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Androgens down-regulate myosin light chain kinase in human prostate cancer cells

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ABSTRACT

Androgens play a major role in the growth and survival of primary prostate tumors. The molecular mechanisms involved in prostate cancer progression are not fully understood but genes that are regulated by androgens clearly influence this process. We searched for new androgen-regulated genes using the Affymetrix GeneChip Human Genome U95 Set in the androgen-sensitive LNCaP prostate cancer cell line. Analysis of gene expression profiles revealed that myosin light chain kinase (MLCK) mRNA levels were markedly down-regulated by the synthetic androgen R1881. The microarray data were confirmed by ribonuclease protection assays. RNA and protein analyses revealed that LNCaP cells express both long (non-muscle) and short (smooth muscle) isoforms, and that both isoforms are down-regulated by androgens. Taken together, these data identify MLCK as a novel downstream target of the androgen signalling pathway in prostate cells.

1. Introduction

The prostate gland serves an essential supporting role in male reproduction and is the most frequent site of cancerous tumor formation in males. Androgens play an important role in prostate gland development, physiology and tumor proliferation. The effects of androgens are mediated by the androgen receptor (AR), a ligand-activated transcription factor of the nuclear receptor superfamily [13]. Androgens bind to the ligand-binding domain of the AR and cause a conformational change that enables the AR to dimerize, bind to the response elements of its target genes, and recruit the coactivators required to activate gene expression.

The biological effects of androgens ultimately result from the actions of the gene products that are regulated by the AR. Consequently, it is of considerable interest to identify androgen-regulated genes in prostate cells. Over the years, a number of these genes have been identified using genome-wide as well as subtractive hybridization or enrichment approaches. Most of these studies were performed in androgen-responsive LNCaP human prostate cancer cells. Our own group has identified four novel androgen-regulated genes and/or transcript variants [17–20]. Collectively these discoveries have revealed that androgens regulate a variety of cellular functions. Interestingly, most of the androgen-regulated genes that have been characterized are up-regulated by androgens whereas relatively few are down-regulated. Whether this truly reflects the biological effects of androgens or is the result of experimental bias is unclear.

Here we report the identification of a new androgen-repressed gene in LNCaP cells. Myosin light chain kinase (MLCK) is a calcium/calmodulin-dependent kinase that is implicated in the regulation of smooth muscle contraction [3,7]. MLCK is also known to be expressed in non-muscle cells but its expression and role in prostate cells have not been characterized. Microarray profiling experiments performed in LNCaP cells revealed that MLCK mRNA is expressed in these cells and that MLCK mRNA levels are down-regulated by androgens. These observations were confirmed by ribonuclease protection assays and immunoblotting. Additional studies were conducted to determine precisely which MLCK isoforms are expressed in LNCaP cells. Together these studies establish a link between the AR and a previously unrelated signalling pathway in prostate cells.

2. Materials and methods

2.1. Cell lines

The source and culture conditions of the cell lines used in this study have been described previously [20].

2.2. Microarray experiment

To deprive cells of androgenic stimuli, LNCaP cells were grown in medium containing 0.25% (w/v) hormone-depleted fetal bovine serum for 6 days (medium was changed every 2–3 days). The

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medium was then replaced with the same medium supplemented or not with the synthetic androgen methyltrienolone (R1881, 0.1 nM). Control and R1881-treated cells were harvested 6 and 18 h later. RNA samples were prepared using TriReagent and shipped to a contract laboratory (Research Genetics) where they were processed to probes and hybridized to Affymetrix Human Genome U95 arrays.

