Adenovirus-mediated transfer of the smooth muscle cell calponin gene inhibits proliferation of smooth muscle cells and fibroblasts

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Abstract Smooth muscle cell calponin (*h1* or basic isoform) is an actin-binding protein that inhibits actomyosin MgATPase activity and is abundantly expressed in differentiated smooth muscle. Western blots showed bovine tracheal (BT) smooth muscle cells in culture expressed only $2 \pm 1\%$ ($n = 8$) of the amount of calponin in tissues, while NIH-3T3 fibroblasts expressed none. We tested the hypothesis that introduction of calponin to cultured BT and 3T3 cells would inhibit cytoskeletal activities associated with cell proliferation. To achieve high-efficiency expression, an adenovirus encoding the CMV–calponin construct (Adv-CaP) was generated by homologous recombination in 293 cells. With greater than 90% of BT and 3T3 cells infected with Adv-CaP, calponin expression (32 and 11 μg/mg total protein, respectively) was similar to that in smooth muscle tissues (51 μg/mg). Cells were infected with Adv-CaP for 48 h, replated at low density and proliferation rates were assessed by cell density and [³H]thymidine incorporation. Cell growth and DNA synthesis by Adv-CaP-infected cells were inhibited to one-third control values for both BT and 3T3 cells. Expressed calponin was localized primarily on stress fibers in both cell types. Calponin may act at the cytoskeletal level to retard signaling pathways that normally lead to tight coupling between cell shape and DNA synthesis.
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Key words: Adenovirus; Calponin; Actin-binding protein; Smooth muscle; Fibroblast

1. Introduction

Smooth muscle cell (SMC) calponin (also *h1* or basic calponin) is a thin filament-associated protein expressed exclusively in smooth muscle cells in adult animals [1,2]. SMC calponin is implicated in actin-linked regulation because it inhibits smooth muscle actin-activated myosin MgATPase and this inhibition can be reversed by phosphorylation or binding of Ca^{2+}/calmodulin in vitro [3–5]. In smooth muscle cells, calponin can also promote actin filament stability in vitro by inhibiting depolymerization [6]. SMC calponin is localized to actin-containing filaments [7–9]. Its enrichment in the cytoskeletal (myosin-deficient) domains, including dense

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Abbreviations: SMC, smooth muscle cell; Adv-CaP, recombinant, replication-deficient adenovirus containing avian SMC calponin cDNA expression cassette; Adv-βgal, recombinant, replication-deficient adenovirus containing β-galactosidase cDNA expression cassette; BT, bovine trachea; CaP, calponin; MOI, multiplicity of infection; pAC-CaP, pAC$_{sk.2}$CMV5 plasmid containing avian SMC calponin cDNA expression cassette; PFU, plaque forming unit

bodies and dense plaques, of chicken gizzard cells suggests that SMC calponin may perform as yet undefined cytoskeletal functions [10,11].

In order to assess the effects of SMC calponin on actin- and myosin-dependent functions in intact smooth muscle cells, we have taken advantage of the fact that SMC calponin is rapidly down-regulated in SMCs placed in culture [7,8]. Introduction of the SMC calponin gene to these cells and to non-muscle cells should yield insights into potential contractile and cytoskeletal functions of the re-expressed calponin. Results may also bear on potential functions of other isoforms of calponin (neutral (*h2*) and acidic) which have a wider tissue distribution and are likely to affect processes other than contraction [12,13]. Interpretation of the effects of exogenous protein on functions in a population of cells depends critically on achieving high-efficiency expression. We have accomplished this by constructing a recombinant, replication-defective adenovirus containing the avian SMC calponin cDNA. The present report documents the utility of this approach for introduction of contractile proteins into smooth muscle cells. In addition, we demonstrate that SMC calponin expression in cultured cells inhibits proliferation, establishing a potential cytoskeletal action for this abundant smooth muscle protein.

2. Materials and methods

2.1. Cell culture

Bovine tracheal (BT) smooth muscle cells were isolated and prepared for primary culture as previously described [14]. BT cells and NIH-3T3 cells were maintained at 37°C, 5% CO_2 in DMEM medium containing 10% fetal bovine serum, 1% antibiotic-antimycotic (GIBCO BRL). BT cells were used at passage 2-3.

