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A novel phosphoglucomutase-related protein is concentrated in adherens junctions of muscle and nonmuscle cells

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SUMMARY

Using five monoclonal antibodies raised against a human uterine smooth muscle extract, we have identified a novel antigen which runs as a closely spaced doublet in SDS-gels. The proteins (60/63 kDa) co-purify, are present in a 1:1 ratio as judged by Coomassie Blue staining, and are immunologically closely related, if not identical. No N-terminal sequence could be obtained from a mixture of the 60/63 kDa proteins, but the sequence of four polypeptides liberated by V8 protease or cyanogen bromide cleavage showed that the proteins are closely related to the glycolytic enzyme phosphoglucomutase type 1. Affinity-purified polyclonal antibodies and three different monoclonal antibodies to the 60/63 kDa proteins cross-reacted with rabbit skeletal muscle phosphoglucomutase type 1, whilst two additional monoclonal antibodies were specific for the 60/63 kDa proteins. Peptide maps of the 60/63 kDa proteins and phosphoglucomutase 1 are markedly different, and the purified proteins have no detectable phosphoglucomutase activity.

Staining of cultured smooth muscle cells and fibroblasts with antibodies to 60/63 kDa proteins showed that the antigen is concentrated in focal contacts at the ends of actin bundles and is also associated with actin filaments. About 60% of the cellular 60/63 kDa proteins were found in the detergent-insoluble fraction, suggesting a physical association with the cytoskeleton. The highest levels of protein immunoreactivity were found in muscles. The antigen is concentrated in muscle adherens junctions, including smooth muscle dense plaques, cardiomyocyte intercalated disks, and striated muscle myotendinous junctions. Among epithelial cells, the 63 kDa isoform of the protein was found only in cultured keratinocytes where immunofluorescent staining was localized in cell-to-cell adherens junctions. Expression of the 60/63 kDa proteins in vascular smooth muscle cells is developmentally regulated and correlates with the differentiated contractile phenotype of these cells.

Key words: 60/63 kDa protein, phosphoglucomutase, adherens junction, smooth muscle

INTRODUCTION

Adhesion of cells to the extracellular matrix and to other cells is widely believed to play a major role in the regulation of cellular morphogenesis, growth and differentiation. Both cell-cell and cell-matrix adhesions involve specialized regions of the cell surface associated with underlying cytoskeletal elements. The most prominent class of such cellular junctions, characterized by their specific association with actin microfilaments, are the adherens junctions (AJs) (Geiger et al., 1987). AJs are found in a wide variety of cell types and are highly dynamic structures undergoing extensive reorganization during cellular morphogenesis, cell division, differentiation and oncogenic transformation. The organization and dynamics of AJs can be modulated by hormones, cytokines, inflammation mediators, and drugs affecting signal transduction and energy metabolism (for review, see Burridge et al., 1988; Geiger and Ginsberg, 1991).

Much interest has focused recently on the molecular architecture and function of the cytoplasmic domain of AJs. Studies on the mechanism of microfilament-plasma membrane linkage have identified a group of proteins specifically associated with the cytoplasmic face of AJs (for review, see Burridge et al., 1988; Geiger and Ginsberg, 1991; Luna and Hitt, 1992; Tsukita et al., 1992). Some of the cytoskeletal proteins of AJs are able to interact in vitro with adhesion receptors (talin/β_1 integrin, α-actinin/β_1 integrin, α-actinin/VCAM, cadherins/α,β-catenins), with actin filaments (α-actinin, tensin, talin, fimbrin,

radixin), and with each other (talin/vinculin, vinculin/vinculin, vinculin/paxillin, vinculin/tensin, vinculin/α-actinin, α-actinin/zyxin, 220 kDa protein/spectrin); it has been postulated that these interactions may be involved in the linkage of the actin cytoskeleton to the plasma membrane (for review, see Burridge et al., 1988; Geiger and Ginsberg, 1991; Luna and Hitt, 1992, Tsukita et al., 1992). Several proteins known to participate in signal transduction such as tyrosine kinases, protein kinase C and the calcium-activated protease calpain II, are also found to co-localize in AJs (for review, see Burridge et al., 1988; Geiger and Ginsberg, 1991; Luna and Hitt, 1992). These findings suggest that the cytoplasmic domains of AJs are highly complex structures containing a variety of structural and regulatory components. However, their role in AJ formation and regulation remains poorly understood and further dissection of the molecular composition of the cytoplasmic domains of these structures is required.

Major progress in understanding the molecular structure of the AJs has come from studies on the smooth muscle cytoskeleton. In smooth muscle cells (SMCs), the actin cytoskeleton is associated with highly developed specialized AJs termed 'dense plaques' (Small and Sobieszek, 1980). The morphological prominence of dense plaques and the abundance of their protein constituents in SMCs greatly facilitated both the biochemical characterization and the ultrastructural localization of a number of AJ proteins. In the present study, we have identified a novel AJ antigen in human uterine smooth muscle that migrates as a closely spaced doublet (60/63 kDa) in SDS-gels, and is structurally and immunologically related to the glycolytic enzyme phosphoglucomutase.

MATERIALS AND METHODS

Purification of the 60/63 kDa proteins from human uterine smooth muscle

A modification of the published procedures for vinculin and metavinculin purification was used (Feramisco and Burridge, 1980; Siliciano and Craig, 1987; Belkin et al., 1988). Human uterine SM tissue (400 g) was homogenized (3×15 seconds) in a Waring Blender at top speed in 2.5 l of deionized H_2O containing 0.5 mM PMSF. The homogenate was centrifuged (10 minutes at 16,000 ***g*** in a JA-10 rotor, Beckman Instruments, Inc, Berkeley, CA) and the pellet was extracted by re-homogenization in H_2O/0.5 mM PMSF. For extraction of SM cytoskeletal proteins, the pellet was resuspended in 2.5 l of buffer containing 20 mM, Tris (pH 9.0), 1 mM EGTA and 0.5 mM PMSF, and incubated with stirring for 60 min at 37°C. The extract was clarified by centrifugation and its pH was adjusted to 7.2 with 0.5 M acetic acid. The extract was then made 10 mM with respect to $MgCl_2$ and subjected to centrifugation to remove F-actin. The supernatant obtained at this step was further fractionated by ammonium sulfate precipitation; 12.2 g $(NH_4)_2SO_4$ was added per 100 ml of the supernatant and the precipitate discarded; 9.6 g $(NH_4)_2SO_4$ per 100 ml was added to the supernatant, the precipitate was collected by centrifugation, and dissolved in, and dialyzed against Buffer A (5 mM Tris-HCl, pH 7.8, 20 mM NaCl, 0.1 mM EDTA, 0.5 mM DTT). The sample was applied to a 2.6 cm × 22 cm Q-Sepharose column (Pharmacia Fine Chemicals, Uppsala, Sweden) equilibrated with Buffer A. The column was washed with Buffer A and bound proteins were eluted using a linear gradient of NaCl (20 mM to 350 mM) in Buffer A. Fractions containing the 60/63 kDa proteins were pooled, dialyzed against Buffer A and applied to a 0.9 cm × 10 cm hydroxylapatite column (Bio-Rad Laboratories, Cambridge, MA). Bound proteins were eluted with a linear potassium phosphate gradient (10 mM to 200 mM) in Buffer A. Fractions containing the purified 60/63 kDa protein doublet were pooled, concentrated, and stored in aliquots at –70°C.

Production of antibodies to the 60/63 kDa proteins

Rabbits were immunized with 1 mg of native or SDS-PAGE-purified protein in complete Freund's adjuvant at multiple intradermal sites. Four weeks later, the rabbits received booster injections of 0.5 mg antigen in the incomplete Freund's adjuvant. Polyclonal antibodies were affinity-purified from the antisera using the 60/63 kDa proteins immobilized on nitrocellulose as described by Talian et al. (1983). Monoclonal antibodies (mAbs) to the 60/63 kDa proteins were produced using a crude fraction of human SM cytoskeletal proteins as antigen. Primary screening of hybridoma supernatants was carried out using an ELISA assay. Positive clones were further tested by immunofluorescent staining of cryostat tissue sections. Five independent clones (VID4, XIVF8, XIVD11, XVB3 and XVE2) were selected for further study. The specificity of the monoclonal antibodies was analyzed by immunoblotting. All five monoclonal antibodies recognized two polypeptides with molecular masses of 60 kDa and 63 kDa in extracts of human uterus.

Human tissue samples

Human fetal tissue was obtained from fetuses aborted either spontaneously or on medical grounds. Child and adult tissues obtained at autopsy were taken within 2-4 hours of death. At least five independent samples were analyzed for each tissue. For immunoblotting experiments, the frozen samples were crushed, dispersed in sample buffer (Laemmli, 1970) and boiled for 5 minutes. Each electrophoretic sample contained 0.15 mg of total protein. For immunofluorescent staining, freshly isolated tissue segments were frozen by immersion into freezing isopentane and used to prepare 5 μm cryostat sections.

Gel electrophoresis and western blot analysis

SDS-PAGE (7.5%, 10% or 5% to 15% gradient polyacrylamide gels) was performed according to the method described by Laemmli (1970), and western blot analysis as described by Towbin et al. (1979). Monoclonal (2-10 μg/ml) or affinity-purified polyclonal (1-2 μg/ml) antibodies against the 60/63 kDa proteins were used as primary antibodies. ^{125}I-labeled goat anti-rabbit IgG (1 μg/ml; 10^7 cpm/μg) was used as a secondary antibody. Immunoblots were extensively washed, dried and exposed to X-ray film for 48-96 hours at –70°C.

Peptide mapping and protein sequencing

The purified 60/63 kDa proteins and rabbit skeletal muscle phosphoglucomutase (Boehringer) were digested using V8 protease as described by Cleveland et al. (1977). The liberated polypeptides were resolved in 15% polyacrylamide SDS-gels and electroblotted to polyvinylidene difluoride (PVDF) membranes for N-terminal sequencing using an Applied Biosystems 470A gas phase sequencer. Cyanogen bromide cleavage of the proteins was performed as described by Gross and Witkop (1961). Liberated peptides were separated on 16.6% polyacrylamide SDS-gels using a tricine buffer (Schagger and von Jagow, 1987), and sequences obtained were compared with those in the Daresbury Segnet Laboratory OWL Composite Protein Sequence Database (version 18.1).

Phosphoglucomutase assay

Phosphoglucomutase activity was measured in a coupled enzymatic reaction assay based on a reduction of NADP to NADPH. The assay buffer contained 40 mM Tris-HCl, pH 7.5, 1 mM glucose 1,6-bisphosphate, 0.2 mM NADP, 10 mM $MgCl_2$, 0.5 unit/ml glucose-6-phosphate dehydrogenase, and 2 mM glucose 1-phosphate. Reactions were started by mixing the buffer with the column fractions or with rabbit skeletal muscle phosphoglucomutase (Boehringer Mannheim, Germany) activated by pre-incubation in 10 mM imidazole buffer, pH

7.5, containing 1 mM Mg^{2+}. The extinction at 340 nm was measured in the samples after a 5 minute incubation at room temperature. Controls in which glucose 1-phosphate was excluded from the assay buffer were included to account for the endogeneous dehydrogenase activity in tissue extracts.