2.3. Northern blot analysis

For mRNA distribution studies we used a Multiple Tissue Northern Blot (Clontech, Palo Alto, CA, USA) containing 2 μg poly(A)$^+$ mRNA from human spleen, thymus, prostate, testis, uterus, small intestine, colon and peripheral blood leukocytes. A 781-bp fragment corresponding to nucleotides 4965–5745 (exons 30–34) of the MLCK variant 1 open reading frame (Genbank accession number NM_053025) was radiolabelled with [α-^{32}P] dCTP using the DECAprimer II DNA labelling kit (Ambion, Austin, TX, USA). The RNA blot was prehybridized 1 h at 42 °C in ExpressHyb hybridization solution (BD Bioscience) with 100 μg/ml of sonicated salmon sperm DNA. Hybridization was done at 42 °C overnight in fresh prehybridization buffer containing 2×10^6 cpm/ml of radiolabelled probe. After hybridization, the blot was washed twice with low stringency buffer (2× SSC) at room temperature and once with high stringency buffer (0.2× SSC containing 2% SDS) at 55 °C for 30 min. The membrane was then exposed to Hyperfilm MP (Amersham Biosciences, Piscataway, NJ, USA). The same blot was hybridized with GLTSCR2 (positive control probe) following the same procedure.

2.4. Ribonuclease protection assay

The probes specific for MLCK exons 10, 11, 30 and 34 correspond to nucleotides 1095–1309, 1310–1516, 4965–5170 and 5554–5745, respectively, of the MLCK variant 1 open reading frame. The corresponding pBluescript plasmids were linearized and probes were synthesized with T3 or T7 RNA polymerase and labelled with [α-^{32}P] UTP using the Riboprobe in vitro Transcription System (Promega Corporation, Madison, WI, USA). Assays were performed with 10 μg total RNA according to the RPA III instruction manual. Samples were analyzed on 6% acrylamide-7 M urea gels and exposed to Hyperfilm HP with intensifying screens at −80 °C.

2.5. Immunoblotting

For immunoblotting experiments, control and androgen-treated LNCaP cells were lysed by sonication in buffer (50 mM Tris–HCl, pH 6.8, 6 M urea, 2% SDS) and protein extracts were quantitated using the DC Protein Assay (Bio-Rad Laboratories, Hercules, CA, USA). Protein samples were resolved on 12% SDS-polyacrylamide gels and transferred to nitrocellulose membranes. MLCK was detected using the K36 mouse monoclonal anti-MLCK antibody (Sigma–Aldrich, St. Louis, MO, USA). The androgen-regulated protein AIbZIP was detected using antibody 150 as described [17] whereas GLTSCR2, which served as a loading control, was detected using the 5A8 mouse monoclonal antibody (Novus Biologicals, Inc., Littleton, CO, USA).

3. Results

3.1. Androgens down-regulate MLCK in prostate cells

To screen for novel androgen-regulated transcripts in prostate cells, cultures of LNCaP cells were deprived of androgens for 6 days and then cultured in medium containing the synthetic androgen R1881 (0.1 nM) or its vehicle for 6 or 18 h. RNA samples from control and R1881-treated cells were hybridized to Affymetrix microarrays. As expected, analysis of the microarray data confirmed that R1881 up-regulated known androgen-regulated genes such as NKX3.1 and PSA in LNCaP cells [8,14]. Interestingly, we also noted that the amount of MLCK mRNA decreased by 80% at 18 h. Given the novelty of this observation, we endeavoured to characterize the regulation of MLCK expression by androgens in greater detail.

The MLCK gene consists of 34 exons that span 272 kb on chromosome 3. Several transcript variants have been described and these fall into three broad categories. The long isoforms, also known as the non-muscle isoforms, are encoded by exons 4–34 and some of these lack exons 11 and/or 30. The short isoforms, also known as the smooth muscle isoforms, are encoded by exons 17–34. Finally, the telokin/KRP isoforms, which lack the catalytic domain of MLCK, are encoded by exons 32–34 (Fig. 1).

