomach calponin (data not shown) it could be demonstrated that neither deliberate nor spontaneous proteolysis of calponin gave rise to polypeptides whose position corresponded to those of the additional spots seen in uterine tissue: the latter polypeptides were therefore not likely products of proteolysis of 34 kDa calponin. Further evidence for the presence of a distinct variant of calponin in uterus came from parallel analysis of benign smooth-muscle derived tumors (leiomyoma). In contrast to those from normal myometrium the leiomyoma samples showed, without exception, no detectable expression of the lower molecular weight calponin variant (Fig. 3b,d). Two variants of caldesmon of high and low molecular weight termed l- and h-caldesmon have been described by Ueki et al. [10]: by analogy we

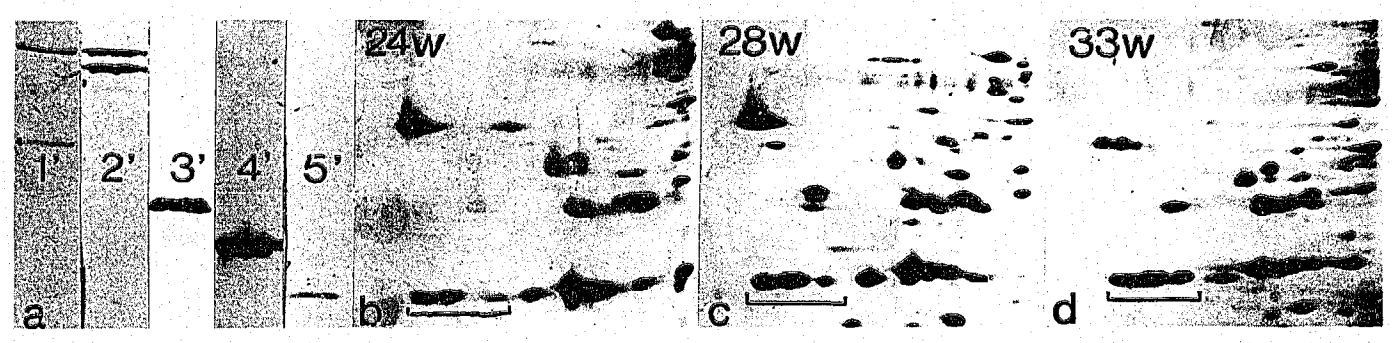

Fig. 5. Smooth muscle specific proteins and calponin isoforms in human fetal development. (a) Immunoblots of fetal bladder (gestational age 24 weeks), with antibodies against: caldesmon (1'), metavinculin/vinculin (2'), desmin (3'), α-smooth muscle actin (4'), and calponin (5'). All smooth muscle specific differentiation markers are present at this stage. b–d: 2D electrophoresis (NEPHGE) of fetal bladder at the gestational ages indicated. Two main isoforms were observed at 24 weeks and three from 28 weeks onwards.

shall refer to the low molecular weight calponin variant as l-calponin.

L-calponin in bladder was also expressed in bladder and vas deferens (not shown) as well as ureter (Fig. 4), suggesting that this variant is mainly confined to muscles of the urogenital tract.

3.3. *Calponin isoforms during differentiation and phenotypic modulation of mammalian smooth muscle*

When we examined human fetal tissue (12–33 weeks of gestation) the muscle or smooth muscle specific proteins caldesmon, metavinculin, desmin, α-smooth muscle actin and calponin could be demonstrated by immunoblotting (Fig. 5a): no trace of l-calponin, however, could be detected. At the different stages examined, changes in the number of calponin isoforms were also evident. At 12, 16 and 24 weeks only two isoforms (the most basic ones of those seen in adult tissue) could be identified in bladder (e.g. Fig. 5b). At 28 and 33 weeks three isoforms were apparent (Fig. 5c,d), as compared to the 4 isoforms characteristic of the adult (Figs. 1, 2 and 3); similar data were obtained for stomach muscle.

Intestinal smooth muscle of fetal mice at the gestational age 15 days showed only one calponin isoform (Fig. 6a), newborn mice two (Fig. 6b) and the adult intestines three (Fig. 6c). No l-calponin could be detected in mouse by immunoblotting.

In chicks it has been shown that the modulation of the phenotype of gizzard cells in culture is accompanied by the loss of calponin expression [11]. We confirmed and extended these findings with cultures of human myometrium. In addition, it could be shown that, apart from the immediate loss of l-calponin, calponin down-regulation occurred via a sequential loss of its isoforms, starting with the most acidic (Fig. 7). This loss occurred, not surprisingly, in the reverse order of appearance seen for the same isoforms, during differentiation in vivo.