2.2. Construction of recombinant adenovirus and cell infection

Adv-CaP, the replication-defective, recombinant adenovirus encoding avian SMC calponin was constructed and prepared according to standard methods [15,16]. The cDNA encoding chicken SMC calponin was placed under transcriptional control of the constitutive cytomegalovirus (CMV) promoter/enhancer present in the pAC$_{sk2}$CMV5 shuttle vector [17]. The pAC$_{sk2}$CMV5 vector was derived from the previously described pAC vector. It contains 8.1 map units of adenoviral genomic sequence (0–1.4 and 9.1–15.8), the pBluescript SK-vector, and the expression cassette from pCMV5 including the CMV promoter, polylinker, and SV40 polyadenylation sequence. A 923 base pair *BglII/ClaI* fragment of the SMC calponin cDNA that contains the entire protein-coding region was subcloned into the pAC$_{sk2}$CMV5 vector. The resulting plasmid, pAC-CaP, was co-transfected with pJM17 into 293 cells (adenovirus E1A-transformed human embryonic kidney cells) by calcium phosphate/DNA co-precipitation. pJM17 is a bacterial plasmid in which a circularized, full-length adenovirus 5 genome is interrupted at 3.7 map units by insertion of the plasmid pBRX. Adv-CaP was generated as a result of homologous recombination between the two plasmids yielding a genome in which the adenovirus early region 1 is replaced by the chimeric gene, causing the recombinant virus to be replication defective. Vectors

pAC$_{sk.2}$CMV5 and pJM17 and adenovirus encoding β-galactosidase (Adv-βgal) were generously provided by Dr. J. Alcorn (UT Southwestern Medical Center). High-titer viral stocks (≥10^9 plaque forming units (PFU)/ml) for Adv-CaP and Adv-βgal were obtained following selection of individual viral plaques, propagation in 293 cells, and purification by CsCl density centrifugation, as described [16]. Titers of viral stocks were determined by plaque assay in 293 cells. Aliquots of concentrated viral stocks were stored at −80°C and thawed for use before each experiment.

BT and 3T3 cells cultured in 60 mm plates to 90% confluence were infected with different concentrations of purified recombinant adenovirus at 0.5 ml/60 mm plate for 1 h. Fresh medium was added and incubation continued for 48–72 h before harvesting cells subsequent to incubation in trypsin/EDTA. Efficiency of gene transfer was assessed by staining Adv-βgal-infected cells with isopropylthio-β-D-galactoside to detect β-galactosidase activity [18].

2.3. Growth assay

Adv-CaP- and Adv-βgal-infected NIH-3T3 and BT smooth muscle cells as well as uninfected cells were plated in duplicate at 4×10^4 cells per well in 24-well plates. Cells were fixed with methyl alcohol and stained with Eosin Y and Thiazine dyes using Diff-Quik Staining Set (Baxter). Cell numbers were counted with results derived from the average of three random fields (1 mm^2) per well, with two wells per condition.

2.4. Immunocytochemistry

Cells were plated on cover slips and infected by Adv-CaP or transfected with pAC-CaP using lipofectamine (GIBCO BRL) according to the recommendations of the manufacturer. After 48 h, cells were fixed and immunofluorescence-stained for calponin and F-actin with monoclonal anti-chicken smooth muscle calponin antibody (Sigma), followed by fluorescein-labeled goat anti-mouse IgG (Sigma) and rhodamine-phalloidin. Fluorescence images were acquired using a cooled CCD camera as previously described [19].

Fig. 2. Proliferation of smooth muscle cells and fibroblasts infected by Adv-CaP. Top panel: Growth curves of uninfected BTC (control) or those infected by Adv-CaP or Adv-βgal at 3×10^9 PFU/ml (>95% efficiency). Bottom panel: Growth curves of uninfected NIH-3T3 cells or those infected by Adv-CaP or Adv-βgal at 9×10^{10} PFU/ml (>80% efficiency).

2.5. Purification of smooth muscle calponin

Tracheal smooth muscle calponin was partially purified from fresh bovine trachealis by a modification of the method described for bovine aorta [4]. All steps were identical with the omission of ion exchange and gel filtration chromatography. This resulted in a protein preparation containing 50% calponin as assessed from densitometry of Coomassie Blue-stained gels. Expressed avian calponin was purified from bacterial lysates as described [17].