Immunofluorescence

Human or rabbit aortic SMCs, keratinocytes, MDBK cells, human endothelial cells or rat embryo fibroblasts cultivated on glass coverslips were washed 3 times with PBS (phosphate-buffered saline) and fixed in methanol for 5 minutes at –20°C. Tissue sections were fixed with acetone for 15 minutes at room temperature, air-dried and additionally fixed with ice-cold methanol as stated above. Incubation of the specimens in 2% bovine serum albumin in PBS was used to prevent nonspecific antibody binding. For double immunofluorescent staining, rabbit affinity-purified polyclonal antibodies or mAb XIVF8 against the 60/63 kDa proteins were used in combination with the anti-vinculin mouse mAb VIIF9 or affinity-purified anti-vinculin polyclonal antibodies (Glukhova et al., 1990) and polyclonal antibodies to cytoplasmic domain of β_1 integrin. Monoclonal antibodies XIVF8 against the 60/63 kDa proteins were also used in combination with rabbit polyclonal anti-talin, anti-actin and α-actinin antibodies or with rabbit polyclonal anti-E-cadherin antibodies. Rabbit polyclonal anti-60/63 kDa proteins antibodies were used with monoclonal anti-PECAM antibody (Ab 4G6). Goat anti-mouse IgG conjugated with rhodamine, and fluorescein-conjugated goat anti-rabbit IgG (Miles Scientific Division, Naperville, IL) were used as secondary antibodies. In some experiments biotinylated goat anti-mouse or biotinylated goat anti-rabbit IgGs and streptavidin-Texas Red (Miles Scientific Division, Naperville, IL) were used to enhance the staining intensity. The stained specimens were mounted in PBS/glycerol (1:1, v/v) and examined in the Zeiss Photomicroscope III. Photographs were taken on the Kodak Tri-X film.

Cell culture

SMCs from human aortic media were isolated by collagenase-elastase digestion and maintained in DME (Flow Laboratories Inc., McLean, VA) supplemented with 10 mM HEPES, 100 μg/ml sodium pyruvate, 50 μg/ml ascorbic acid, 0.6 μg/ml L-glutamine, 50 μg/ml gentamycine sulfate, and 10% heat-inactivated human serum (Chamley-Campbell et al., 1979). Rat embryo fibroblasts (REF52) were grown in DMEM supplemented with 10% FBS. The human Asch-7 astrocytoma cell line and human neurons were a generous gift from Dr Y. Balabanov (Institute of Experimental Medicine, St Petersburg). Umbilical vein endothelial cells were a gift from Dr L. Romer (UNC, Chapel Hill) and the hepatoma Hep G-2 cell line from Dr V. Kosykh (Cardiology Research Center, Moscow). Human primary keratinocytes were kindly donated by Dr V. Belkin (Institute of Biological and Medical Chemistry, Moscow) and by Dr E. O'Keefe (UNC, Chapel Hill). Madin-Darby bovine kidney cells (MDBKs) were from the American Tissue Culture Collection (ATCC). For immunoblotting or immunofluorescent staining, freshly isolated rabbit and human aortic SMCs were seeded on plastic dishes or glass coverslips at high (15×10^4 cells/cm^2) and low (0.2×10^4 cells/cm^2) density as described by Shirinsky et al. (1991). The cells attached and spread within two days after seeding. The dense and sparse primary cultures of rabbit SMCs were used for immunofluorescence and immunoblotting experiments after 2 or 10 days of culture. In some experiments, human aortic SMCs (7- to 10-day-old primary cultures) were extracted with a buffer containing 50 mM PIPES, pH 6.9, 50 mM KCl, 1 mM $MgCl_2$, 2 mM EGTA, 0.1 mM PMSF and 0.2% Triton X-100. The Triton-extracted proteins were precipitated with 9 vol. of ice-cold acetone and redissolved in the SDS-gel sample buffer prior to SDS-PAGE. The Triton-insoluble material enriched in the cytoskeleton-associated proteins was solubilized in the same buffer.

RESULTS

Purification of the 60/63 kDa proteins from human uterine smooth muscle

A number of membrane and cytoskeletal proteins associated with AJs have been previously purified from smooth muscle tissues (Feramisco and Burridge, 1980; Siliciano and Craig, 1987; Belkin et al., 1988). Using a modification of the published procedures, we extracted human uterine tissue with a low salt buffer, pH 9.0, and after precipitation of the bulk of the actin, subjected the extracts to ammonium sulfate fractionation to obtain a fraction enriched in dense plaque proteins. Using this material for immunization, we have described a mAb XVE2 that reacts in immunoblots with 60 kDa and 63 kDa proteins, and specifically stains intercalated disks in cardiomyocytes (Koteliansky et al., 1989). Chromatographic fractionation of the uterus extracts on Q-Sepharose (Fig. 1A) yielded a series of fractions considerably enriched in proteins migrating on SDS-gels as a doublet with apparent molecular masses of 60 kDa and 63 kDa (Fig. 1B). The peak of these proteins eluted from the column in 120 mM NaCl, slightly behind the vinculin peak. The 60 kDa and 63 kDa proteins demonstrated identical chromatographic behavior, and both reacted with XVE2 antibody in western blots (Fig. 1C). The 60/63 kDa proteins were separated from major contaminants by a re-chromatography on a smaller Q-Sepharose column, and finally purified on a hydroxylapatite column (Fig. 1D). Additional chromatography of the purified material on FPLC Mono Q ion exchange column was performed in some experiments. Using this procedure, we obtained 3-5 mg of 60/63 kDa proteins (90-95% purity) from 400 g of the wet tissue.

Purified 60/63 kDa proteins appeared on 5% to 15% gradient gels as a doublet of closely apposed bands present in a ratio of approximately 1:1, as judged by Coomassie Blue staining (Fig. 1E, lane a). Both bands were recognized by an affinity-purified polyclonal antibody to the 60/63 kDa proteins (Fig. 1E, lane b) as well as by five diferent monoclonal antibodies (Fig. 1E, lanes c-g). The ratio of the two bands remained unaltered through the course of purification of the 60/63 kDa proteins, and upon storage of the purified proteins.

The 60/63 kDa proteins are structurally and immunologically related to phosphoglucomutase type 1

To establish the identity of the purified proteins, we attempted to determine N-terminal amino acid sequence. No sequence was obtained from the 60/63 kDa protein doublet, presumably because both proteins have a blocked N terminus. Limited digestion of the undenatured 60/63 kDa proteins with *Staphylococcus aureus* V8 protease produced two major fragments of approximately 25 and 36 kDa (Fig. 2C). Attempts to sequence the 25 kDa fragment failed, suggesting that this peptide also contained a blocked N terminus. However, a sequence of 17 amino acids was determined on the 36 kDa fragment (Fig. 2D, fragment A). A search of the databases showed that this sequence was very similar to a fragment of the glycolytic enzyme phosphoglucomutase 1 (PGM1) (Ray et al., 1983; Whitehouse et al., 1992), with the exception of one conservative substitution and one unidentified residue. The sequence of three additional polypeptides generated from the 60/63 kDa proteins

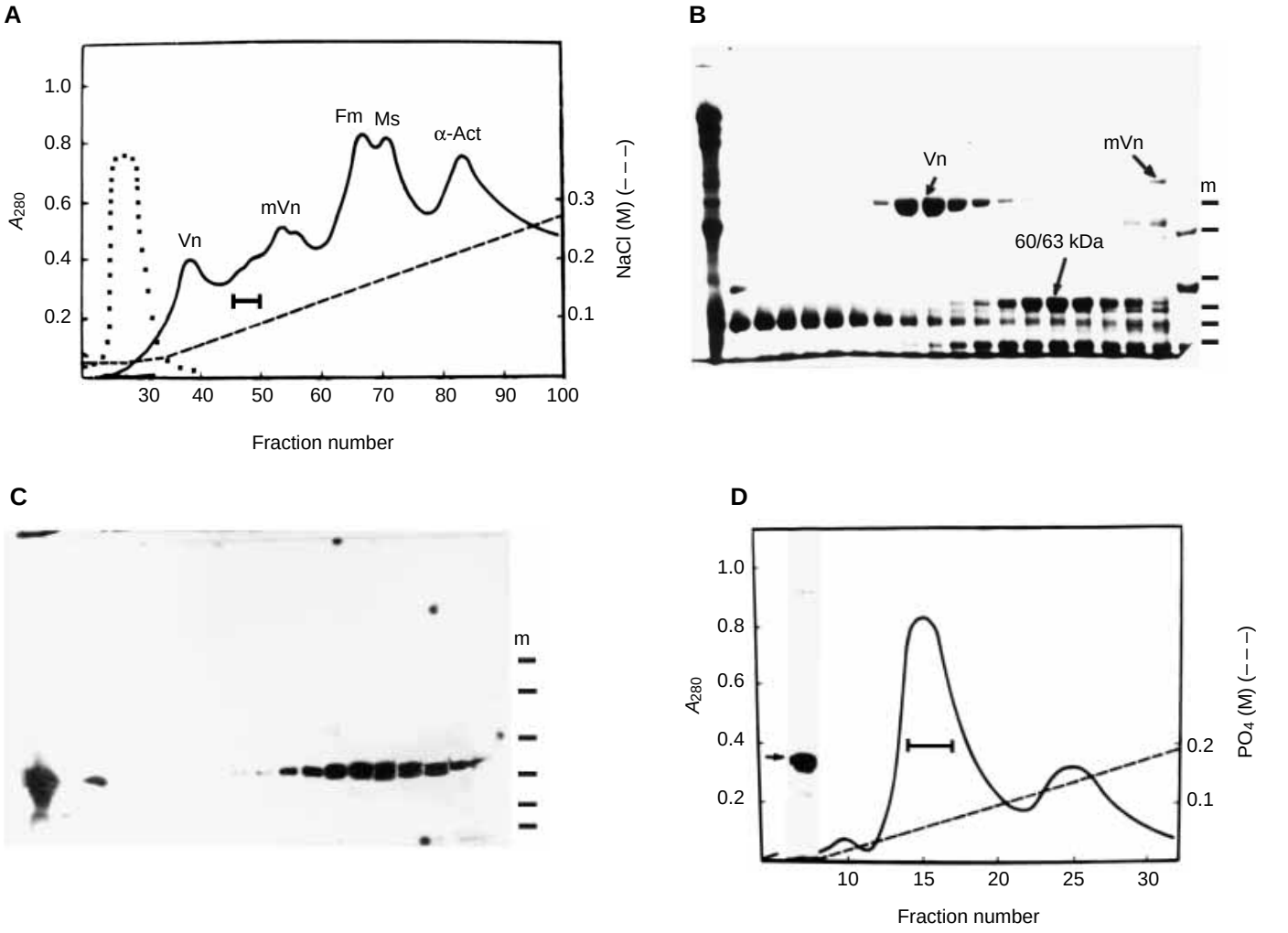

Fig. 1. Purification and initial characterization of the 60/63 kDa proteins. (A) Chromatography of the low-salt SM extract on Q-Sepharose. The peak fractions containing vinculin (Vn), meta-vinculin (mVn), filamin (Fm), myosin heavy chains (Ms), and α-actinin (α-Act) are labeled. The bar shows fractions collected for further purification of the 60/63 kDa proteins. Note that the material containing PGM activity (···) eluted from the column much earlier than the 60/63 kDa proteins. (B,C). Coomassie Blue staining (B) and immunoblotting (C) with the XVE2 mAb of the fractions adjacent to and including the ones containing the 60/63 kDa proteins from chromatography of the low-salt extract (left lane) on Q-Sepharose. m, molecular markers. From top to bottom: vinculin (116 kDa), α-actinin (102 kDa), transferrin (76 kDa), pyruvate kinase (57 kDa), actin (42 kDa), carbonic anhydrase (29 kDa). (D) Purification of the 60/63 kDa proteins on hydroxylapatite. Inset: SDS-PAGE and Coomassie Blue staining of the pooled fractions (indicated by the the bar) containing purified the 60/63 kDa proteins. (E) The purified 60/63 kDa proteins resolved by SDS-PAGE in a 5% to 15% gradient gel: lanes a, stained with Coomassie Blue; b-g transferred to nitrocellulose and probed with polyclonal antibody to the 60/63 kDa proteins (b) and with five different monoclonal antibodies produced against a crude SMC cytoskeletal fraction (c-g).

by cleavage with cyanogen bromide also revealed close similarities to the sequence of PGM1 (Fig. 2D, fragments B, C and D). However, the sequences of the proteins are clearly distinct from each other, suggesting that the 60/63 kDa proteins are not encoded by the PGM1 gene. No PGM activity was detected in the purified 60/63 kDa protein preparations. Analysis of the fractions obtained at different stages of the protein purification showed that most of the PGM activity was solubilized and thus separated from the 60/63 kDa proteins upon extraction of the smooth muscle tissue with water (data not shown). Residual PGM activity was eluted ahead of the 60/63 kDa proteins during chromatography on Q-Sepharose (Fig. 1A).

