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Proteomic Analysis of Androgen-Regulated Protein Expression in a Mouse Fetal Vas Deferens Cell Line

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During sex differentiation, androgens are essential for development of the male genital tract. The Wolffian duct is an androgen-sensitive target tissue that develops into the epididymis, vas deferens, and seminal vesicle. The present study aimed to identify androgen-regulated proteins that are involved in development of Wolffian duct-derived structures. We have used male mouse embryos transgenic for temperature-sensitive simian virus 40 large tumor antigen at 18 d of gestation, to generate immortalized mouse fetal vas deferens (MFVD) parental and clonal cell lines. The MFVD parental and clonal cell lines express androgen receptor protein and show features of Wolffian duct mesenchymal cells. Clonal cell line MFVD A6 was selected for proteomic analysis and cultured in the absence or presence of androgens. Subsequently, two-dimensional gel electrophoresis was performed on total cell lysates. Differentially expressed proteins were analyzed by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry and two androgen-regulated proteins were identified as mElfin and CArG-binding factor-A (CBF-A). CBF-A and mElfin are known to bind to cytoskeletal F-actin. Both proteins appeared to be regulated by androgens at the posttranslational level, possibly involving phosphorylation. Posttranslational modification of mElfin and CBF-A by androgens may be associated with a cytoskeletal change that is involved in androgen-regulated gene expression. (*Endocrinology* 144: 1147–1154, 2003)

ANDROGENS ARE ESSENTIAL for development and differentiation of the male genital tract. Androgen action is mediated by the androgen receptor (AR) that functions as a transcription factor by binding to specific DNA sequences in promoter and enhancer regions of target genes, thereby regulating their transcriptional activity (1). During male embryonic development, testicular testosterone stimulates the Wolffian ducts to develop into epididymides, vasa deferentia, and seminal vesicles. In the urogenital sinus, 5α-reductase converts testosterone into the more potent 5α-dihydrotestosterone, which stimulates the urogenital sinus to develop into the prostate gland and external genitalia (2). The importance of AR signaling during male sexual differentiation is demonstrated in certain pathological situations, in particular androgen insensitivity syndrome (reviewed in Ref. 3).

In the mouse Wolffian duct, AR expression can be detected in mesenchymal cells from d 12.5 of gestation [embryonic day (E) 12.5] onwards (4, 5) shortly before testicular testosterone secretion starts (6). In contrast, epithelial AR expression first appears in a temporal, cranial-to-caudal fashion, starting in the efferent ductules at E16, immediately after morphological differentiation of the Wolffian duct has started. AR protein is not expressed in epididymis and vas deferens epithelium before E19, and in seminal vesicle epithelium before postnatal d 1 (4, 5). The transition from AR negative to AR positive epithelium between E18 and E19 in the mouse is similar to what is found for fetal reproductive tract development in the rat (7). Because androgen-dependent development of the genital tract occurs before epithelial cells express AR, it was postulated that androgen action in epithelium is mediated by paracrine influences from mesenchymal cells (8), suggesting an important role for AR expressing mesenchymal cells in the development of the genital tract. Thus, androgen action in the developing Wolffian duct is mediated solely by the AR-positive mesenchymal cells until E18, and from E19 onwards by both the mesenchymal and the epithelial cells. Tissue recombination experiments have proven to be successful model systems for investigating mesenchymal-epithelial cell interactions in the reproductive tract and were used to identify growth factors as androgen-induced paracrine factors (8, 9). However, it is largely unknown which target genes, or other processes, in the mesenchymal cells are regulated by androgens.

To study androgen regulation of protein expression in mesenchymal cells, we developed androgen target cell lines derived from E18 Wolffian duct mesenchymal tissue. We used a transgenic mouse line that harbors the DNA sequence encoding thermolabile simian virus (SV) 40 large tumor (T) antigen under the direct control of an β-actin promoter. A clonal mouse fetal vas deferens (MFVD) cell line was used for proteomic analysis. Comparative two-dimensional gel electrophoresis (2DE) was performed, and androgen-regulated proteins were identified using matrix-

Abbreviations: ACN, Acetonitrile; AR, androgen receptor; CBF-A, CArG-binding factor-A; DCC, dextran-coated charcoal; 2DE, two-dimensional gel electrophoresis; FCS, fetal calf serum; IEF, isoelectric focusing; IPG, immobilized pH gradient; large T, large tumor antigen; LIM, cysteine rich consensus sequence ($CX_2CX_{17-19}HX_2CX_2CX_2CX_{16-20}CX_2C/D/H$); MALDI-TOF-MS, matrix-assisted laser desorption/ionization time-of-flight mass spectrometry; MFVD, mouse fetal vas deferens; m/z, mass-to-charge ratio; MSDB, Matrix Science database; OHF, hydroxyflutamide; pI, isoelectric point; PSF, penicillin/streptomycin/fungizone; SDS, sodium dodecyl sulfate; SV, simian virus.

assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF-MS).