The Affymetrix probe set for MLCK on the U95 array (32847_at) spans exons 31–34 of the MLCK gene. Consequently, as many as nine different MLCK transcripts could potentially be regulated by androgen. To determine which MLCK variants are expressed in LNCaP cells and which of these are androgen-regulated, we designed four exon-specific cRNA probes. The exon 34 probe was designed to detect all known MLCK variants whereas the exon 10 probe detects only the long isoforms. The exons 11 and 30 probes detect subsets of the long isoforms. LNCaP cells were exposed to increasing concentrations (0.001–10 nM) of R1881 for 24 h and RNA samples were analyzed by ribonuclease protection assay.

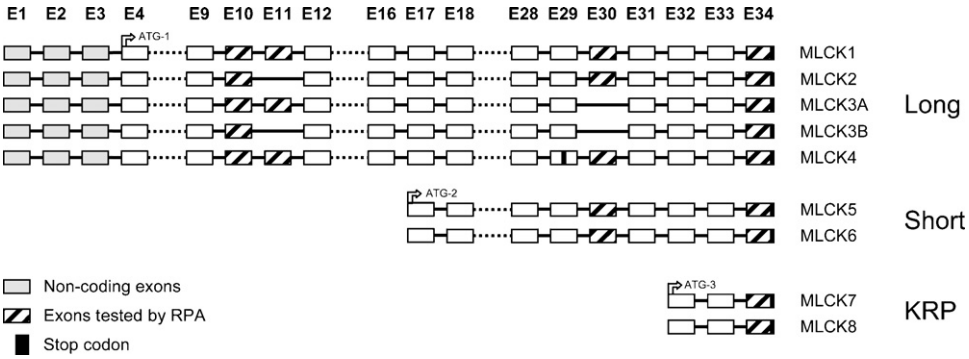

Fig. 1. MLCK isoforms. The domain structure of the longest MLCK isoform (MLCK1) is shown at the top. The exon composition of the different MLCK mRNA variants is shown below. The 34 exons are depicted as boxes (not to scale) where black boxes correspond to the non-coding regions, white boxes correspond to the open reading frame, and hatched boxes (exons 10, 11, 30 and 34) identify the exons that were detected using exon-specific cRNA probes. The three translation initiation sites in exons 4 (ATG-1), 17 (ATG-2) and 32 (ATG-3) are represented as well as the usual (exon 34) and premature (exon 29) stop codons. MLCK 2, 3a, 3b and 4 are splice variants of MLCK1 and correspond to long MLCK. Isoforms 5 and 6 encode the short MLCK isoform and only differ from the AAG codon deletion in exon 33. The C-terminal part of MLCK, also referred to as telokin or kinase-related protein (KRP), is encoded by variants 7 and 8.

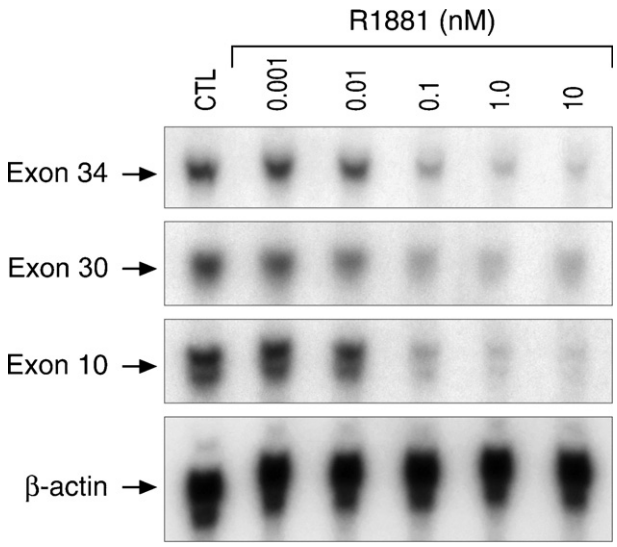

Fig. 2. Down-regulation of MLCK transcripts by androgens in LNCaP cells. Following 6 days in hormone-depleted culture medium, LNCaP cells were incubated with the indicated concentrations of R1881 for 24 h. Ribonuclease protection assays were performed using 10 μg of total RNA and the exons 10, 30 and 34 MLCK cRNA probes. A β-actin probe served as a loading control. Similar results were observed in two other independent experiments.