2.6. [³H]Thymidine incorporation assay

Infected and control cells were plated at 4×10^4 cells per well in 24-well plates. After 48 h, [³H]thymidine was added to the medium followed by a 2-h incubation. Cells were then washed twice with phosphate-buffered saline (PBS; GIBCO BRL), and treated with 5% trichloroacetic acid for 30 min. Cells were harvested by trypsin/EDTA and lysed by adding 0.1 M NaOH for 30 min. [³H]Thymidine incorporation into DNA was measured by liquid scintillation spectroscopy.

2.7. Western blot analysis

Cells were collected in SDS sample buffer and solubilized proteins subjected to electrophoresis in 10% or 3–20% polyacrylamide gels as described previously [14]. Protein concentrations were determined with the BCA assay (Pierce). For detection of calponin, proteins separated by 10% PAGE were transferred to nitrocellulose and blotted with monoclonal anti-human calponin antibody (Sigma) for BT tissues and cells as well as NIH-3T3 fibroblasts, or with monoclonal anti-chicken smooth muscle calponin antibody (Sigma) for detection of avian calponin in Adv-CaP-infected BT and NIH-3T3 cells. For detection of α-actin, myosin heavy chains and caldesmon, proteins separated by 3–20% PAGE were transferred to nitrocellulose and blotted with monoclonal anti-smooth muscle α-actin (Sigma) or polyclonal antibodies raised in this laboratory against bovine tracheal smooth muscle myosin or chicken gizzard caldesmon. Immunoreactive bands were visualized by chemiluminescence (ECL, Amersham) following treatment with secondary antibodies conjugated to horseradish peroxidase. Immunoblots were subjected to scanning laser densitometry for quantification.
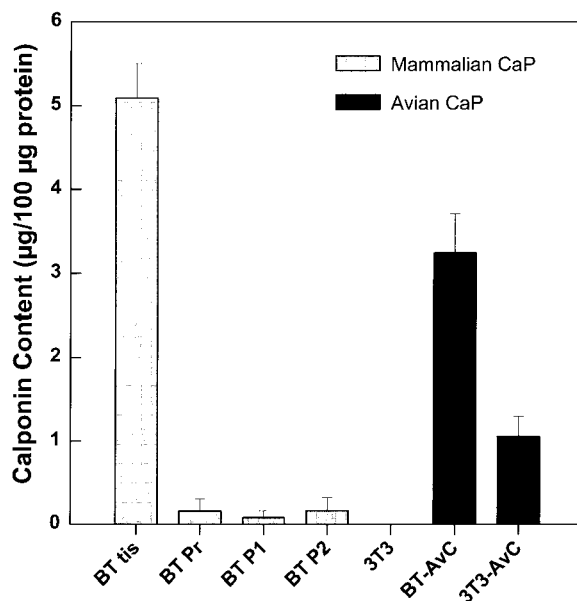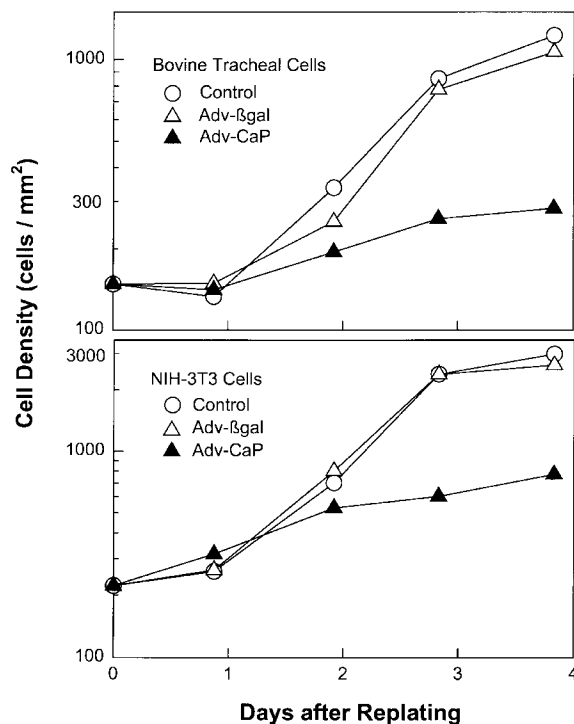