Materials and Methods

Materials

All media (Glutamax-supplemented DMEM/F12 nutrient mix and Hanks' balanced salt solution), HEPES, collagenase, trypsin, TRIzol reagent, multiwell tissue culture plates (NUNC, Roskilde, Denmark), and filtertop culture flasks were purchased from Invitrogen Ltd. (Paisley, UK). Fetal calf serum (FCS) was obtained from Greiner (Frickenhausen, Germany). A mixture of penicillin, streptomycin, and fungizone was obtained from BioWhittaker (Walkersville, MD). Deoxyribonuclease and complete protease inhibitors were from Roche (Basel, Switzerland). NEN Life Science Products (Boston, MA) supplied R1881 (methyltrienolone). Schering (Bloomfield, NJ) provided hydroxyflutamide (OHF). Antibodies used were SV 40 T Ag (Pab 101; Santa Cruz Biotechnology, Inc., Santa Cruz, CA), antivimentin (clone VIM 3B4; Roche Molecular Biochemicals, Mannheim, Germany), and anticalponin (clone hCP; Sigma, St. Louis, MO). PROTRAN nitrocellulose transfer membranes were from Schleicher & Schuell (Keene, NH). West-Pico chemiluminescence substrate was purchased from Pierce Chemical Co. (Rockford, IL). Sequencing grade trypsin was from Promega Corp. (Madison, WI). 10× Tris-glycine-sodium dodecyl sulfate (SDS) electrophoresis buffer, TEMED (*N*,*N*,*N*′,*N*′-tetra-methyl-ethylenediamine), RC DC Protein Assay and prestained precision protein standard were from Bio-Rad Laboratories, Inc. (Hercules, CA). Immobiline dry immobilized pH gradient (IPG) strips (pH 3–10 nonlinear, 18 and 24 cm), IPG buffer pH 3–10, and PlusOne chemicals for isoelectric focusing (IEF) and 2DE and 12.5% wt/vol acrylamide SDS precast gels were purchased from Amersham Biosciences (Uppsala, Sweden). Duracryl (30% vol/vol acrylamide/0.8% vol/vol BIS) was obtained from Genomic Solutions (Chelmsford, MA). Colloidal Blue Staining Kit was from Novex/Invitrogen (Carlsbad, CA). α-Cyano-4-hydroxycinnamic acid and peptide calibration mix were from Bruker-Daltonica (Bremen, Germany).

Animals

Young adult mice, age 10–12 wk (B10/CBA strain) were purchased from Harlan Winkelmann GmbH (Bohren, Germany). Dr. E. Dzierzak (Department of Cell Biology, Erasmus MC, Rotterdam, The Netherlands) kindly provided the SV 40 large T transgenic Tag 5 mice. Both mice strains were housed under standard animal housing conditions in accordance with the NIH Guide for the Care and Use of Laboratory Animals. This study was approved by the Dutch experimental animal committee, protocol no. 124-98-03.

Preparation of primary cell cultures

Timed pregnancies were performed by placing Tag 5 transgenic male mice in individual cages overnight with B10/CBA females. Day 0 (E0) of gestation was determined by the presence of a vaginal plug in the morning. On d E18, females were killed, uteri were removed, and fetuses were dissected in a sterile hood in DMEM/F12 medium containing 25 mM HEPES, 5% vol/vol dextran-coated charcoal (DCC)-treated FCS, and a mixture of penicillin/streptomycin/fungizone (PSF). Wolffian duct-derived tissues were isolated and vasa deferentia from six animals were pooled and washed twice in Ca^{2+}- and Mg^{2+}-free PBS. The tissues were incubated in Hanks' balanced salt solution with 150 U/ml collagenase and 1 μg/ml deoxyribonuclease at 37 C for 30 min and were further dislodged into a single-cell suspension by pipetting up and down. This cell suspension was washed once with DMEM/F12 medium, supplemented with 5% (vol/vol) DCC-FCS and PSF, and the cell number was determined. Cells were subsequently seeded into 24-well plates at a density of 5×10^5 cells per well and cultured at 33 C in DMEM/F12 supplemented with 5% DCC-FCS, PSF, and 10 nM synthetic androgen R1881.

Establishment of vas deferens cell lines

After 1 wk of culture, medium from the cells was collected, centrifuged, and sterile filtered through a 0.22-μm filter. This conditioned medium was obtained freshly every week. Cells were washed with Ca^{2+}-, Mg^{2+}-free PBS, detached with 0.25% (vol/vol) trypsin/0.05% (wt/vol) EDTA by incubating at 33 C for 5 min and washed once with medium. Subsequently, cells were seeded at a lower density of 5×10^4 cells/cm^2 in medium as described above containing 20% (vol/vol) conditioned medium, until cell crisis was reached. During cell crisis, cell density was kept high, 2.5×10^5 cells/cm^2, to promote cell proliferation. Once the primary culture started proliferating again, it was considered immortal. These cells were seeded at a density of 5×10^5 cells/80-cm^2 culture flask. The immortalized cell line was designated parental MFVD. To obtain clonal cell lines, limited dilution cloning was performed on the MFVD cell line (passage 10) in a 48-well plate at an average density of 1 cell/well. After 1 wk of culture, colony formation had started. Medium was replaced weekly, until the cell number in the well was high enough to be passaged to larger plates. From this point on, addition of conditioned medium was omitted and the R1881 concentration was lowered to 1 nM. The immortalized cell cultures were passaged once a week. Three clonal cell lines were obtained from the parental MFVD cell line, designated clones A5, A6, and E2.

Growth studies

Growth studies were performed on the MFVD clone A6. At d 0, cells were seeded, in duplicate, into 24-well plates at a density of 2×10^4 cells per well. The cells were grown for 7 d in the presence of vehicle (0.1% vol/vol ethanol), 1 nM R1881, or 100 nM antiandrogen OHF, at 33 and 39 C. On d 2, 5, and 7, cells were trypsinized and cell numbers were determined using a hemocytometer.