To characterize the structural relationship between the 60/63 kDa proteins and PGM1 in more detail, we compared their antigenic properties and peptide maps. Three of the five monoclonal antibodies which recognize the 60/63 kDa proteins (Fig. 2A, lanes 1-3), as well as an affinity-purified polyclonal antibody (Fig. 2A, lane 6), cross-reacted with a commercial preparation of rabbit skeletal muscle PGM1 in western blots. However, the mAbs XIVF8 and XIVD11 did not react with

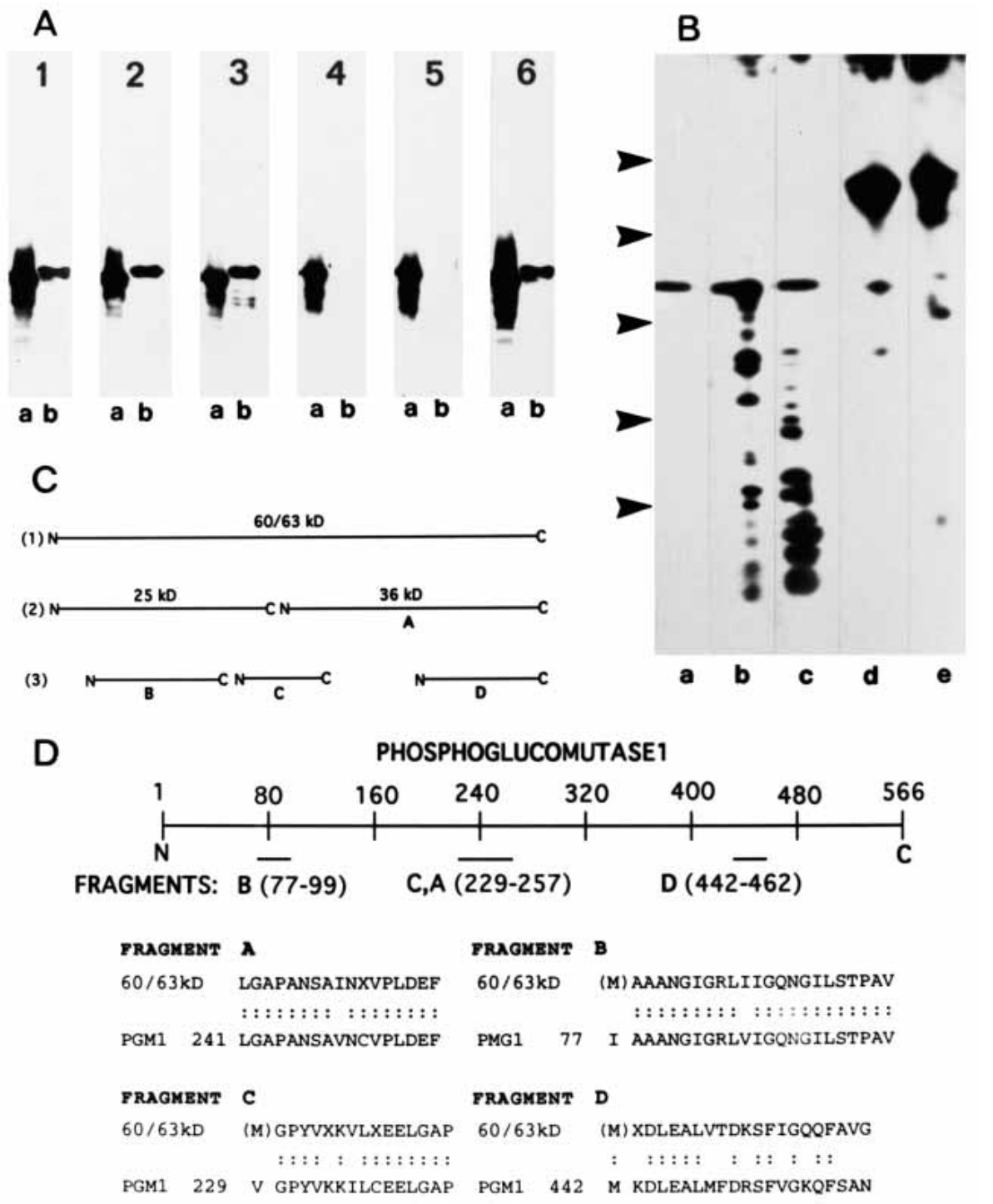

Fig. 2. Comparative immunoblotting (A), peptide mapping (B) and amino acid sequence analysis (C,D) of the 60/63 kDa proteins and rabbit skeletal muscle PGM1. (A) The 60/63 kDa proteins (lanes a) and rabbit skeletal muscle PGM1 (lanes b) were run in parallel lanes and immunoblotted with mAbs VID4 (lanes 1, a and b), XVB3 (lanes 2, a and b), XVE2 (lanes 3, a and b), XIVD11 (lanes 4, a and b), XIVF8 (lanes 5, a and b) or polyclonal antibodies (lanes 6, a and b). mAbs XIVD11 and XIVF8 recognized 60/63 kDa protein (lane 4a and lane 5a) but did not react with the PGM1 band (lane 4b and lane 5b). (B) Equal amounts of the 60/63 kDa proteins and rabbit skeletal muscle PGM1 were subjected to in-gel digestion by the *S. aureus* V8 protease. The gel was stained with silver nitrate. (a) *S. aureus* V8 protease; (b,c) polypeptides liberated from the (b) the 60/63 kDa proteins or (c) PGM1 by V8 protease; (d,e) the intact 60/63 kDa proteins and PGM1. Arrowheads indicate the positions of molecular mass standards (from top to bottom): bovine serum albumin, 67 kDa; ovalbumin, 43 kDa; carbonic anhydrase, 29 kDa; soybean trypsin inhibitor, 21.5 kDa; lysozyme, 14.4 kDa. (C,D) Amino acid sequence analysis on fragments of the 60/63 kDa proteins. The partial amino acid sequence of a 36 kDa polypeptide (fragment A, line 2) liberated by V8 protease and three cyanogen bromide fragments (B-D, line 3) is shown aligned with the sequence of human PGM1 (D, upper panel) (Whitehouse et al., 1992). The N terminus of all three cyanogen bromide polypeptides is assumed to be a methionine residue (M). Unassigned residues (X) are inferred to be cysteine residues.

PGM1 (Fig. 2A, lanes 4b and 5b). Digestion of the purified 60/63 kDa protein doublet and PGM1 with *S. aureus* V8 protease under denaturing conditions demonstrated that the proteins have markedly different peptide maps (Fig. 2B). We have found several clusters of peptides difference between 37 kDa and 32 kDa, between 28 kDa and 25 kDa, between 21 kDa and 14 kDa, and between 12 kDa and 7 kDa. The first two peptide clusters, are more characteristic for 60/63 kDa proteins; another two clusters for PGM1. In summary, the amino acid sequence, immunological properties and peptide maps of the 60/63 kDa proteins we have purified from human uterine smooth muscle are similar to, but quite distinct from, those of PGM1.

Analysis of the cell type and tissue distribution of the 60/63 kDa proteins

Using western blotting and the mAb XIVF8, which is specific for the 60/63 kDa proteins, we have detected immunoreactive proteins of identical size in several human tissues and cell types (Fig. 3). The 60/63 kDa proteins were most abundant in muscle tissues. Visceral and vascular SM (uterus and aorta) contained the highest level of the proteins, while striated muscles contained intermediate amounts of the antigen. Other tissues, including liver, kidney, skin and brain contained a low level of the proteins (Fig. 3A). Of a number of cell types examined, only SMCs in primary (3-5 days old) culture displayed a high level of 60/63 kDa protein immunoreactivity (Fig. 3B, lane c); nonmuscle cultured cells, including fibroblasts, keratinocytes and astrocytoma cells contained low amount of 60/63 kDa proteins (Fig. 3B, lanes a, b and d); the 60/63 kDa proteins were barely detectable, if present, in neurons, endothelial cells, Hep G-2 hepatoma cells,platelets (Fig. 3B, lanes e-h) and various epithelial cell cultures (data not shown). The 63 kDa protein was detected in all tissues and cells which were immunoreactive with mAb XIVF8. The 60 kDa protein was detected only in smooth and cardiac muscle and was apparently absent from skeletal muscle and non-muscle tissues and cells. Immunocytochemical studies of various human tissues showed that the 60/63 kDa proteins were mainly expressed in smooth and striated muscles. Epithelial cells from different organs, endothelium and connective tissue fibroblasts did not react with antibodies to these proteins (data not shown). However, the presence of 60/63 kDa protein immunoreactivity in cultured fibroblasts and keratinocytes suggests that culture conditions can induce the expression of 60/63 kDa proteins in particular cell types.

Subcellular localization of the 60/63 kDa proteins in cultured cells

No immunofluorescent staining of non-permeabilized cells with the 60/63 kDa protein antibodies was detected, indicating that the antigen is not expressed on the cell surface. In fixed and permeabilized rat embryo fibroblasts, the mAb XIVF8 (which is specific for the 60/63 kDa proteins), produced bright staining of plaque-like and needle-like structures, mostly adjacent to the cell periphery (Fig. 4A,B), indicating that the antigen could be associated with the actin cytoskeleton. The staining produced with the polyclonal and monoclonal antibodies could be blocked by the purified undenatured 60/63 kDa proteins (data not shown). Double staining of the cells for 60/63 kDa proteins (Fig. 4C) and F-actin (Fig. 4D) and for 60/63 kDa proteins (Fig. 4E) and actin-binding protein α-actinin (Fig. 4F) revealed that the 60/63 kDa proteins were associated with actin filaments and with the terminal segments of the stress fibers. The distribution of the staining along the stress fibers varied in length from compact plaques apparently associated with ends of fibers (Fig. 4B,E) to a more elongated, needle-like pattern, extending over a considerable part of the length of a fiber (Fig. 4A,C,E). A delicate, web-like periodic staining, seemingly associated with the plasma membrane, was also observed in some of the cells (Figs 4A-C,E; 6A,C,E).