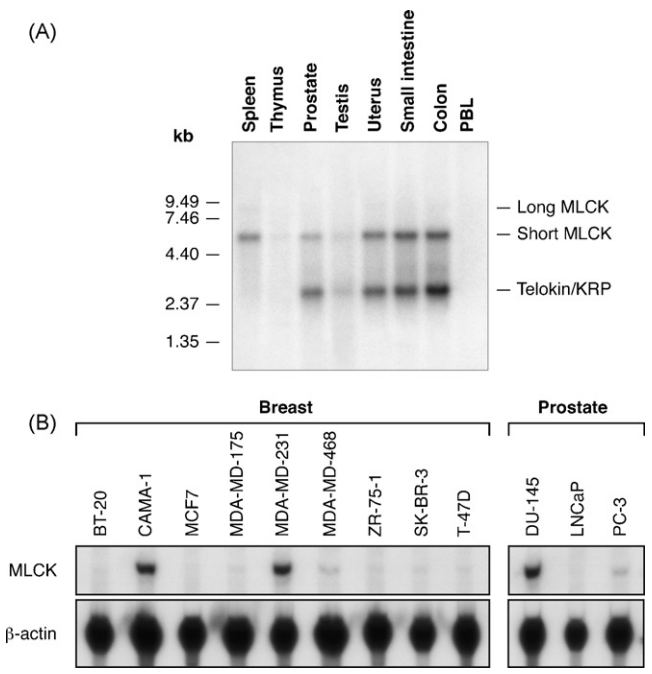

Fig. 3. Expression profile of MLCK mRNA in human tissues and various cancer cell lines. (A) Tissue-specific distribution of MLCK expression was performed using CLONTECH™ human multiple tissue northern blots (2 μg polyadenylated RNA/lane). The positions of the long MLCK (8.1 kb), short MLCK (5.8 kb) and Telokin/KRP (2.4 kb) transcripts are indicated. (B) MLCK mRNA expression in breast and prostate cancer cell lines was determined by ribonuclease protection assays using the exon 34 cRNA probe 1. β-actin was used as a loading control.

As shown in Fig. 2, the exons 10, 30 and 34 cRNA probes detected MLCK transcripts in control (untreated) LNCaP cells. Addition of R1881 caused a dose-dependent decrease in the amount of MLCK mRNA detected by each probe. A maximal decrease in MLCK mRNA levels was achieved with 0.1 nM R1881. On the other hand, the exon 11 probe did not detect any transcript (data not shown), which indicates that MLCK isoforms 1, 3A and 4 are not expressed in LNCaP cells. Taken together, these results indicate that the long MLCK isoforms 2 and/or 3B are expressed in LNCaP cells. However, these experiments do not exclude the possibility that short MLCK variants are also expressed and androgen-regulated in LNCaP cells.

3.2. *MLCK expression in normal prostate and cancer cell lines*

The long (non-muscle), short (smooth muscle) and telokin mRNAs produced by the MLCK gene are easily distinguishable by their size (8.1, 5.8 and 2.6 kb, respectively) [12]. Lazar and Garcia demonstrated that long MLCK is expressed in human heart, brain, placenta, lung, liver, kidney and pancreas but not in skeletal muscle. They also pointed out that the MLCK2 isoform is the predominant transcript expressed in these tissues [12]. To extend these observations to the human prostate and other tissues that had not been previously examined, we probed a multiple tissue Northern blot with a probe mapping to the 3′ end of the MLCK1 open reading frame (exons 30–34). As shown in Fig. 3A, a 5.8 kb-mRNA corresponding to short MLCK variants was highly abundant in spleen, prostate, uterus, small intestine and colon, weakly expressed in thymus and testis, and undetected in peripheral blood leukocytes. A shorter 2.4 kb transcript corresponding to telokin/KRP transcripts was also detected in several tissues such as prostate, testis, uterus, small intestine and colon but not in spleen, thymus and peripheral blood leukocytes. Although long MLCK transcripts were detected in LNCaP cells by ribonuclease protection assay, the corresponding 8.1-kb mRNA was not visible in normal prostate. This is most likely due to the lesser sensitivity of the Northern blot assay but it could also reflect a difference between normal and cancerous prostate.