Western blotting and immunoprecipitation

MFVD parental and clonal cell lines were grown in 80-cm^2 culture flasks for 7 d in the presence of vehicle or 1 nM R1881 at 33 and 37 C or 39 C. On d 4, medium and hormone were replaced. On d 7, the confluent cell layer was washed twice with PBS, and cells were subsequently collected by scraping on ice in 0.5 ml lysis buffer [40 mM Tris-HCl, 1 mM EDTA (pH 7.4), 10% glycerol (vol/vol), 1% Triton X-100 (vol/vol), 0.08% SDS (wt/vol), 0.5% sodium desoxycholate (wt/vol), 10 mM dithiothreitol, and complete protease inhibitors]. The obtained lysate was sonicated for 5 sec to decrease viscosity and centrifuged 10 min at 4 C, 50,000 rpm. Total protein lysate (25 μg) was subjected to SDS-PAGE, and the proteins were blotted onto a nitrocellulose membrane. The AR protein was detected with monoclonal antibody F39.4.1 (10). Marker proteins were detected using monoclonal antibodies against SV 40 T Ag, vimentin, and calponin. In addition, AR protein was immunoprecipitated from 100 μg total cell lysate with F39.4.1 and detected on blot with polyclonal antiserum against synthetic peptide SP 213 (10). Proteins were visualized by chemiluminescence detection, according to the instructions of the manufacturer.

Northern blot analysis

MFVD parental and clonal cell lines were cultured in 80-cm^2 culture flasks for 7 d at 33 C. Total RNA was isolated using TRIZOL reagent according to instructions of the manufacturer. Fifteen micrograms of RNA of each sample were applied on a formamide gel and blotted overnight to a PROTRAN nitrocellulose membrane. The Hoxa11 probe was obtained using a 300-bp *Bam*HI-*Bgl*II fragment of the mouse Hoxa11 cDNA (11) and was labeled with α^{32}P-deoxy-ATP. Hybridization was performed at 63 C overnight. RNA was visualized by autoradiography.

2DE

2DE was performed on protein lysates of MFVD A6 cells between passages 12 and 18, which were cultured in 80-cm^2 culture flasks as described above in the presence of either vehicle (0.1% ethanol), 1 nM R1881, or 10 nM testosterone for 24 h. After 7 d of culture, cells were washed twice with PBS and collected in PBS. Cells from five 80-cm2 culture flasks from the same passage were pooled and centrifuged. Pellets were solubilized with 2D lysis buffer containing 7 M urea, 2 M thiourea, 4% wt/vol CHAPS (3-[3-(cholamidopropyl)-dimethyl-ammonio]-1-propanesulfonate), 40 mM Tris-HCl, 10 mM dithiothreitol, and complete protease inhibitors. This lysate was sonicated and centrifuged, and protein concentration was determined as described above. For analytical gels 100 μg, and for preparative gels, 500 μg protein lysate were

mixed with rehydration buffer (8 M urea; 2% wt/vol CHAPS; 0.5% vol/vol IPG buffer, pH 3–10; and a trace of bromophenol blue). Immobiline dry IPG gel strips of 18 cm for analytical (n = 4) and 24 cm for preparative gels (n = 1) with a nonlinear pH range of 3–10 were actively rehydrated overnight with sample containing rehydration buffer applying a voltage of 30 V/h (Vh). IEF was performed with an IPGphor system (Amersham Biosciences) according to the manufacturer's instructions, until 92 kVh was reached. After IEF, proteins in the gel strips were reduced in equilibration buffer (6 M urea; 30% vol/vol glycerol; 2% wt/vol SDS; 50 mM Tris-HCl, pH 8.8) with 60 mM dithiothreitol for 20 min, and subsequently carbamidomethylated in the same buffer, containing 280 mM iodoacetamide, for 2 min. Resolution in the second dimension was carried out in an Ettan-Dalt II system (Amersham Biosciences) on 10% wt/vol Duracryl gels (analytical) or 12.5% wt/vol SDS precast gels (preparative). The gels were run 20 min with a constant power of 3 W and subsequently 3.5 h with a constant power of 180 W. During the run the temperature was kept constant at 25 C. Prestained protein standards were run together with the strips. The gels were fixed overnight in 5% vol/vol phosphoric acid/40% vol/vol methanol, and protein spots were visualized by colloidal Coomassie stain according to the manufacturer's instructions. Analytical gels were subsequently silver stained according to Morrissey (12).

In-gel trypsin digestion and mass spectrometry

Protein spots of interest were excised out of the gel, were washed briefly in MilliQ water, destained twice for 20 min in 37% acetonitrile (ACN)/2.5 mM ammonium hydrogen carbonate, washed again, vacuum-dried for 30 min, digested overnight with sequencing grade trypsin (0.7 U per protein spot), and rehydrated in 50% vol/vol ACN/0.1% vol/vol trifluoroacetic acid. The sample was mixed with a saturated α-cyano-4-hydroxycinnamic acid matrix solution in 100% ACN (one part sample and four parts matrix), and 0.5 μl of this mixture was spotted on an anchorchip plate (Bruker-Daltonica). Digested peptide fragments were analyzed in a MALDI-TOF-MS in a positive reflectron ion mode using a Biflex III apparatus (Bruker-Daltonica). The obtained peptide mass fingerprint spectra were analyzed searching the Matrix Science database (MSDB) nonredundant protein database (National Center for Biotechnology Information) with MASCOT software (http://www.matrixscience.com), allowing one missed cleavage of trypsin per peptide. After protein identification, the database was further searched allowing the peptides to be modified for carbamidomethylation, oxidation of methionine and phosphorylation. Peptide mass accuracy was set to a maximum of 200 ppm. Upon protein identification, mass spectra of different isoforms of one and the same protein were compared with investigate the presence of modified peptides.

Results

Development of vas deferens cell lines and establishment of their growth characteristics

An immortalized MFVD cell line was developed from vasa deferentia from E18 mouse fetuses as described in *Materials and Methods*. From this parental cell line, clonal cell lines designated A5, A6, and E2 were derived. All cell lines were continuously cultured at 33 C, the permissive temperature of large T. Higher temperatures (37–39 C) cause degradation of the large T protein and are therefore nonpermissive. It was described for other immortalized cell lines that cell proliferation at large T permissive conditions will stop, whereas cell differentiation will be initiated (13, 14).