The plaque-like staining suggested a concentration of the

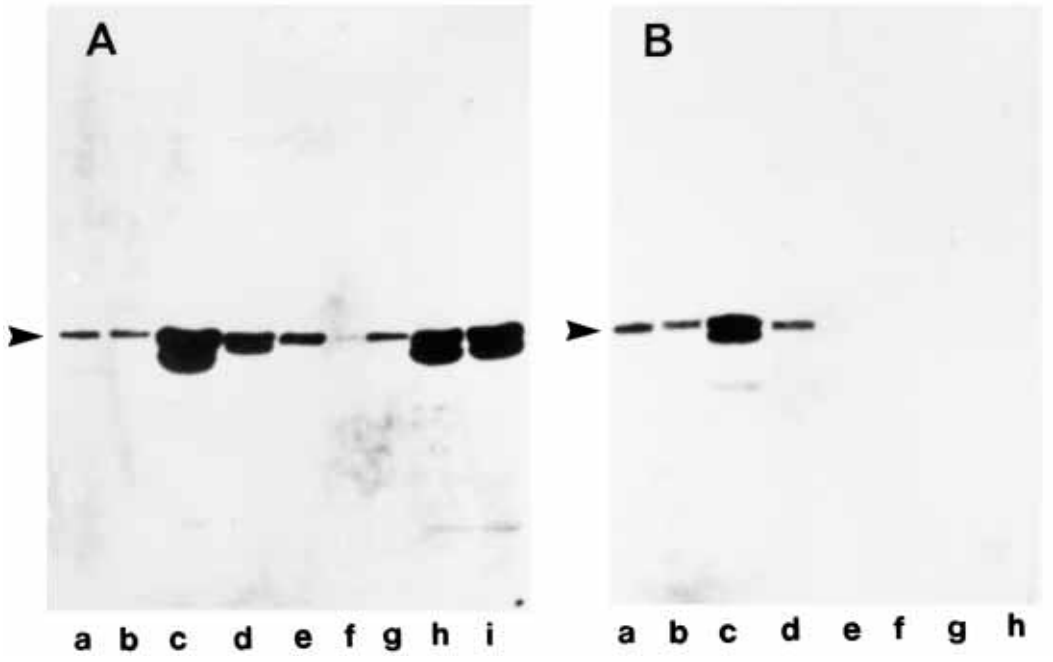

Fig. 3. Immunoblot analysis of 60/63 kDa protein expression in human tissues and cultivated cells. Proteins (150 μg) were resolved by SDS-PAGE (5% to 15% gradient gel), blotted to nitrocellulose and the 60/63 kDa proteins (arrowheads) detected with the mAb XIVF8, which is specific for these proteins, as described in Materials and Methods. (A) Tissues: lanes a, kidney; b, liver; c, uterus; d, cardiac muscle; e, skeletal muscle; f, brain; g, skin; h, aortic media; i, aortic intima. (B) Cultivated cells: lanes a, keratinocytes; b, skin fibroblasts; c, primary SMCs (5 days in culture); d, astrocytoma Asch-7; e, neurons; f, endothelial cells; g, hepatoma Hep G-2; h, platelets.

60/63 kDa proteins in focal contacts. To test this, we have compared the cellular distribution of the 60/63 kDa proteins with focal contacts visualized by interference reflection microscopy, and with several AJ markers known to localize in focal contacts. As shown in Fig. 5 (A and B, C and D), the fraction of 60/63 kDa proteins was found to co-localize with focal contacts. Double-immunofluorescence experiments show that the distribution of the proteins (Fig. 6A,C,E) overlaps with those of the typical focal contact proteins including vinculin (Fig. 6B), talin (Fig. 6D), and β_1 integrin (Fig. 6F). However,

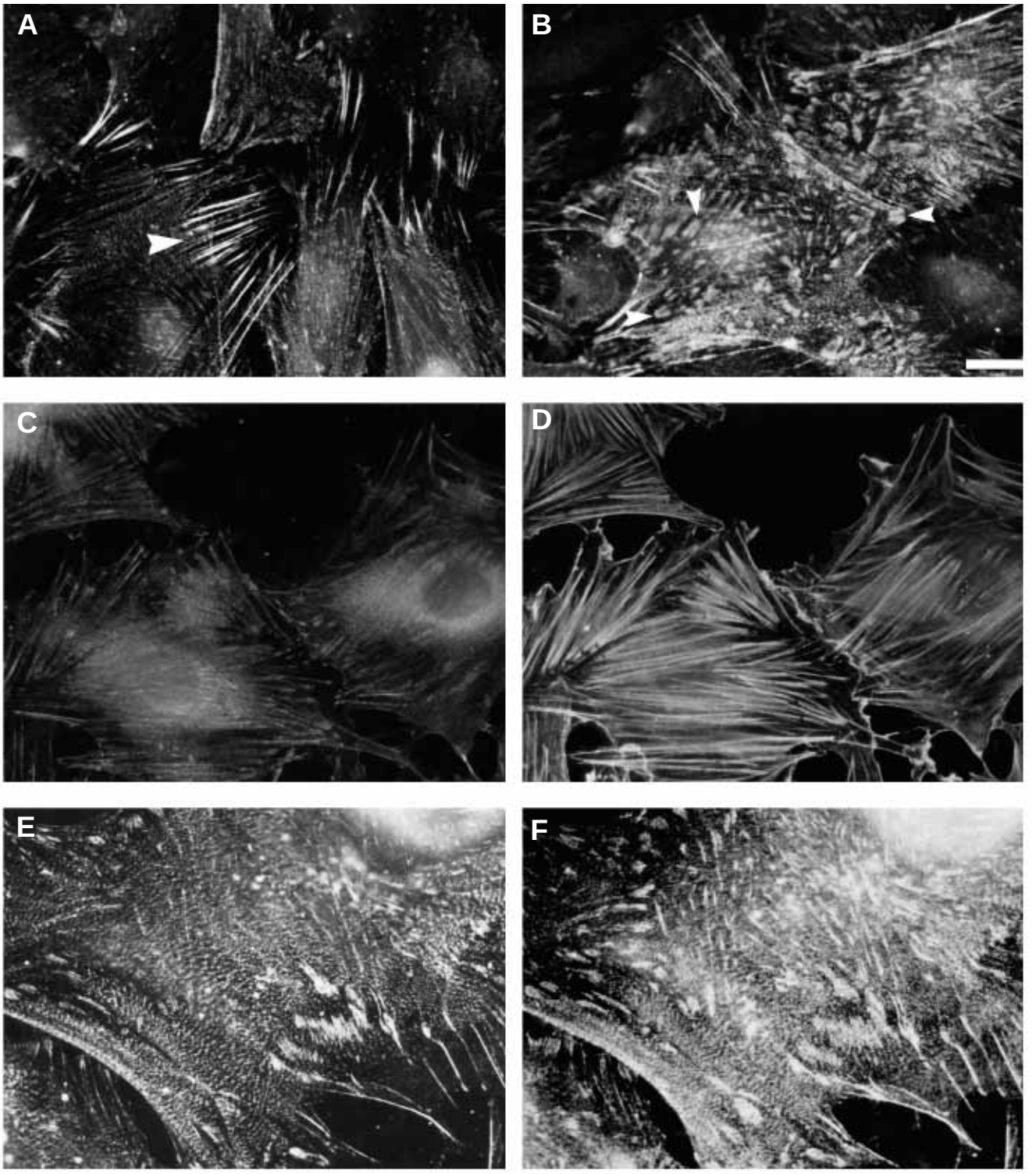

Fig. 4. Immunofluorescent staining of cultured fibroblasts with the anti-60/63 kDa protein antibodies. Cultures of rat embryo fibroblasts (REF52) were stained with anti-60/63 kDa protein mAb XIVF8 (A,B,C and E) and double labeled with polyclonal anti-actin (D) and anti-α-actinin (F) antibodies. Arrowheads point to the needle-like (A) and plaque-like (B) 60/63 kDa protein-positive structures. Bar, 10 μm.

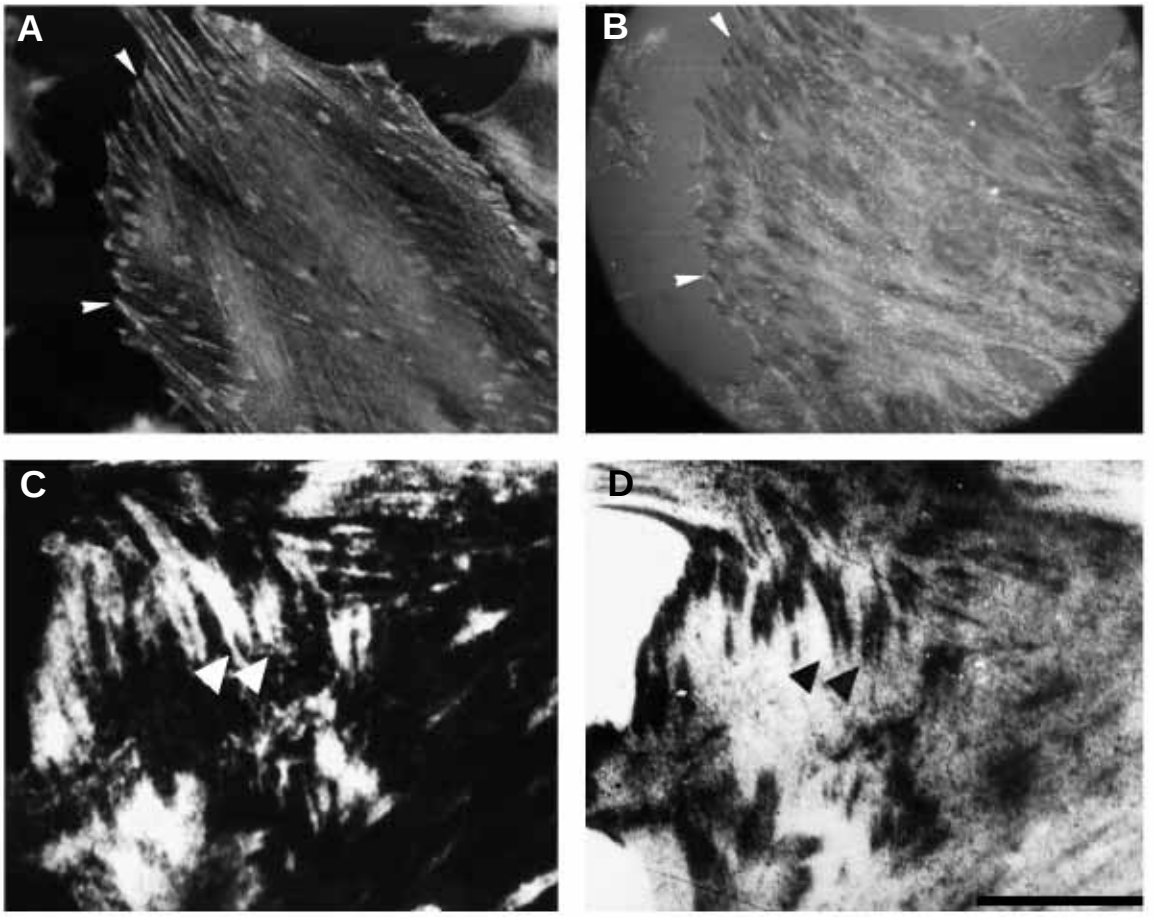

Fig. 5. Immunolocalization of 60/63 kDa proteins at focal contacts of fibroblasts and smooth muscle cells. The 60/63 kDa proteins detected with mAb XIVF8 (A,C) co-distributed with the focal contacts visualized by interference reflection microscopy (B,D) (arrowheads). (A,B) Rat embryo fibroblasts; (C,D) human aortic SMCs. Bar, 10 μm.

the 60/63 kDa protein antigen was apparently absent from some focal adhesions, particularly from those adjacent to cell margins. The same contacts were found to show positive immunostaining for vinculin, talin and β_1 integrin. In addition to the microfilament and focal contact localization, some of the 60/63 kDa protein staining was found in patch-like cytoplasmic structures showing no apparent similarity to focal contacts.