Fig. 1. Calponin expression in smooth muscle cells and fibroblasts. Endogenous CaP content was measured in bovine tracheal tissue homogenates (BT tis), tracheal cells in primary culture (BT Pr), tracheal cells following one or two passages (BT P1, BT P2, respectively) and in cultured NIH-3T3 fibroblasts (3T3). Content of exogenous calponin expressed 72 h after infection with Adv-CaP was measured in pass 3 BT cells (BT-AvC) and NIH-3T3 cells (3T3-AvC). Tracheal cells were infected at 3×10^9 PFU/ml and 3T3 cells at 9×10^{10} PFU/ml. n = 3–6 dishes collected in three separate experiments.
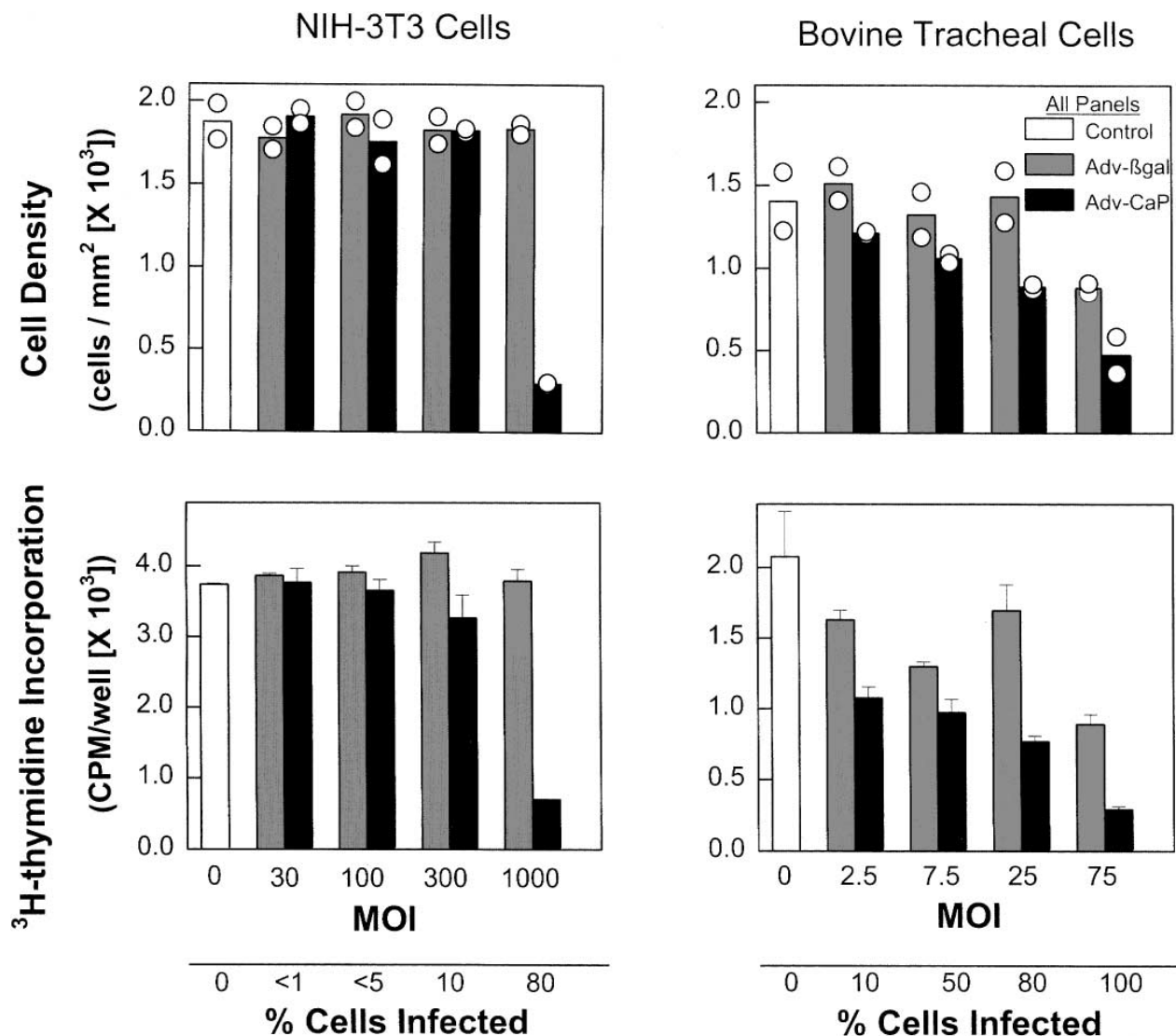
Fig. 3. Concentration dependence of smooth muscle and fibroblast cell growth on Adv-CaP. Cell growth was assessed by measurements of cell density and [³H]thymidine incorporation. Cells were infected by indicated amounts of Adv-CaP or Adv-βgal. After 72 h cells, including uninfected (control) cells, were replated at equivalent number per plate. Cell densities were measured after 4 days. [³H]thymidine incorporation was assessed after 48 h. Data points in top panels represent measurements on duplicate plates; data in lower panels are mean ± SE for three plates.