Initially, the MFVD parental and clonal cell lines were continuously cultured under permissive conditions in the presence of methyltrienolone (R1881), a synthetic androgen. Growth studies were performed under variable culture. For this purpose, the MFVD clonal cell line A6 was cultured for 7 d at 33 and 39 C in the absence of AR ligand, in the presence of 1 nM R1881, or in the presence of 100 nM of the OHF. Microscopic analysis revealed a difference in morphology between cells cultured at 33 and 39 C (Fig. 1, A and B). At 33 C, MFVD A6 cells were found to grow to a high density, reaching confluency (Fig. 1A). At 39 C, the cells developed a much larger and flatter appearance and entered a more quiescent stage, although cell growth had not completely stopped (Fig. 1B). Quantification of cell growth showed a difference in growth between cells cultured at 33 and 39 C (Fig. 1C). Culturing under large T permissive conditions provided sufficient stimuli for cell growth (Fig. 1C, *open symbols*). However, raising the culture temperature to 39 C resulted in a dramatic reduction of cell proliferation (Fig. 1C, *closed symbols*). A reduction in cell growth was observed to a lesser extent at 37 C (data not shown). Cell growth at 33 and 39 C was neither stimulated by R1881, nor inhibited by OHF. Western blot analysis showed a correlation between growth and large T protein expression. As expected, large T protein was highly expressed at 33 C in all cell lines (Fig. 1D, lanes 1, 3, 5, and 7). Shifting the temperature to 39 C (or 37 C; data not shown) resulted in a decline of large T protein expression in the MFVD parental cell line, in MFVD clone A5, and most prominent in MFVD clone A6 (Fig. 1D, lanes 2, 4, and 6). MFVD clone E2, however, did not show any large T regulation, as the protein level remained high under non-permissive conditions (Fig. 1D, lane 8). This may be due to a varying basal level of large T protein between the cell lines and to the turnover rate of the large T protein. It can be concluded that differentiation of the MFVD A6 clone in particular is dependent on large T protein expression.

MFVD cells are of Wolffian duct stromal origin

To select for the stromal cells in our vas deferens cultures, the MFVD parental and clonal cells were cultured in serum-containing medium. To confirm the stromal origin of MFVD cells, some marker proteins were analyzed. On Western blots, all clonal cell lines were positive for two mesenchymal cell markers, vimentin, and calponin (Fig. 2A, lanes 1–3; Refs. 15 and 16). In contrast, the cells were negative for the epithelial cell marker pan-keratin (a mixture of all cytokeratins; Ref. 17). Chinese hamster ovary cells were used as a positive control for mesenchymal cells (Fig. 2A, lane 4). The prostate cancer cell line LNCaP was used as a control cell line for epithelial cells expressing pan-keratin (Fig. 2A, lane 5).

To determine the vas deferens origin of these stromal cells, Northern blot analysis was performed with a Hoxa11 probe. This member of the Hoxa complex of homeotic genes is expressed mainly in developing limbs and the embryonic kidney (not in adult kidney), and in stromal cells surrounding the Müllerian and Wolffian ducts (11). MFVD parental and clonal cell lines expressed Hoxa11 mRNA (Fig. 2B, lanes 1–4), as did the embryonic kidney (positive control; Fig. 2B, lane 5) but not the adult kidney (negative control; Fig. 2B, lane 6). From these experiments, we conclude that the MFVD parental and clonal cell lines have features of fetal vas deferens mesenchyme.