The presence of the 60/63 kDa proteins in cell-to-cell adherens junctions of cultured epithelial and endothelial cells was studied by immunofluorescence labeling of these cells with anti-60/63 kDa proteins antibodies as well as antibodies to typical cell-to-cell adherens junction proteins uvomorulin (E-cadherin) and PECAM. The 60/63 kDa staining was concentrated at the cell-to-cell contacts of cultured human epidermal keratinocytes similarly to the localization of E-cadherin (Fig. 7A,B); focal contacts were not stained with anti-60/63 kDa proteins antibody. In cultured epithelial MDBK cells and human umbilical vein endothelial cells the staining of the 60/63 kDa proteins was not detected (Fig. 7C,E).

To test whether the 60/63 kDa proteins are physically associated with the cytoskeleton, we analyzed the partitioning of the proteins between soluble and insoluble fractions upon detergent extraction of the SMCs. Cells were extracted with Triton X-100-containing buffer, and the 60/63 kDa protein content in the detergent-soluble and detergent-resistant fractions was determined by western blotting. Under conditions of this experiment (10% polyacrylamide gel, presence of Triton X-100 in the sample buffer, 7-day-old primary culture of human aortic SMCs), only a single immunoreactive band was revealed in the detergent-soluble and -resistant fractions. About 60-70% of the total cellular 60/63 kDa proteins resisted extraction and were found in the detergent-insoluble fraction (Fig. 8B). The 60/63 kDa proteins were more resistant to detergent extraction than the AJ cytoskeletal proteins vinculin and meta-vinculin (data not shown).

Subcellular localization of the 60/63 kDa proteins in muscle

Experiments on cultured cells have demonstrated that 60/63 kDa proteins are associated with actin filaments and concentrated in focal and cell-to-cell contacts. To determine whether the proteins are also a component of the naturally occurring AJs, we have studied their distribution in muscle tissues expressing enhanced levels of the 60/63 kDa proteins, and having morphologically prominent specialized AJs. Double staining of uterine tissue for the 60/63 kDa proteins and vinculin showed that the 60/63 kDa protein antibody staining was concentrated in visceral and vascular SMC, and it was

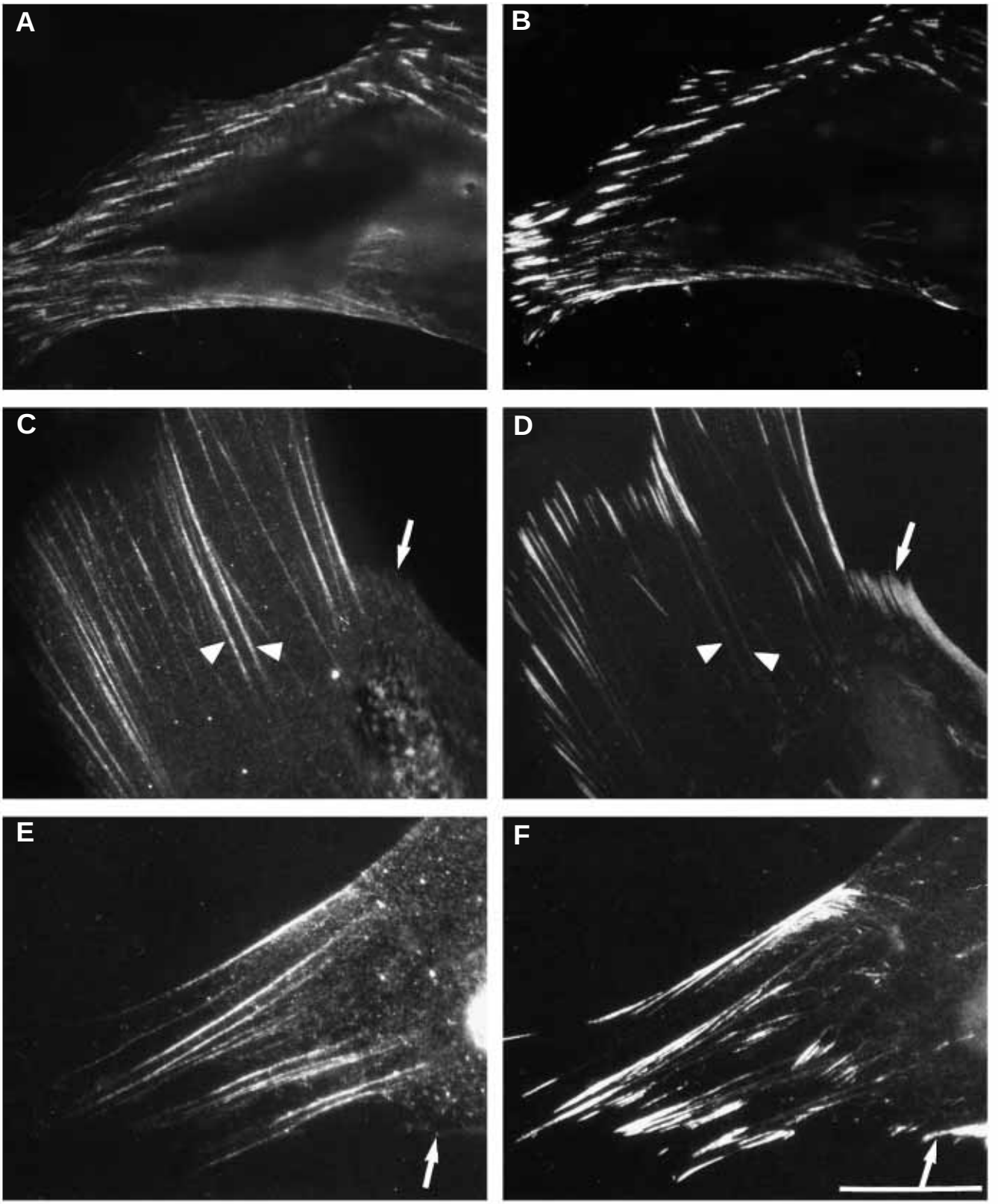

Fig. 6. Comparative localization of the 60/63 kDa proteins and AJ markers in cultured SMCs. Primary cultures of SMCs were double-stained for the 60/63 kDa proteins using mAb XIVF8 (A,C,E) and polyclonal antibodies against vinculin (B), talin (D), or β_1 integrin subunit (F). Note apparent absence of the 60/63 kDa proteins from some focal adhesions (arrows in C,E). Arrowheads in (C) and (D) point to the needle-like, 60/63 kDa protein-positive structures, apparently corresponding to the terminal segments of the stress fibers. Bar, 10 μm.

barely detectable in the connective tissue (Fig. 9A). Both SMCs and connective tissue were brightly stained with the anti-vinculin antibodies (Fig. 9B). Comparison of the 60/63 kDa proteins and vinculin-staining patterns in the uterine SMCs at higher magnification has revealed their co-localization in dot-like structures apparently associated with the plasma membrane (Fig. 9C,D), and previously shown to correspond at the light microscopy resolution level to the AJs of SMCs, or dense plaques (Geiger et al., 1981; Small, 1985; Belkin et al., 1988; Draeger et al., 1989).

In frozen sections of human cardiac muscle, anti-60/63 kDa protein antibodies produced bright immunofluorescent staining of the cardiomyocyte cell-cell junctions (intercalated disks) (Fig. 10A). A considerably weaker staining apparently associated with costameres, the lateral microfilament-plasma membrane attachment sites, could be observed at higher magnification (Fig. 10C). In both intercalated disks and costameres the 60/63 kDa protein staining (Fig. 10A,C) co-localized with that of vinculin (Fig. 10B,D).

On longitudinal sections of a skeletal muscle, the 60/63 kDa protein antibody produced bright staining localized at the ends of the muscle fibrils (Fig. 11A), and apparently associated with

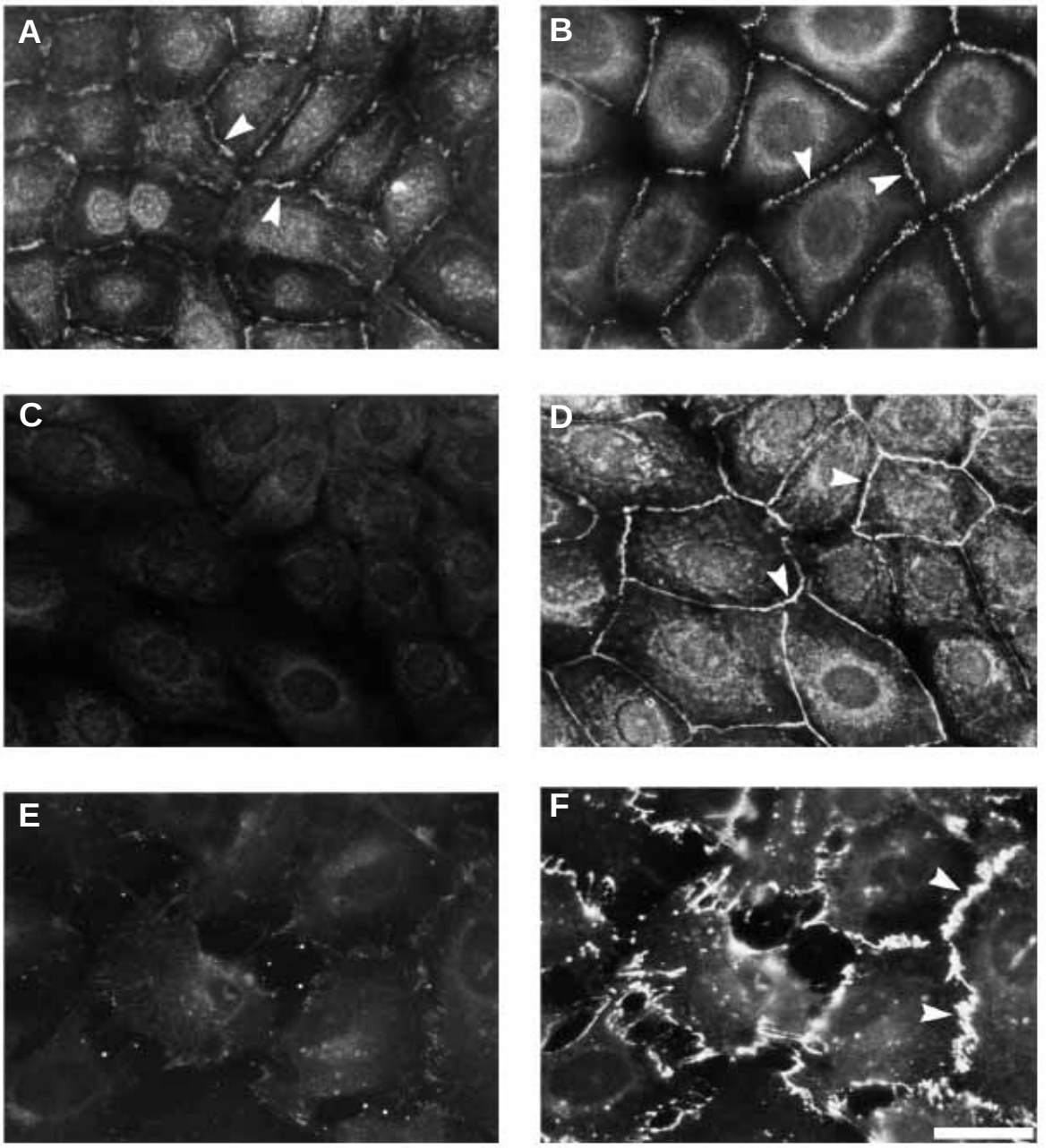

Fig. 7. Comparative localization of the 60/63 kDa proteins and cell-to-cell AJ markers in epithelial and endothelial cell cultures. Human skin keratinocytes (A and B) and epithelial MDBK cells (C,D) were labeled with anti-60/63 kDa protein mAb XIVF8 (A,C) and with polyclonal anti-E-cadherin antibodies (B,D). Human umbilical vein endothelial cells (E,F) were labeled with polyclonal anti-60/63 kDa protein antibodies (E) and monoclonal anti-PECAM antibodies (F). Arrowheads in (A,B,D,F) point to cell-to-cell AJ. Bar, 10 μm.