To gain additional information on the expression pattern of MLCK in tumors derived from hormone-sensitive tissues, we analyzed MLCK mRNA levels in a panel of human breast and prostate cancer cell lines (Fig. 3B). The cells used for this experiment were grown under standard culture conditions and MLCK mRNA was detected by ribonuclease protection assay using the exon 34 probe. High levels of MLCK mRNA were detected in the DU 145 prostate cancer cell line as well as in CAMA-1 and MDA-MB-231 cells. MLCK was not detected in LNCaP cells, an observation that is consistent with the fact that the cells used for this experiment were grown in standard androgen-containing medium. It is interesting to note that MLCK expression did not correlate with AR expression. Indeed, very low levels of MLCK were detected in AR-null PC-3 prostate cells whereas high levels were observed in AR-expressing CAMA-1 cells as well as in AR-null MDA-MB-231 cells. This suggests that factors besides the AR play an important role in the regulation of MLCK expression. Based on our analysis, it appears that MLCK is expressed in a higher percentage of prostate cell lines than in breast cancer cells.

3.3. *Regulation of MLCK mRNA by androgen and antiandrogens in LNCaP cells*

To further characterize the regulation of MLCK by androgens we evaluated the kinetics of MLCK down-regulation. As mentioned previously, microarrays detected an 80% decrease in MLCK mRNA levels after an 18 h exposure to R1881. LNCaP cells were deprived of androgens and then treated with R1881 (0.1 nM) or vehicle for 1 to 72 h. Total RNA samples were analyzed by ribonuclease protection assay using the exon 34 probe and MLCK mRNA levels were normalized using β-actin. As shown in Fig. 4A, MLCK mRNA levels remained constant in untreated (control) cells. On the other hand, R1881 caused a progressive decrease in MLCK mRNA levels which decreased by 70% and 90%, respectively, at 24 and 48 h. Based on these results and those shown in Fig. 2, we conclude that androgen regulation of MLCK expression is dose- and time-dependent in LNCaP cells.