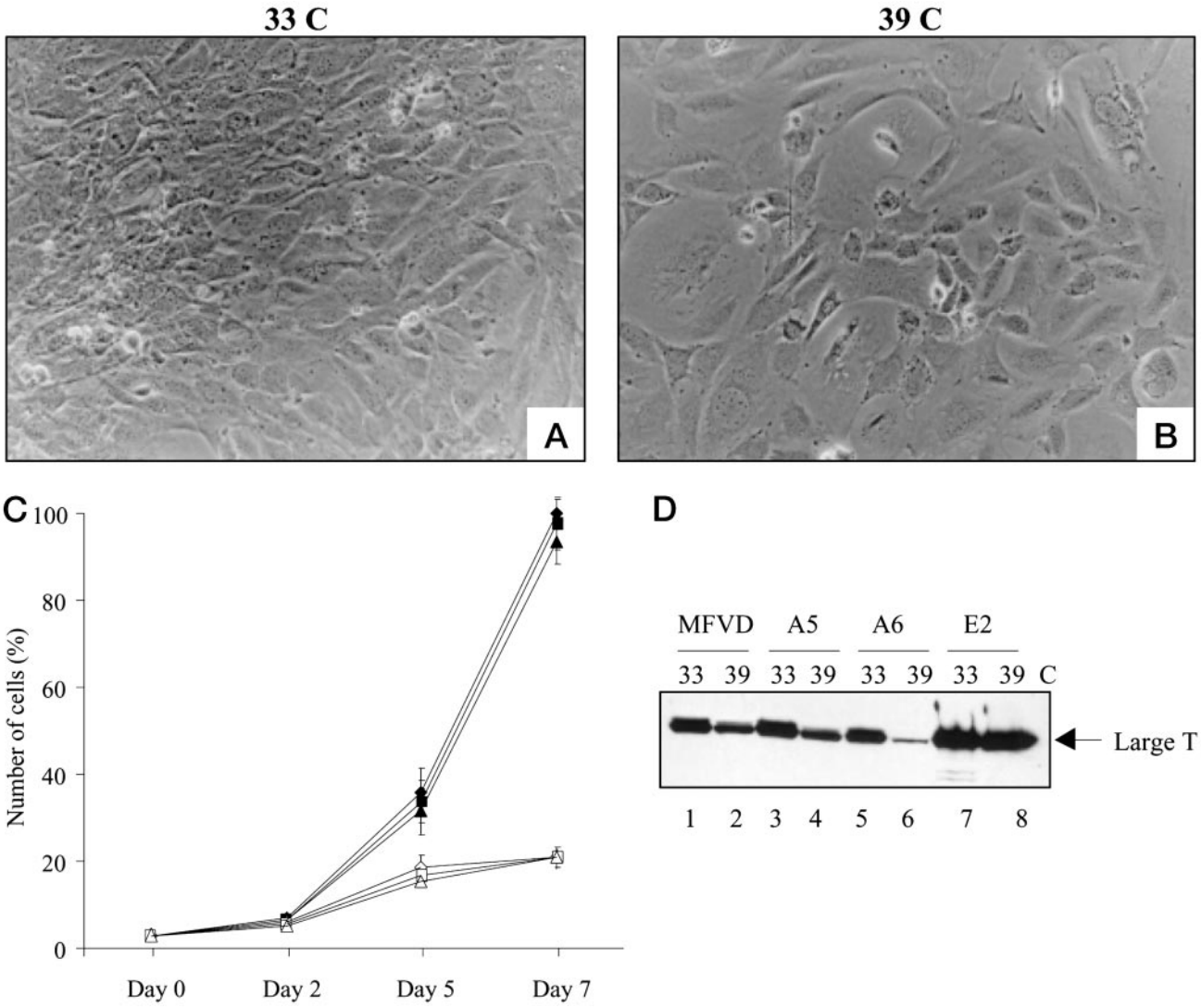

FIG. 1. Phase-contrast microscopy photographs of MFVD clonal cell line A6 at passage 20. The cells were cultured for 7 d at 33 C (A) and 39 C (B). Magnification, ×200. Note the difference in morphology between cells cultured at 33 and 39 C. C, Growth curve of MFVD A6 clonal cell line cultured at 33 C (*closed symbols*) and 39 C (*open symbols*) in the presence of vehicle (0.1% ethanol; *squares*), 1 nM R1881 (*rectangles*), or 100 nM OHF (*triangles*). Each *point* represents the SE of the mean (+/− SEM) of three individual experiments performed in duplicate. The number of cells grown for 7 d in the absence of hormone at 33 C was set at 100%, and all individual points were calculated relative to this value. D, Western blot of large T protein expression in MFVD parental and clonal cell lines.

AR protein is expressed in MFVD parental and clonal cell lines

Because cell growth in MFVD cell lines was not stimulated by androgens, we investigated the expression level of the AR protein. For that purpose, immunoprecipitation of AR protein from MFVD parental and clonal cell lines, cultured at 33 and 39 C, was performed (Fig. 3A). The parental cell line showed AR protein expression at both large T permissive and nonpermissive temperatures (Fig. 3A, lanes 1 and 2). All three clonal cell lines showed AR expression. Whereas AR expression at 33 C was rather low (Fig. 3A, lanes 3, 5, and 7), it was clearly higher at 39 C (Fig. 3A, lanes 4, 6, and 8) when cells were not under the control of large T and thus in a differentiating state. In all our further studies, MFVD cells were cultured at 37 C instead of 39 C, to provide a more physiological temperature. AR expression was followed in the MFVD A6 clone during a culture period of 20 passages, by performing Western blots on whole cell lysates at passages 10 and 20. Figure 3B shows a relatively low AR expression at 33 and 37 C in the absence of R1881 (lanes 1, 3, 5, and 7). However, addition of R1881 to the cells resulted in a marked increase in AR protein level (Fig. 3B, lanes 2, 4, 6, and 8). This increase is most probably due to stabilization of the receptor protein in the presence of ligand (18). In cells cultured at 37 C, AR protein expression in the presence of R1881 was more pronounced than at 33 C. The AR protein expression was reduced at passage 20 (Fig. 3B, lanes 5–8) compared with passage 10 (Fig. 3B, lanes 1–4). AR expression was lost at passage 30 (results not shown). From these experiments, it was concluded that the AR protein in the MFVD A6 cell line is stabilized in the presence of ligand and that AR protein expression is lost after 30 passages.

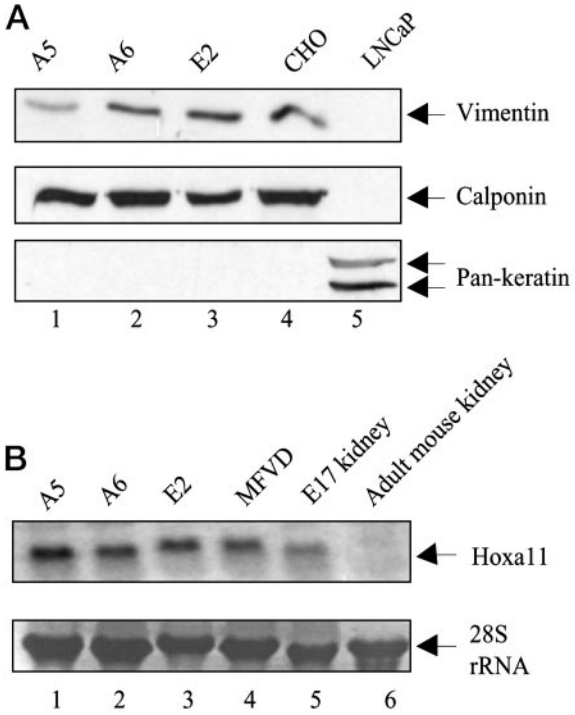

FIG. 2. Characterization of MFVD parental and clonal cell lines. A, Protein expression of two mesenchymal cell markers (vimentin and calponin) and one epithelial cell marker (pan-keratin). Chinese hamster ovary cells were used as mesenchymal control cells. LNCaP cells were used as epithelial control cells. B, Hoxa11 mRNA expression in MFVD clonal cell lines and mouse fetal kidney. Mouse adult kidney was used as a negative control tissue.