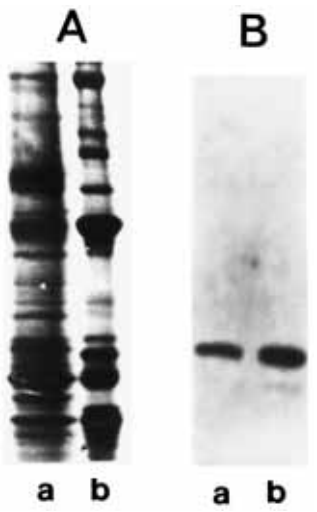

Fig. 8. Association of the 60/63 kDa proteins with the detergent-insoluble cytoskeletal fraction of smooth muscle cells. Cultured SMCs were extracted with buffer containing Triton X-100 as described in Materials and Methods, and the detergent-soluble (a) and detergent-insoluble (b) fractions were analysed by SDS-PAGE (10% gel). (A) Proteins stained with Coomassie Blue; (B) proteins stained with antibodies against the 60/63 kDa proteins.

the talin-positive myotendinous junctions (Fig. 11B). On transverse sections, the 60/63 kDa protein, as well as vinculin staining was sharply confined to the sarcolemma (Fig. 11C,D).

60/63 kDa protein expression in SMCs is regulated during differentiation and phenotypic modulation

The differentiation of SMCs is accompanied by assembly of the specialized AJs (dense plaques), and by specific alterations in the expression and subcellular distribution of a number of AJ-associated proteins (Volberg et al., 1986; Belkin et al., 1990; Glukhova et al., 1990). Since the 60/63 kDa proteins are particularly enriched in SMCs, we attempted to study whether their expression depends on the differentiation state of SMCs. We have analyzed the content of the 60/63 kDa proteins in the SMCs of developing human aorta and compared it with the expression of a SM-specific differentiation marker, meta-vinculin. As shown in Fig. 12A, the level of the 60/63 kDa proteins was very low in the aortas of the 10- to 12-week-old fetuses and progressively increased in 24-week fetal, 6-month and 1.5-year-old child aortic SM. The expression of the 60/63 kDa proteins in the 12-year-old child aortic wall reached levels typically found in the adult aorta. Interestingly, the increase in antigen content was accompanied by a change in electrophoretic pattern from a single 63 kDa band, which is present in the SMCs of all aortas, to the 60 kDa and 63 kDa doublet appearing in aortic SMCs only in the postnatal period. The pattern of expression of the 60 kDa component was similar to that observed for meta-vinculin (Fig. 12A; and Glukhova et al., 1990). Like meta-vinculin, the 60/63 kDa proteins may be characteristically expressed in differentiated SMCs.

Modulation of the SMC phenotype is accompanied by a specific loss of several cytoskeletal-associated proteins and was shown to be significantly retarded in SMC cultures maintained at high cell density (Glukhova et al., 1988; Campbell et al., 1989; Thyberg et al., 1990; Shirinsky et al., 1991). We analyzed the level of 60/63 kDa protein expression in the primary cultures of rabbit aortic SMCs plated at high and low

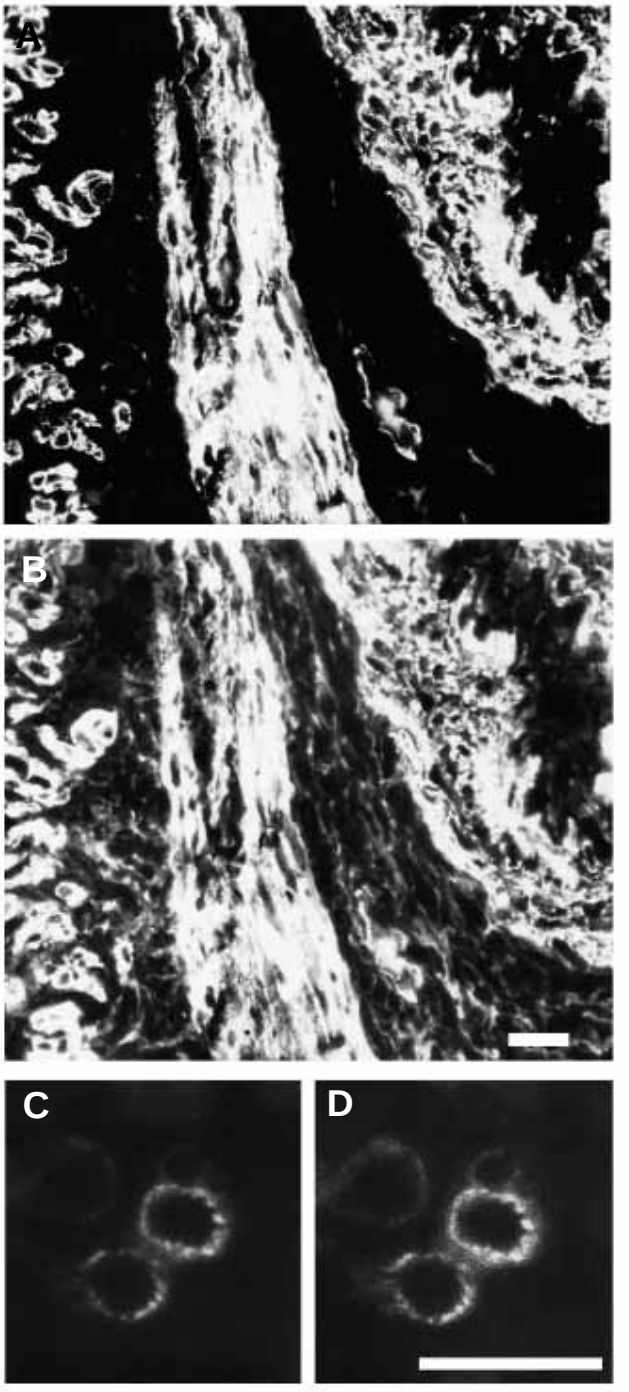

Fig. 9. Immunofluorescent localization of the 60/63 kDa proteins in uterine tissue. Cryostat sections of the human uterus were double-stained with anti-60/63 kDa protein mAb XIVF8 (A,C) and polyclonal anti-vinculin (B,D) antibodies. The 60/63 kDa proteins and vinculin were both present in uterine SMCs, while the connective tissue appeared negative for the 60/63 kDa proteins. On the cross-sections of the SMCs viewed at a higher magnification (C,D), the 60/63 kDa proteins and vinculin co-localized in the submembranous dot-like structures tentatively identified as SMC dense plaques. Bars, 20 μm.

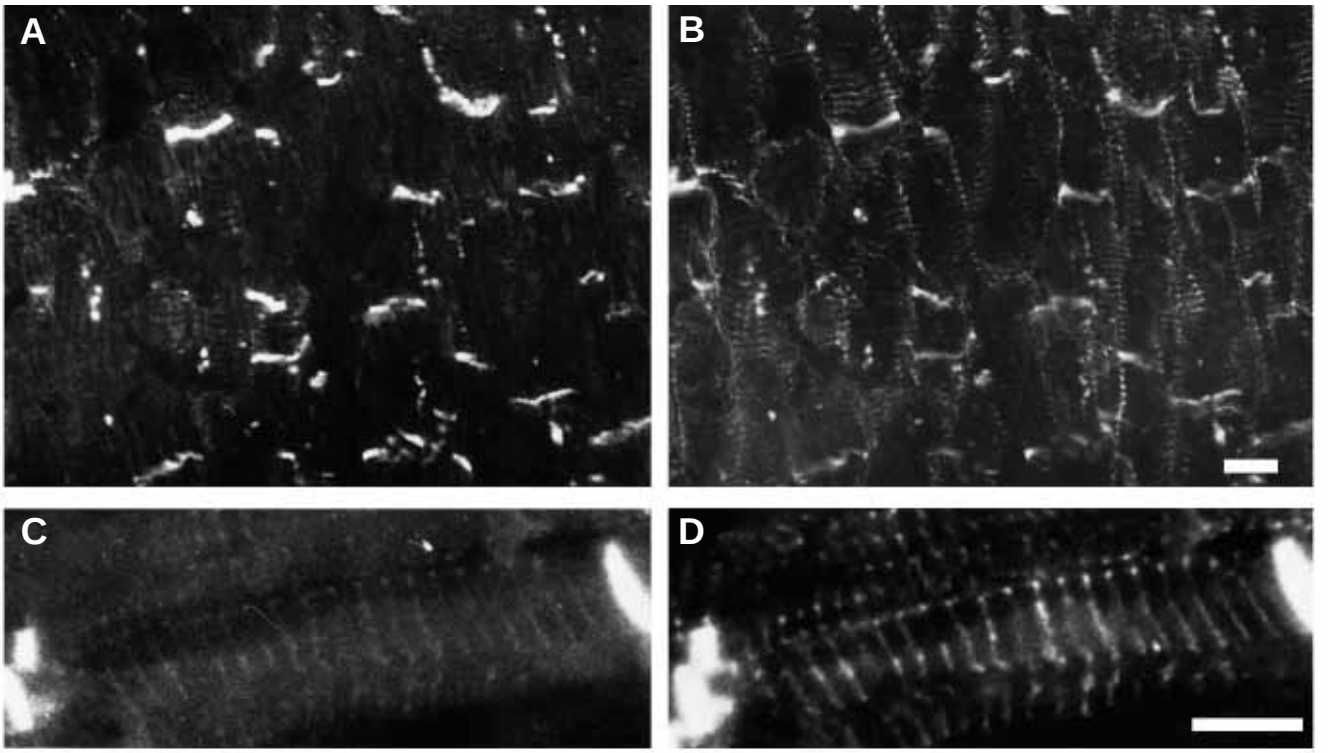

Fig. 10. Localization of the 60/63 kDa proteins in cardiac muscle. Longitudinal sections of cardiac muscle were double stained with anti-60/63 kDa protein mAb XIVF8 (A,C) and polyclonal anti-vinculin antibodies (B,D). Staining for the 60/63 kDa proteins was most prominent in the cardiomyocyte intercalated disks (A). At higher magnifications, some weak 60/63 kDa protein staining was also detected in costameres (C). Bars, 20 μm.

density, and used after the 2nd and the 10th days of culture. After 2 days in culture, regardless of the initial seeding density, the cells displayed a high 60/63 kDa protein content, close to that found in the intact rabbit aortic media (Fig. 12B, lanes a-c). A prominent decrease in 60/63 kDa protein expression, paralleled by a disappearance of the 60 kDa immunoreactive form, was observed in the 'sparse' cultures after 10 days of cultivation (Fig. 12B, lane e). There was a similar decrease in the level of meta-vinculin. However, the cells in the 'dense' cultures continued to express almost unchanged levels of the 60/63 kDa protein doublet (Fig. 12B, lane d). The decrease in 60/63 kDa protein content in the sparse SMC cultures was paralleled by its disappearance from focal contacts. No significant decrease in the intensity of the 60/63 kDa protein staining was noticed in the SMCs from the high density cultures after 10 days of cultivation (data not shown).