3. Results

The infective efficiency of recombinant adenovirus was assessed in BT smooth muscle cells and NIH-3T3 fibroblasts following treatment by different multiplicities of infection (MOI) with Adv-βgal. Cells showed maximal staining for β-galactosidase activity at 48–72 h after infection. BT smooth muscle cells were more sensitive to infection than NIH-3T3 fibroblasts, with 50% infected cells occurring at MOIs of 10 and 300 PFU/cell, respectively. High infective efficiency was achieved with both BT smooth muscle cells (75 PFU/cell for 100% efficiency) and NIH-3T3 fibroblasts (1000 PFU/cell for 80–90% efficiency).

Contents of endogenous calponin in bovine tracheal tissue, cultured cells and NIH-3T3 fibroblasts were compared to amounts of avian calponin expressed in smooth muscle cells and fibroblasts infected with Adv-CaP (Fig. 1). Calponin content was assessed by Western blotting with monoclonal antibodies specific for mammalian calponin or avian calponin.

Amounts were calculated according to standard curves obtained with purified bovine tracheal calponin or bacterially expressed chicken gizzard calponin as appropriate. Consistent with results of others [7,8], calponin expression was diminished in cultured smooth muscle cells as compared to tissue (Fig. 1). Cultured BT cells expressed a small fraction (0.026, $n = 9$) the amount of calponin in tissue, while NIH-3T3 cells expressed none. Serum withdrawal from cultured BT cells did not promote re-expression of calponin. Infection of BT cells resulted in increases in calponin to amounts similar to those in tracheal tissues (64%; Fig. 1). Infected NIH-3T3 cells expressed about one-third the amount calponin as BT cells (Fig. 1).