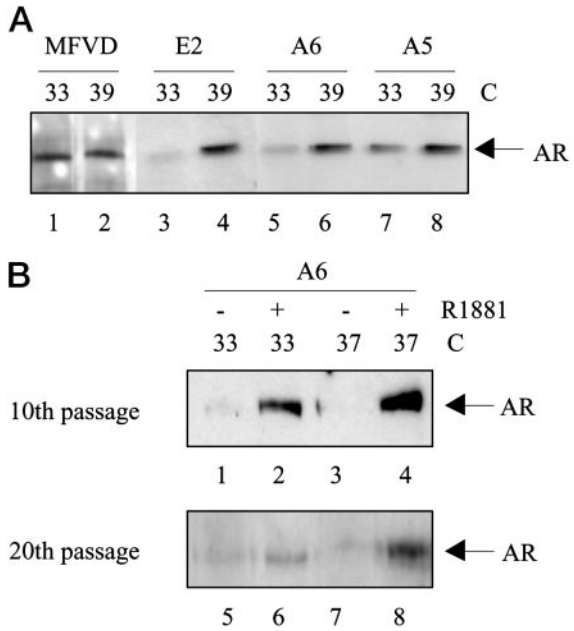

FIG. 3. AR protein expression. A, AR immunoprecipitation in MFVD parental and clonal cell lines cultured at 33 and 39 C. B, AR protein expression in A6 cell line at passages 10 and 20. A6 cells were cultured at 33 or 37 C in the presence of vehicle (0.1% ethanol) or 1 nM R1881, as indicated in the figure.

CArG-binding factor-A (CBF-A) and mElfin are differentially expressed in MFVD A6 cells

To identify proteins that are regulated by androgens, the MFVD A6 cell line was used for proteomic analysis. MFVD A6 protein lysates from four separate experiments were used to generate analytical 2DE gels. Typically, silver-stained 2DE gels showed approximately 1500 protein spots. Comparison of protein expression patterns of control and androgen-treated cells revealed only minor qualitative changes, except for one part of the gels which showed, repeatedly (n = 4), a differential protein expression in a region focused between pH 6 and 7 and between a molecular mass of 30–35 kDa. To identify these differentially expressed proteins by MALDI-TOF-MS, preparative 2DE gels were run. MFVD A6 cells were either treated for 24 h with vehicle, 1 nM R1881, or 10 nM testosterone. A colloidal Coomassie-stained gel from cells treated with vehicle is shown in Fig. 4A. Approximately 400 protein spots could be visualized on these gels. Protein expression patterns of the cells cultured under different hormonal conditions were much alike, except for the region that is boxed in Fig. 4A. A more detailed representation of this part of the gel is shown in Fig. 4B, for all three culture conditions. Protein spots a, b, c, and d represent nonchanging anchors that were identified by MALDI-TOF-MS (Table 1). Protein spots 1 and 2 present in the vehicle situation are subject to changes upon androgen treatment. While spot 1 is present in the vehicle situation, this protein disappears and three other proteins appear at positions 1a, 1b, and 1c, upon treatment with either R1881 or testosterone. The exposure to R1881 or testosterone resulted in migration of protein spot 2 to position 2a and position 2b, respectively. Protein spots 1, 1a, 1b, and 1c could all be identified with MALDI-TOF-MS as CBF-A (Table 1). Protein spots 2, 2a, and 2b were all found to represent mElfin (Table 1). From the gels it is apparent that addition of either 10 nM of the natural ligand testosterone or 1 nM of the synthetic androgen R1881 has the same effect on the position of the protein spots representing CBF-A. However, 1 nM R1881 appears to have a different effect on the 2DE migration of mElfin compared with the effect of 10 nM testosterone. No change in position of CBF-A and mElfin was observed when cells were treated with 1 nM testosterone (preliminary results, data not shown), which may be the consequence of the lower affinity of testosterone for the AR compared with dihydrotestosterone or R1881. Furthermore, addition of 100 nM of an AR antogonist, hydroxyflutamide, together with 1 nM R1881, blocks the effect of R1881 (preliminary results, data not shown).

Proteins a–d in Fig. 4B and e–o *encircled* in Fig. 4A were also identified to provide a further characterization of MFVD A6 cells and are all listed in Table 1, together with their molecular mass, theoretical and experimental isoelectric point (pI), and accession number. In some cases, proteins did not run in the gel according to their theoretical mass or pI, and these proteins may have undergone modifications that change their position in the gel. Using this proteomic approach, we have performed a small-scale analysis to further characterize the MFVD A6 cell line. Several identified proteins (protein disulfide isomerase precursor, laminin A, laminin C, ATP-synthase β-chain, and mitochondrial stress protein-70) have been reported to be expressed in mouse fibroblasts (19). The presence of these proteins in the MFVD