DISCUSSION

In this paper we describe the identification and purification of a novel AJ antigen from human uterine smooth muscle that migrates as a closely spaced doublet (60/63 kDa) in SDS-gels. A number of pieces of evidence suggest that the 60 kDa and 63 kDa proteins are very similar if not identical; (i) they show identical chromatographic behavior; (ii) both proteins cross-react with five different monoclonal antibodies; (iii) the N terminus of the 60 kDa and 63 kDa proteins is blocked; (iv) immunofluorescence studies of muscles show that both proteins are concentrated in AJs. In visceral smooth muscle, which expresses both proteins, the only structures stained with cross-reacting antibodies were the membrane-associated dense plaques. The most obvious explanation for these results is that the two proteins are isoforms that arise via either post-translational modification or alternative splicing, although we do not exclude the possibility that they might be closely related proteins encoded by separate genes. At present we are unable to distinguish between these various possibilities.

Partial amino acid sequencing has shown that the 60/63 kDa proteins are closely related to phosphoglucomutase 1 (PGM1), an enzyme that converts glucose 1-phosphate to glucose 6-phosphate in the initial stage of glycolysis. The sequences of both the rabbit (Ray et al., 1983) and human PGM1 isoform (Whitehouse et al., 1992) have been published, and the sequence of a second PGM isoform suggests that it arises from the same gene by alternative splicing (Lee et al., 1992). The primary sequences of these two isoforms differ over the first 81 amino acids, but are otherwise identical. The amino acid sequence we have determined on the human uterine smooth muscle 60/63 kDa proteins strongly suggests that they are not encoded by the PGM1 gene. The first sequence we obtained was from the N terminus of a 36 kDa polypeptide liberated from the 60/63 kDa proteins by V8 protease cleavage of the native protein. The sequence of 17 residues was very similar to that of residues 241-257 of PGM1. Interestingly, PGM1 also contains a V8 protease cleavage site in an identical position,

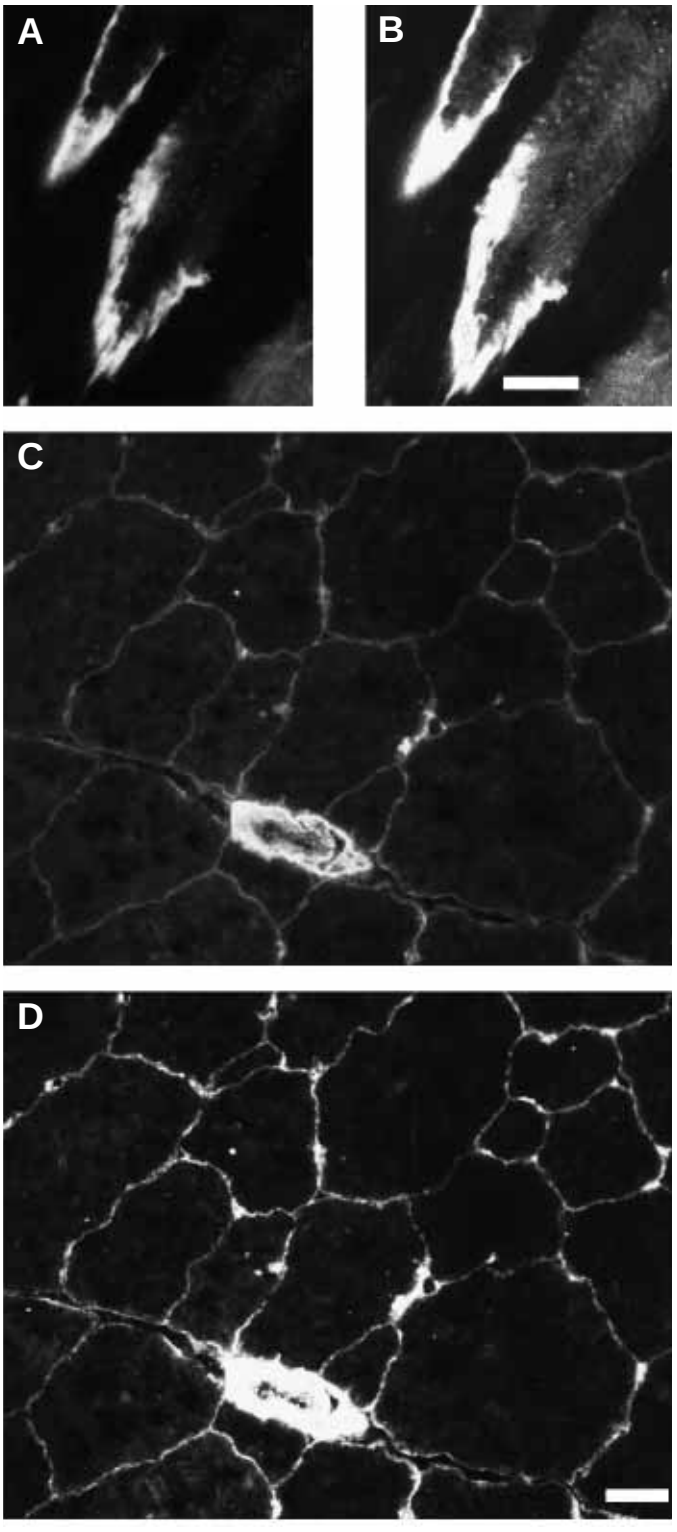

Fig. 11. Localization of the 60/63 kDa proteins in skeletal muscle. Longitudinal (A,B) and transverse (C,D) cryostat sections of a skeletal muscle were double stained with anti-60/63 kDa protein mAb XIVF8 (A,C) and polyclonal anti-talin (B) or anti-vinculin (D) antibodies. On the longitudinal sections, the 60/63 kDa proteins (A) was found to co-localize with talin (B) in the myotendinous junctions. On the transverse sections, the 60/63 kDa protein staining was confined to the sarcolemma (C). Bars, 20 μm.

suggesting that the domain structure of the proteins is similar. Further sequence data generated on cyanogen bromide fragments derived from the 60/63 kDa proteins confirmed the homology with PGM1, but clearly demonstrated that these proteins are distinct from PGM1. The greatest sequence divergence was found in a polypeptide derived from the C-terminal region of the 60/63 kDa proteins where only 10 out of 19 residues were identical to human PGM1. These results suggest that we have identified a novel PGM isoform, a conclusion further substantiated by the results of immunoblotting experiments. Thus, affinity-purified polyclonal antibodies raised against the purified 60/63 kDa proteins and three mAbs recognizing the 60/63 kDa proteins were found to cross-react with rabbit skeletal muscle PGM1, whereas two mAbs recognizing the 60/63 kDa protein did not react with the rabbit PGM1. The comparative V8 peptide maps of the denatured 60/63 kDa proteins and rabbit skeletal muscle PGM1 are also markedly different. The amino acid sequences of rabbit and human PGM1 are 97% identical (Whitehouse, 1992) with all potential V8 protease cleavage sites fully conserved in both proteins. The pronounced dissimilarities between the peptide maps of PGM1 and the 60/63 kDa proteins are unlikely therefore to represent species-specific sequence differences between the rabbit and human PGM1, and indicate that the 60/63 kDa proteins are distinct from PGM1. Whether the 60/63 kDa proteins we have characterized represent muscle-specific isoforms of the PGM family of enzymes, which are encoded by at least three independent genes (McAlpine et al., 1990), remains to be established.

Tissue distribution analysis has shown that the 60/63 kDa proteins are mostly expressed in muscle tissues and cells, and are particularly enriched in smooth muscles, while nonmuscle tissues contain only trace amounts of the antigen. We also found that 63 kDa polypeptide has broader tissue distribution compared to 60 kDa polypeptide, which expression is sharply confined to smooth and cardiac muscle cells. Therefore, the expression pattern of the 63/60 kDa proteins differs from those of ubiquitous AJ markers, including vinculin, talin, etc., suggesting a specific role for these PGM-related polypeptides in the organization of AJs in muscle cells. At the same time, some nonmuscle cells in culture apparently express the 63 kDa polypeptide, although at much lower levels. This fact probably reflects induction of the 63 kDa protein synthesis under culture conditions and shows that different types of nonmuscle cells may significantly vary in regard to 63 kDa polypeptide expression.

Immunofluorescent staining of tissue sections and cultured cells with antibodies to the 60/63 kDa proteins has revealed a pattern of antigen distribution consistent with an association with the actin cytoskeleton and AJs. In cultured SMCs and fibroblasts, the staining was concentrated along the termini of the stress fibers and in the focal contacts where it co-localized with ubiquitous AJ components, talin, β_1 integrin and vinculin. In cultured keratinocytes 60/63 kDa proteins were localized in cell-to-cell AJs, while there was no labeling at the cell-to-substratum AJs. On tissue sections, the antigen appeared specifically associated with several types of naturally occuring AJs including SMC dense plaques, cardiomyocyte intercalated disks, and myotendinous junctions. The unusual feature of the intracellular distribution of the antigen was the needle-like patterns found in cultured cells. Sometimes, the 60/63 kDa

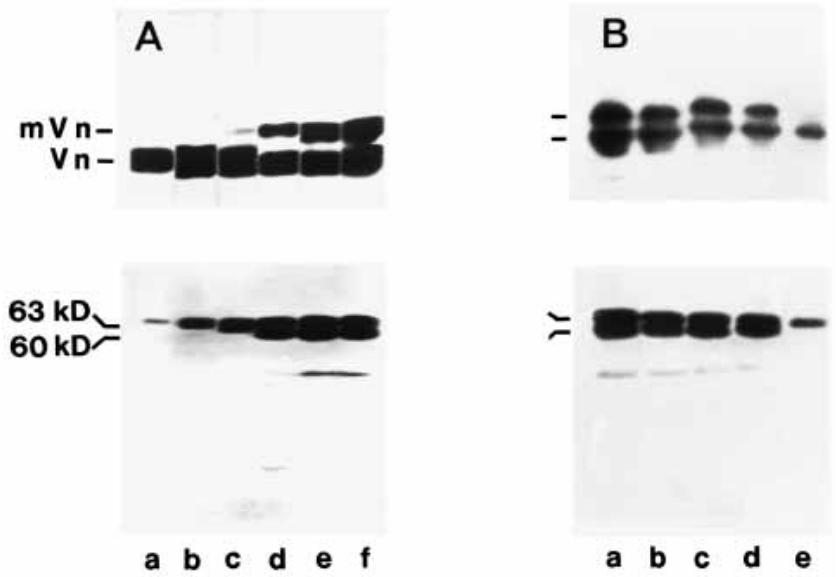

Fig. 12. Expression of the 60/63 kDa proteins, vinculin and meta-vinculin in the smooth muscle cells of developing human aorta, and during phenotypic modulation of rabbit aortic smooth muscle cells. Tissue samples were processed for SDS-PAGE (5% to 15% gradient gel) and immunoblotting as described in Materials and Methods. The 60/63 kDa proteins, vinculin and meta-vinculin were detected in the same blot using mAb XIVF8, which is specific for the 60/63 kDa proteins, and an antibody to human vinculin which cross-reacts with meta-vinculin. The positions of meta-vinculin (mVn), vinculin (Vn) and the 63 kDa and 60 kDa proteins are indicated. (A) SMCs of developing human aorta. Lanes a, 9- to 10-week-old fetal aorta; b, 24-week-old fetal aorta; c, aorta from a 3-month-old child; d, aorta from a 6-month-old child; e, aorta from a 1.5-year-old child; f, aortic media from a 12-year-old child. (B) Phenotypic modulation of rabbit aortic SMCs. Lanes a, intact rabbit aortic media; b, dense primary culture of SMCs after 2 days of cultivation; c, sparse culture after 2 days of cultivation; d, dense culture after 10 days of cultivation; e, sparse culture after 10 days of cultivation.