Expression of exogenous calponin had no significant effect on the contractile protein 'phenotype' of infected SMCs as assessed by Western blots for smooth muscle α-actin, myosin heavy chains and caldesmon. Antibody reagents for these proteins were smooth muscle-specific (monoclonal anti-α-actin) or cross-react with both smooth and non-muscle isoforms
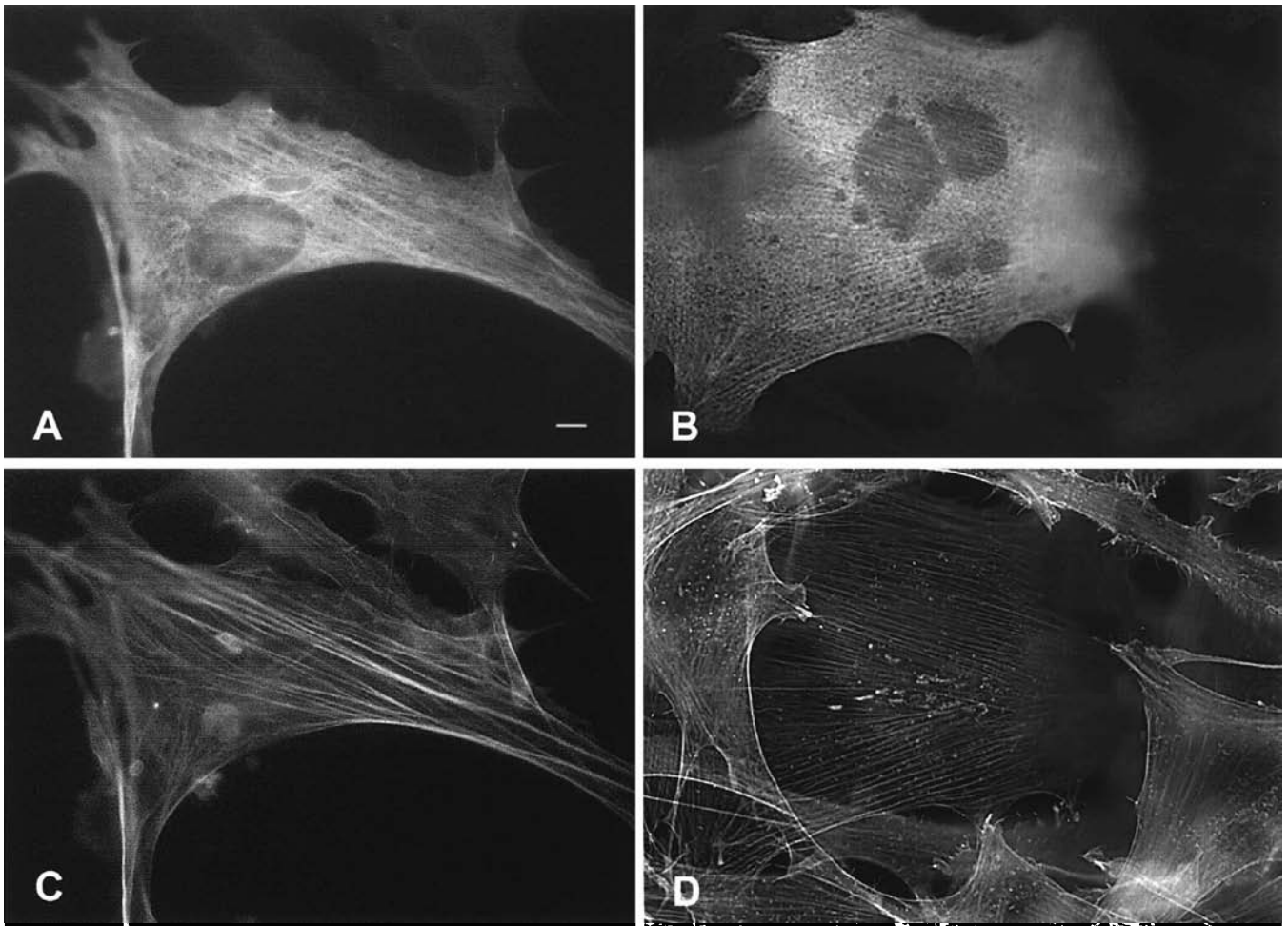
Fig. 4. Localization of CaP expressed in smooth muscle cells and fibroblasts. Image of Adv-CaP-infected BT cell shows distribution of CaP by fluorescein staining anti-CaP (A) and F-actin by rhodamine-phalloidin staining (C). Image of NIH-3T3 cell transfected with pAC$_{sk.2}$CMV5-CaP shows distribution of CaP by fluorescein staining anti-CaP (B) and F-actin by rhodamine-phalloidin staining (D). Bar for all panels = 20 μm.

(polyclonal anti-myosin heavy chain and caldesmon). The low molecular weight isoform of caldesmon was the only form detected in either control or infected BT cells (pass 2). The ratios of protein contents in calponin-expressing SMCs with respect to control cells were 1.07 ± 0.06 for α-actin, 0.93 ± 0.04 for myosin heavy chains and 0.97 ± 0.14 for caldesmon ($n = 4$).

To evaluate cytoskeletal actions of calponin, we compared the proliferation of cultured smooth muscle cells and NIH-3T3 fibroblasts infected by Adv-CaP to Adv-βgal-infected and uninfected cells. Cells were infected, then collected after 48-72 h and replated at equal density in 24-well plates. Cell density was assessed daily following fixation and staining over 4 days. The rate of increase in cell density for both smooth muscle cells and NIH-3T3 cells was significantly diminished by Adv-CaP compared to control or Adv-βgal conditions (Fig. 2). Doubling times ($0.693/k$) were calculated from exponential growth rate constants (k, day^{-1}) obtained in three separate experiments. Doubling times were similar for both BT cells and 3T3 cells with respective control values of 0.97 ± 0.14 and 0.93 ± 0.14 days. These values increased to 3.2 ± 1.3 and 2.5 ± 0.6 days, respectively, for Adv-CaP infected cells.