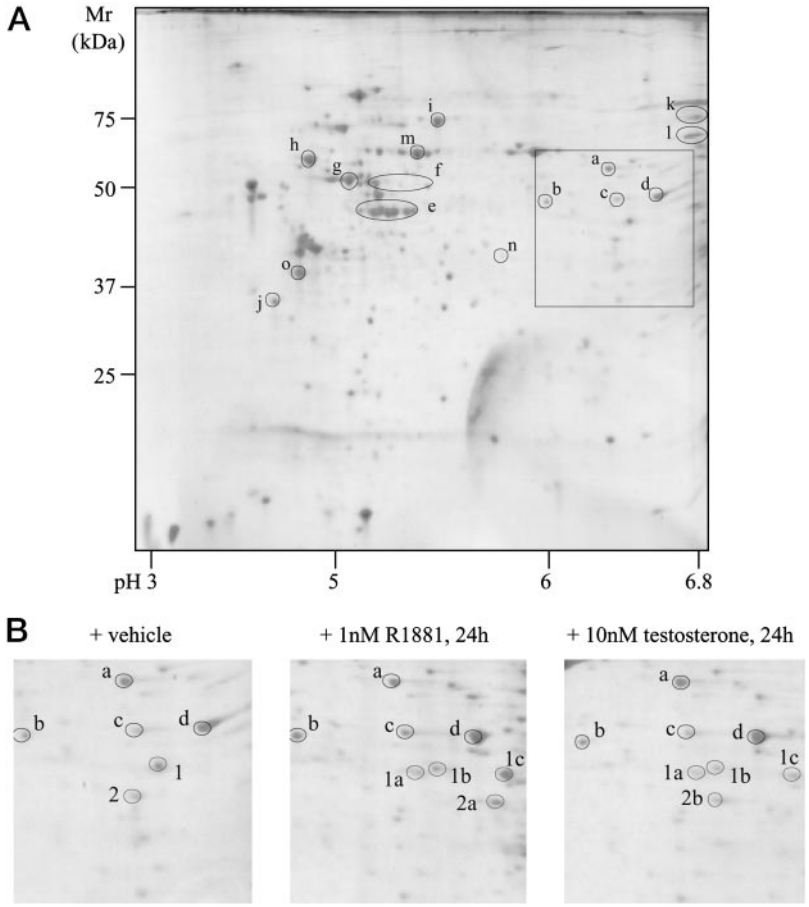

FIG. 4. Colloidal Coomassie-stained 2DE gel of MDVD A6 cell lysate. A, Part of a 2DE gel from cells cultured in the presence of vehicle (0.1% ethanol) for 24 h. Proteins identified by MALDI-TOF-MS are *encircled* and *labeled a–o*. *Boxed* is a region with differential protein expression. B, The *boxed* region from panel A is shown in more detail. The same region is shown in detail for 2DE gels from cells cultured in the presence of 1 nM R1881 or 10 nM testosterone. Differentially expressed proteins are numbered as 1 and 2, for cell incubations without ligand (vehicle). In the gels representing the cells incubated with either R1881 or testosterone, the differentially expressed proteins are *numbered as 1a, 1b, 1c, and 2a or 2b*, respectively.

TABLE 1. Peptide mass fingerprinting by MALDI-TOF-MS

Spot[a]	Protein[b]	MM (kDa)	tpI	epI	Accession no.
a	Chaperonin containing TCP-1β-chain[c]	57	6.0	6.3	BAA81874
b	Ornithine amino transferase precursor	47	6.1	6.1	AAH08118
c, d	α-Enolase	47	6.4	6.4–6.6	P17182
e	β-Actin	41	5.3	5.1–5.4	P02570
f	Vimentin	54	5.1	5.2–5.5	P20152
g	ATP synthase β-chain	56	5.0	5.1	P56480
h	Protein disulfide isomerase precursor	57	4.8	4.8	P09103
i	Mitochondrial stress-70 protein precursor	73	5.5	5.5	P38647
j	Tropomyosin fibroblast isoform 1	33	4.6	4.6	P46901
k	Laminin A	74	6.5	6.7–6.8	P48678
l	Laminin C	64	6.4	6.7–6.8	P11516
m	Mitochondrial matrix precursor protein P1	60	5.4	5.4	P19226
n	γ-Aminobutyric acid A receptor, α-6	32.9	5.7	5.7	AJ222970
o	Proliferating cell nuclear antigen	28.8	4.7	4.7	P17918
1, 1a, 1b, 1c	CArG-binding factor-A[d]	30.8	7.7	6.4–6.8	JQ0448
2, 2a, 2b	mElfin	35.7	6.4	6.4–6.6	AAH04809

[a] Index according to Fig. 4.
[b] Proteins identified with a significance of $P < 0.05$ (MASCOT software, Matrix Science).
[c] TCP, t-complex polypeptide.
[d] CArG box = 10 bp CC (A + T rich)$_6$ GG sequence.
MM, Molecular mass; tpI, theoretical pI as calculated from the SWISS-PROT ExPASy database; epI, experimental pI measured from the gels. Accession no. according to National Center for Biotechnology Information Blast.

A6 cell line emphasizes the common mesenchymal characteristics.

The presence of both CBF-A and mElfin under different culture conditions but at changing positions in the gels strongly suggests that these proteins undergo posttranslational modification upon androgen treatment. Comparison of the peptide mass fingerprint spectra of CBF-A isoforms 1, 1a, 1b, and 1c revealed some differential peptide peaks. In isoform 1, 1a, and 1b, a peptide with a mass-to-charge ratio (m/z) of 1939.71 was observed that was not present in isoform 1c. On the other hand, isoform 1c showed a peptide with a m/z of 1539.79 that could not be observed in isoform

1, 1a, or 1b. According to the MSDB database search, which is based on the peptide masses only and not on the precise sequence, both these peptide masses match with CBF-A amino acid residues 259–274, assuming that the 1939.71 peptide has an extra mass that corresponds with 5 phosphate residues (Table 2). Similarly, a putative phosphopeptide was found with a m/z of 1189.58, corresponding to amino acid residues 90–97 with two phosphorylated residues. For mElfin, a putative phosphopeptide with m/z 1159.75 was found only in isoform 2. Other predicted phosphopeptides that were found in mElfin are listed in Table 2, together with the putative phosphorylation sites.