antigen was found distributed along the terminal parts of stress fibers outside the limits of focal contacts. This could mean that the 60/63 kDa proteins have a role in the organization of microfilament bundles in regions adjacent to the plaque domains of AJs. The localization of the proteins at the ends and along the stress fibers closely resembles that previously described for F-actin cross-linking proteins, α-actinin and variant of filamin, found in cultured cells (Pavalko et al., 1989). We have revealed that more than half of the total cellular pool of the 60/63 kDa proteins present in SMCs could not be solubilized upon extraction with Triton X-100, and remained associated with the detergent-resistant fraction enriched in cytoskeletal components. This observation, along with the intracellular localization of the proteins, suggests that they are physically associated with microfilaments. Therefore, 60/63 kDa proteins exhibit major characteristic features of cell-to-substratum and cell-to-cell AJ components, but in cultivated cells they may have broader distribution pattern compared to typical AJ proteins.

We have shown that expression of the 60/63 kDa proteins in human aortic smooth muscle gradually increases during development, most significantly in the postnatal period, reaching a maximum in the mature smooth muscle. The increase in 60/63 kDa protein levels correlates with the increase in the expression of meta-vinculin, the muscle-specific variant of the AJ protein vinculin. Conversely, in cultured SMCs undergoing transition from a contractile to synthetic state, 60/63 kDa protein levels decrease, again correlating with the decreased expression of meta-vinculin. The dependence of 60/63 kDa protein expression on SMC seeding density was also very similar to that found for meta-vinculin and the classical SM marker α-actin (Campbell et al., 1989; Shirinsky et al., 1991), suggesting that cell-to-cell interactions may be important in the co-ordinated expression of SM-specific proteins. The 60 kDa polypeptide is expressed only in differentiated SMCs, while 63 kDa variant is also detected in fetal (immature) and phenotypically modulated, dedifferentiated SMCs. Differentiation of SMCs towards the contractile phenotype in developing SM tissue is accompanied by a complex reorganization of their cytoskeleton, including formation of the specialized AJs (dense plaques) and the co-ordinated expression of a set of proteins specifically associated with the AJs of differentiated SMCs (Volberg et al., 1986; Belkin et al., 1990; Glukhova et al., 1990; Gimona et al., 1990). On the other hand, expression of these proteins decreases in parallel with the reorganization of the SMC contractile apparatus during transition of the cells from contractile to synthetic phenotype (Glukhova et al., 1988; Shirinsky et al., 1991; Gimona et al., 1990). The pronounced changes in the 60/63 kDa protein expression during SMC differentiation and phenotypic modulation, as well as the correlation with expression of the muscle-specific AJ protein meta-vinculin, suggest that 60/63 kDa protein may be involved in the formation and assembly of the dense plaques in smooth muscle cells.

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REFERENCES

Belkin, A. M., Ornatsky, O. I., Glukhova, M. A. and Koteliansky, V. E. (1988). Immunolocalization of meta-vinculin in human smooth and cardiac muscles. *J. Cell Biol.* **107**, 545-553.

Belkin, V. M., Belkin, A. M. and Koteliansky, V. E. (1990). Human smooth muscle VLA-1 integrin: purification, substrate specificity, localization in aorta, and expression during development. *J. Cell Biol.* **111**, 2159-2170.

Burridge, K., Fath, K., Kelly, T., Nuckolls, G. and C. Turner (1988). Focal

adhesions: Transmembrane junctions between the extracellular matrix and the cytoskeleton. *Annu. Rev. Cell Biol.* **4**, 487-525.

Campbell J. H., Kosher, O., Skalli, O., Gabbiani, J. and Campbell, G. R. (1989). Cytodifferentiation and expression of alpha-smooth muscle actin mRNA and protein during primary culture of aortic smooth muscle cells. Correlation with cell density and proliferative state. *Arteriosclerosis* **9**, 633-643.

Chamley-Campbell, J. H., Campbell., G. R. and Ross, R. (1979). The smooth muscle cell in culture. *Physiol. Rev.* **59**, 1-61.

Cleveland, D. W., Fisher, S. G., Kirschner, M. W. and Laemmli, U. K. (1977). Peptide mapping by limited proteolysis in sodium dodecyl sulphate by gel electrophoresis. *J. Biol. Chem.* **252**, 1102-1106.

Draeger, A., Stelzer, E. H. K., Herzog., M. and Small, J. V. (1989). Unique geometry of actin membrane anchorage sites in avian gizzard smooth muscle cells. *J. Cell Sci*. **94**, 703-711.

Feramisco, J. R. and Burridge, K (1980). A rapid purification of α-actinin, filamin, and 130, 000 dalton protein from smooth muscle. *J. Biol. Chem*. **255**, 1194-1199.

Geiger, B., Dutton, A. H., Tokuyasu, K. T. and Singer, S. J. (1981). Imunoelectron microscope studies of membrane microfilament interactions: distribution of α-actinin, tropomyosin, and vinculin in intestinal epithelial brush border and gizzard smooth muscle cells. *J. Cell Biol.* **91**, 614-628.

Geiger, B., Volk, T., Volberg, T. and Bendori, R. (1987). Molecular interactions in adherens-type contacts. *J. Cell Sci. Suppl. 8*, 251-272.

Geiger, B. and Ginsberg, D. (1991). The cytoplasmic domain of adherens-type junctions. *Cell Motil. Cytoskel*. **20**, 1-6.

Gimona, M., Herzog, M., Vandekerckhove, J. and Small, J. V. (1990). Smooth muscle specific expression of calponin. *FEBS Lett.* **274**, 159-162.

Glukhova, M. A., Kabakov, A. E., Frid, M. G., Ornatsky, O. I., Belkin, A. M., Mukhin, D. N., Orekhov, A. N., Koteliansky, V. E. and Smirnov, V. N. (1988). Modulation of human aorta smooth muscle cell phenotype. A study of muscle-specific variants of vinculin, caldesmon, and actin expression. *Proc. Nat. Acad. Sci. USA* **85**, 9542-9546.

Glukhova, M. A., Frid, M. G. and Koteliansky, V. E. (1990). Developmental changes in expression of contractile and cytoskeletal proteins in human aortic smooth muscle. *J. Biol. Chem.* **265**, 13042-13046.

Gross, E. and Witkop, B. (1961). Selective cleavage of the methionyl peptide in ribonuclease with cyanogen bromide. *J. Amer. Chem. Soc*. **83**, 1510-1517.

Koteliansky V. E., Belkin, A. M., Ornatsky, O. I., Vasilevskaya, T. D. and Glukhova, M. A. (1989). Identification and immunolocalization of a new component of human cardiac muscle intercalated disc. *J. Mol. Cell. Cardiol. Suppl.* **21**, 23-29.

Lee, Y. S., Marks, A. R., Gureckas, N., Lacro, R., Nadal-Ginard, B. and Kim, D. H. (1992). Purification, characterization, and molecular cloning of a 60-kDa phosphoprotein in rabbit skeletal sarcoplasmic reticulum which is an isoform of phosphoglucomutase. *J. Biol. Chem.* **267**, 21080-21088.

Laemmli, U. K. (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **227**, 680-685.

Luna, E. J. and Hitt, A. L. (1992). Cytoskeleton-plasma membrane interactions. *Science* **258**, 955-964.

McAlpine, P. J., Stranc, L. C., Boucheix, C. and Shows, T. B. (1990). The 1990 catalog of mapped genes and report of the nomenclature committee. *Cytogenet. Cell Genet.* **55**, 5-76.

Pavalko, F., Otey, C. A. and Burridge, K. (1989). Identification of a filamin isoform enriched at the ends of stress fibers in chicken embryo fibroblasts. *J. Cell Sci.* **94**, 109-118.

Ray, W. J. Jr, Hermodson, M. A., Puvathingal, J. M. and Mahoney, W. C. (1983). The complete amino acid sequence of rabbit skeletal muscle phosphoglucomutase. *J. Biol. Chem*. **258**, 9166-9174.

Schagger, H. and von Jagow, G. (1987). Tricine sodium dodecyl-sulfate polyacrylamide gel electrophoresis for the separation of proteins in the range from 1 kDa to 100 kDa. *Anal. Biochem.* **166**, 368-379.

Shirinsky, V. P., Birukov, K. G., Koteliansky, V. E., Glukhova, M. A., Spanidis, E., Rogers, J. D., Campbell, J. H. and Campbell, G. R. (1991). Density-reglated expression of caldesmon and vinculin in cultured rabbit aortic smooth muscle cells. *Exp. Cell Res.* **194**, 1351-1354.

Siliciano, J. D. and Craig. S. W. (1987). Properties of smooth muscle metavinculin. *J. Cell Biol.* **104**, 473-482.

Small, J. V. (1985). Geometry of actin-membrane attachments in the smooth muscle cell: the localization of vinculin and α-actinin. *EMBO J.* **4**, 45-49.

Small, J. V. and Sobieszek. A. (1980). The contractile apparatus of smooth muscle. *Int. Rev. Cytol*. **64**, 241-306.

Talian, J. C., Olmsted, J. B. and Goldman. R. D. (1983). A rapid procedure for preparing fluorescein-labeled specific antibodies from whole antiserum:its use for analysis of cytoskeletal architecture. *J. Cell Biol.* **97**, 1277-1282.

Thyberg, J., Heldin, U., Sjorund, M., Palmberg, L. and Bottger, B. A. (1990). Regulation of differentiation properties and proliferation of arterial smooth muscle cells. *Arteriosclerosis* **10**, 966-990.

Towbin, H., Staehelin, T. and Gordon, J. (1979). Electrophoretic transfer of proteins from acrylamide gels to nitrocellulose sheets: procedure and some applications. *Proc. Nat. Acad. Sci. USA* **76**, 4350-4354.

Tsukita, Sh., Tsukita, S., Nagafuchi, A. and Yonemura. Sh. (1992). Molecular linkage between cadherins and actin filaments in cell-cell adherens junctions. *Curr. Opin. Cell Biol.* **4**, 834-839.

Volberg, T., Sabanay, H. and Geiger. B. (1986). Spatial and temporal relationships between vinculin and talin in the developing chicken gizzard smooth muscle. *Differentiation* **32**, 34-43.

Whitehouse, D. B., Putt, W., Lovegrove, J. U., Morrison, K., Hollyoake, M. Fox, M. F., Hopkinson, D. A. and Edwards, Y. H. (1992). Phosphoglucomutase 1: Complete human and rabbit mRNA sequences and direct mapping of this highly polymorphic marker on human chromosome 1. *Proc. Nat. Acad. Sci. USA* **89**, 411-415.

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