4. DISCUSSION

We have identified a protein with an identical isoelectric focussing pattern (pH region 8.4 to 9.1 [17]) and a slightly lower molecular weight than calponin that is most abundant in smooth muscles of the human urogenital tract. This low molecular weight variant of calponin may correspond to the smaller isoform described in a preliminary report on chick calponin cDNAs by Takahashi et al. [18]. The appearance of phenotypically-specific proteins during the differentiation of smooth muscle is not synchronized, but temporally separated [19]. Whereas α-smooth muscle actin and smooth muscle specific myosin isoforms belong to the early differentiation markers [3,4], h-caldesmon and metavinculin appear comparatively late in fetal development [19]. The increase in number of calponin isoforms, up to at least 4 in the human adult, occurs gradually throughout fetal development, but l-calponin has been detected only

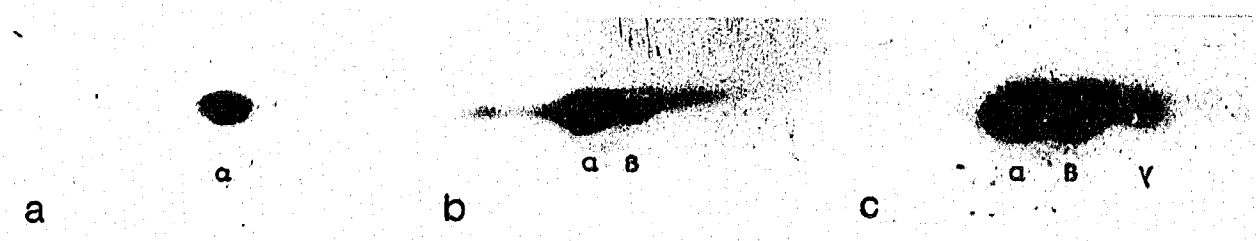

Fig. 6. Calponin isoforms during development of murine smooth muscle. Immunoblots with a calponin antibody of samples of mouse intestine show sequential appearance of calponin isoforms. a, 15 days gestation; b, newborn mouse; c, adult.

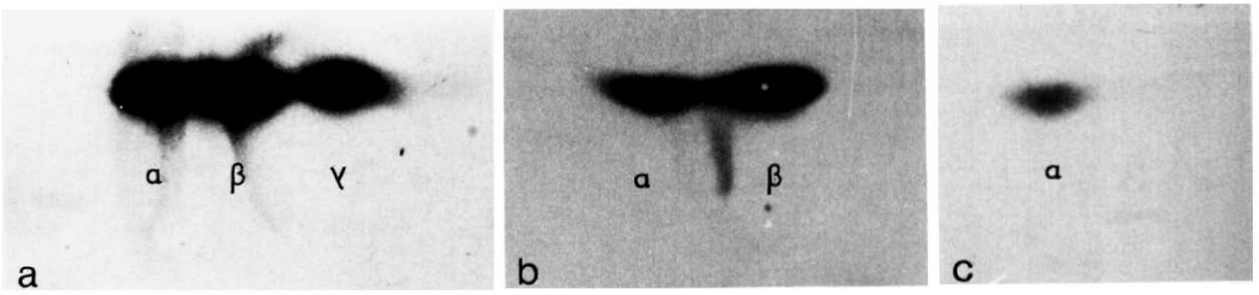

Fig. 7. Disappearance of calponin isoforms during phenotype modulation in tissue culture. Primary cultures of human myometrium (a,b) showing the dependence of calponin isoform expression on seeding density. The γ-isoform is retained up to confluency in primary cultures obtained by seeding cells at a high density (a). Low seeding density leads to downregulation of the γ-isoform at confluency (b). After the first passage the β-isoform is no longer expressed (c), and the α-isoform disappears after further population doublings.

in differentiated smooth muscle. We conclude that this variant may thus be a very late differentiation marker in the smooth muscles in which it is expressed. Not surprisingly, it is also the first to be down-regulated in tissue culture and in smooth muscle-derived tumors.

The present of l-calponin in muscle of the urogenital tract raises the question as to whether it serves a specific function related to this system. Uterine tissue is subject to natural hypertrophy during pregnancy: however, the expression of l-calponin is apparently unrelated to this property. Thus, rat bladder which lacks l-calponin (Draeger and Malmqvist, unpublished data) does not exhibit synthesis of this variant after hypertrophy is experimentally induced [20]; the significance of l-calponin is therefore unclear. It is however of interest to note that the smooth muscles of the urogenital tract derive from the intermediate mesoderm [21] and that this feature may predetermine specific expression patterns.

In conclusion, we show here the expression of calponin isoforms during differentiation of human smooth muscle and the late and organ specific expression of a calponin variant in the urogenital tract. From recent studies [22,23] calponin has been attributed a regulatory role in the smooth muscle contractile apparatus. The reason for the requirement of several isoforms of this protein as well as a smaller variant in some tissues must be clarified by future work. In parallel studies (Gimona et al., in preparation) we have already shown, however, that the calponin isoforms in pig and chicken are encoded by a single messenger RNA species and do not arise via differential phosphorylation.

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