Discussion

The present study addresses the development and characterization of conditionally immortalized clonal cell lines from mouse fetal vas deferens. Immortalized cell lines from mouse urogenital ridge (20) and prepubertal mouse vas deferens epithelium (21, 22) have been reported previously. Other studies were performed using primary epithelial cells from adult (23) and primary mesenchymal cells from fetal reproductive tract (24). However, so far no clonal cell line was described originating from fetal vas deferens mesenchymal cells. The MFVD A6 cell line is homogeneous because of its clonal background, it is of stromal origin, and expresses a functional androgen receptor. Therefore, these cells provide a cellular background that is suitable to study androgen regulation of gene and protein expression in relation to fetal genital tract development.

In addition to cDNA microarrays, proteomics is becoming a widely used approach to study cellular mechanisms. The study of proteins and posttranslational modifications adds to what can be learned from genomic studies. Androgenic effects on gene and protein expression have been studied for prostate cancer (25–27). However, the work presented herein is the first proteomics study on the effects of androgens in relation to genital tract development. In the MFVD A6 cell line, we have identified CBF-A and mElfin as two proteins that are regulated by androgens at the posttranslational level. It is evident that a genomics approach would not have provided information about such a functional change in protein expression.

In the differential 2DE analysis of androgen-treated MFVD A6 cells *vs.* untreated cells, we primarily focused on qualitative differences. It was found that both CBF-A and mElfin were present in the stimulated and nonstimulated cells but as different protein isoforms. Comparing the peptide mass fingerprint spectra of the different protein isoforms resulted in identification of peptide masses that were present in either one or the other protein isoform. Peptide phosphorylation could account for these differences in mass, and possible phosphopeptides were identified using the MSDB database. In the case of the m/z 1939.71 peptide, the predicted presence of 5 phosphorylated amino acid residues is remarkable. It can, however, not be excluded that some other kind of modification, *e.g.* glycosylation, could result in a peptide of the same mass as the putative phosphopeptide.

The MALDI-TOF-MS analysis of 2DE gels shows that posttranslational modification of CBF-A and mElfin occurs upon androgen treatment and that peptide mass fingerprints can provide clues as to what kind of modification is taking place. Thus far, no androgenic regulation of CBF-A and mElfin at the posttranslational level has been described.

CBF-A belongs to a subfamily of highly homologous A/B-type heterogeneous nuclear ribonucleoproteins and functions in both transcriptional and posttranscriptional processes of gene regulation (28). CBF-A was originally described as a ubiquitously expressed protein, which binds to CArG-box motifs and to single-stranded DNA, and functions as a transcriptional repressor (29). In a different context, the protein was found to interact with the SP6κ promoter as a coactivator of transcription (30). In addition, CBF-A activates transcription of the Ha-ras promoter in mammary cells and appears to bind the Ha-ras element 1 with higher affinity than the CArG box (31). Furthermore, it was postulated that CBF-A is subject to posttranslational modification (30), and in a recent study it was found that multiple modified isoforms of CBF-A associate with actin and are involved in nuclear-cytoplasmic shuttling of mRNA (32). In MFVD A6 cells, posttranslational modification of CBF-A might influence the expression of specific androgen target genes.

mElfin is a family member of the enigma proteins that possess a PDZ domain at the amino terminal and a LIM domain at the carboxyl terminal, which is expressed in a variety of tissues (33). Expression of mElfin is found as early as E8 in the developing heart of mouse embryos (34). LIM domains are defined by cysteine-rich sequences ($CX_2CX_{17-19}HX_2CX_2CX_2CX_{16-20}CX_2C/D/H$) that form two zinc fingers, and are involved in protein-protein interactions (reviewed in Ref. 35). LIM domains are found in a variety of proteins with different cellular functions, especially in many key regulators of developmental pathways (35). mElfin was found to associate to the F-actin-rich cytoskeleton by interacting with α-actinin via its PDZ domain, which in turn binds to F-actin (33, 36, 37). It was suggested that mElfin functions as an adapter protein by recruiting signaling kinases to the cytoskeleton via its protein-protein interaction motifs (34, 36). A functional relationship between mElfin and the AR pathway can be similar to what has been observed for FHL2, a four-and-a-half LIM domain protein that was found to be

TABLE 2. Proposed phosphopeptides according to differential MALDI-TOF-MS

Protein	Modified peptide m/z	Unmodified peptide m/z	Sequence	Residues	Candidate phosphorylation sites
CBF-A	1939.71	1539.70	GSGGGQGSTNYGKSQR	259–274	S260, S266, T267, Y269, S272
	1189.58	1029.52	DLKDYFTK	90–97	Y94, T96
mElfin	1159.75	1079.73	CGTGIVGVFVK	261–271	T263
	1805.94	1565.74	IKGCADNMTLTVSR	70–83	T78, T80, S82
	2116.89	1796.82	GDADNMTLTVSRSEQK	72–87	T87, T80, S82, S84

expressed preferentially in the heart (38). FHL2 is also expressed in epithelial cells of the prostate, where it overlaps with nuclear AR expression and functions as an AR-specific, ligand-dependent coactivator of transcription (39).

Another functional link to AR signaling may involve the binding of CBF-A and mElfin to F-actin. It was described that several proteins initially characterized as actin-binding proteins or complexes were found to coactivate transcriptional regulators, including AR. The F-actin binding protein filamin has been found to interact with the AR and is involved in nuclear translocation of the AR (40). Likewise, β-catenin, which plays a pivotal role in cell-cell adhesion by linking cadherins to α-catenin and the actin cytoskeleton, and which is a downstream effector of the Wnt signaling pathway, was found to enhance androgen dependent transcriptional activity of the AR (41). It is tempting to postulate that posttranslational modification of mElfin and CBF-A in the presence of androgens may link a change in cytoskeletal architecture to androgen-regulated gene transcription.

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