Inhibition of cell growth by Adv-CaP was confirmed by [3][H]thymidine incorporation which mirrored cell density in response to viral infection in a concentration-dependent man-

ner (Fig. 3). Specific inhibition of cell growth was observed for NIH-3T3 cells at 80% infection, where Adv-CaP reduced cell number as well as DNA synthesis to one-fifth control values, while Adv-βgal had no effect. At similar infection efficiency BT cells also showed specific depression of cell growth by Adv-CaP, which reduced cell number and DNA synthesis to 40–50% that observed with Adv-βgal. With the greatest MOI of Adv-βgal, non-specific depression of BT cell growth was observed.

Immunocytochemical staining of BT and 3T3 cells infected with Adv-CaP showed calponin localized primarily on fibers, although some diffuse cytoplasmic staining was observed (Fig. 4). Overlaid images of calponin staining and phalloidin binding showed that calponin was localized on actin-containing stress fibers in these cells. In general Adv-CaP-infected cells appeared enlarged compared to control cells.

4. Discussion

Smooth muscle cells in culture express minute amounts of calponin compared to those in tissues [7,8]. In the present study we made use of this smooth muscle calponin 'knockout' model to investigate the effects of re-expression of SMC calponin on cell proliferation. In both cultured bovine tracheal

smooth muscle cells and NIH-3T3 fibroblasts, which contain no endogenous SMC calponin, cell proliferation and DNA synthesis were inhibited by calponin. The cell cycle is coupled to the cytoskeleton at the G1/S transition by minimally understood anchorage and shape-dependent signaling pathways [20,21]. In addition, progression through mitosis is dependent upon the activities of actomyosin in cell rounding, cytokinesis and post-mitotic cell spreading [22]. The present results suggest that the actin-binding protein SMC calponin may act to retard signaling that normally leads to cell division.

Efficient transfer of DNA into smooth muscle and fibroblast cells was accomplished by use of replication-defective adenovirus recombined with cDNA expression cassettes encoding calponin or β-galactosidase. Recombinant adenovirus has been previously shown to yield high-efficiency expression of β-galactosidase in both cultured vascular and tracheal myocytes [17,23,24], far exceeding efficiencies achieved with conventional methods of transfection [16]. The observed difference in sensitivity to infection between BT and 3T3 cells may arise from differences in density of viral receptors required for attachment or in density of αv integrins required for internalization [23].

SMC calponin in smooth muscle tissues is enriched in the cytoskeletal (myosin-deficient) domain that contains non-muscle β-actin [10,11]. In addition, calponin has also been localized to dense bodies and dense plaques in these cells [10]. Our results demonstrate localization of expressed calponin on actin-containing stress fibers in both BT and 3T3 cells. The effects of calponin likely arise from its association with actin; calponin expression had no detectable effect on amounts or isoforms of contractile proteins studied in these cells. Calponin brought about inhibition of proliferation similarly in both BT smooth muscle cells and NIH-3T3 fibroblasts. In each case cell density and DNA synthesis were proportionally diminished, indicating a generalized slowing of the cell cycle. These results suggest that calponin may inhibit the normal signaling between cell attachment, shape and spreading and transition to S-phase. While the cytoskeleton is an integral component of this signaling pathway, details remain to be elucidated [20,21]. The high content of SMC calponin in quiescent cells in tissues, its intracellular localization and the fact that SMC calponin is rapidly down-regulated in proliferating smooth muscle cells in vitro and in vivo circumstantially support the hypothesis that calponin suppresses proliferation in vivo [25].

Calponin may also depress proliferation rates by inhibiting actomyosin-dependent processes such as cytokinesis or respreading. Consistent with its inhibitory effect on actomyosin MgATPase, SMC calponin decreases the rate of actin filament translocation over immobilized myosin in in vitro motility assays, and reduces shortening velocity or force when introduced into permeabilized smooth muscle fibers or cells [3,5]. A less emphasized, but potentially important function of SMC calponin may relate to its stabilizing effect on thin filaments [6] that might result in resistance to cytoskeletal rearrangements stimulated by exposure to mitogens.

The adenovirus gene delivery system results in efficient transfer of contractile proteins to cultured smooth muscle that facilitates studies on populations of cells. We have shown that SMC calponin inhibits cell proliferation in both smooth and non-muscle cells, an effect that may result from inhibition of signaling, actomyosin activity, thin filament turnover or an interplay of these.

References

[1] Miano, J.M. and Olson, E.N. (1996) J. Biol. Chem. 271, 7095–7103.

[2] Samaha, F.F., Ip, H.S., Morrisey, E.E., Seltzer, J., Tang, Z., Solway, J. and Parmacek, M.S. (1996) J. Biol. Chem. 271, 395–403.

[3] Winder, S.J. and Walsh, M.P. (1993) Cell. Signal. 5, 677–686.

[4] Takahashi, K., Hiwada, K. and Kokubu, T. (1988) Hypertension 11, 620–626.

[5] Gimona, M. and Small, J.V. in: Bárány, M. (Ed.), Biochemistry of Smooth Muscle Contraction, Academic Press, San Diego, CA, 1996, pp. 91–103.

[6] Kake, T., Kimura, S., Takahashi, K. and Maruyama, K. (1995) Biochem. J. 312, 587–592.

[7] Gimona, M., Herzog, M., Vanderkerckhove, J. and Small, J.V. (1990) FEBS Lett. 274, 159–162.

[8] Birukov, K.G., Stepanova, O.V., Nanaev, A.K. and Shirinsky, V.P. (1991) Cell Tiss. Res. 266, 579–584.

[9] Walsh, M.P., Carmichael, J.D. and Kargacin, G.J. (1993) Am. J. Physiol. 265, C1371–C1378.

[10] North, A.J., Gimona, M., Cross, R.A. and Small, J.V. (1994) J. Cell Sci. 107, 437–444.

[11] Mabuchi, K., Li, Y., Tao, T. and Wang, C.L.A. (1996) J. Muscle Res. Cell Motil. 17, 243–260.

[12] Strasser, P., Gimona, M., Moessler, H., Herzog, M. and Small, J.V. (1993) FEBS Lett. 330, 13–18.

[13] Applegate, D., Feng, W., Green, R.S. and Taubman, M.B. (1994) J. Biol. Chem. 269, 10683–10690.

[14] Taylor, D.A. and Stull, J.T. (1988) J. Biol. Chem. 263, 14456–14462.

[15] Graham, F.L. and Prevec, L. (1991) Methods Mol. Biol. 7, 128.

[16] Becker, T.C., Noel, R.J., Coats, W.S., Gomez-Foix, A.M., Alam, T., Gerard, R.D. and Newgard, C.B. (1994) Methods Cell Biol. 43, 161–189.

[17] Tang, D.-C., Kang, H.-M., Jin, J.-P., Fraser, E.D. and Walsh, M.P. (1996) J. Biol. Chem. 271, 8605–8611.

[18] Sanes, J.R., Rubenstein, J.L.R. and Nicolas, J.-F. (1986) EMBO J. 5, 3133–3142.

[19] Luby-Phelps, K., Hori, M., Phelps, J.M. and Won, D. (1995) J. Biol. Chem. 270, 21532–21538.

[20] Folkman, J. and Moscona, A. (1978) Nature 273, 345–349.

[21] Koyama, H., Raines, E.W., Bornfeldt, K.E., Roberts, J.M. and Ross, R. (1996) Cell 87, 1069–1078.

[22] Mitchison, T.J. and Cramer, L.P. (1996) Cell 84, 371–379.

[23] Guzman, R.J., Lemarchand, P., Crystal, R.G., Epstein, S.E. and Finkel, T. (1993) Circulation 88, 2838–2848.

[24] Clesham, G.J., Browne, H., Efstathiou, S. and Weissberg, P.L. (1996) Circ. Res. 79, 1188–1195.

[25] Takahashi, K. in: Kohama, K. and Saida, K. (Eds.), Smooth Muscle Contraction, Karger, Basel, 1995, pp. 145–155.