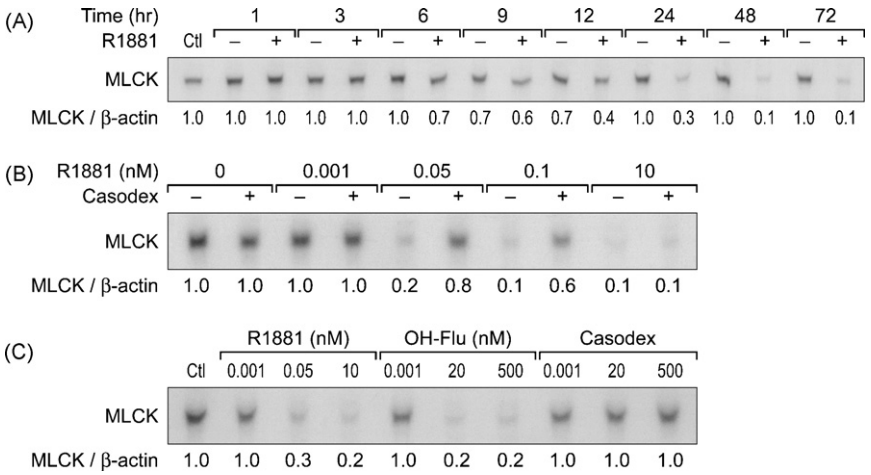

Fig. 4. Characterization of MLCK regulation by androgens. (A) Time course of MLCK down-regulation by R1881 in LNCaP cells. LNCaP cells were cultured for 6 days in hormone-depleted medium and then treated with R1881 (0.1 nM) for 1–72 h. In all three panels, RNA was isolated from control and treated cultures and analyzed by ribonuclease protection assay using cRNA probes for MLCK exon 34 and β-actin (not shown). MLCK mRNA levels are expressed as MLCK/β-actin ratios (the ratio in control cells was assigned a value of 1.0). (B) Effect of increasing doses of R1881 on MLCK mRNA levels in LNCaP cells. LNCaP cells were cultured for 10 days in medium containing R1881 alone (0.001–10 nM) or in combination with the antiandrogen casodex (3 μM). (C) The antiandrogen flutamide down-regulates MLCK in LNCaP cells. LNCaP cells were cultured for 10 days with the indicated concentrations of R1881, hydroxyflutamide (OH-FLU) or casodex and analyzed as described.

To verify that MLCK down-regulation results from AR activation, we evaluated the effect of the non-steroidal antiandrogen casodex on R1881-induced MLCK down-regulation. Following 3 days in steroid-depleted medium, LNCaP cells were cultured for 10 days in the presence of increasing doses of R1881 (0.001–10 nM) alone or in combination with casodex (3 μM). RNA samples were analyzed by ribonuclease protection assay using the exon 34 probe. As illustrated in Fig. 4B, the higher concentrations of R1881 (0.05–10 nM) down-regulated MLCK mRNA. Casodex partially blocked the effect of 0.05 and 0.1 nM R1881 but had no effect on 10 nM R1881. Together, these observations confirm that androgen-induced down-regulation of MLCK proceeds through the AR.

The AR expressed in LNCaP cells contains a threonine to alanine substitution at codon 877 in its ligand-binding domain which alters the receptor's ligand binding specificity [6]. In addition to being activated by androgens, the mutant AR is also activated by other steroid hormones and by antiandrogens such as hydroxyflutamide. To determine if the conformation of the hydroxyflutamide-bound T877A AR is able to repress MLCK expression, LNCaP cells were first cultured for 3 days in steroid-depleted serum following which they were grown in medium containing R1881 (0.001–10 nM), hydroxyflutamide (0.001–500 nM) or casodex (0.001–500 nM) for 10 days. Total RNA samples were analyzed by ribonuclease protection assay using the exon 34 probe. As expected, MLCK expression was down-regulated in the presence of R1881 and unaffected by increasing doses of casodex (Fig. 4C). As observed with R1881, the antiandrogen hydroxyflutamide also down-regulated MLCK mRNA levels, indicating that the mutated androgen receptor bound to hydroxyflutamide is able to repress MLCK expression.

3.4. Androgens down-regulate MLCK protein levels

Having confirmed that androgens down-regulate MLCK mRNA in LNCaP cells, we then sought to determine if MLCK protein levels were similarly affected. LNCaP cells were cultured for 6 days in steroid-depleted medium and then for 72 h with increasing doses (0.001–10 nM) of R1881. The long (non-muscle), short (smooth muscle) and telokin isoforms have apparent molecular weights of 210, 150 and 17 kDa, respectively [15,22]. The most abundant MLCK polypeptide detected in steroid-depleted cells had an apparent molecular weight of 150 kDa, consistent with that of short MLCK (Fig. 5A). The long MLCK isoforms were also detected albeit at much lower levels. R1881 caused a dose-dependent decrease in the abundance of MLCK proteins. The long MLCK isoforms were narrowly detected at the dose of 0.1 nM R1881 whereas a marked decrease in short MLCK was observed at the 10 nM concentration. In the same experiment, R1881 caused a dose-dependent increase in the levels of the androgen-induced protein AIbZIP.

To further confirm that androgens down-regulate MLCK protein, we compared MLCK protein levels in LNCaP cells cultured in

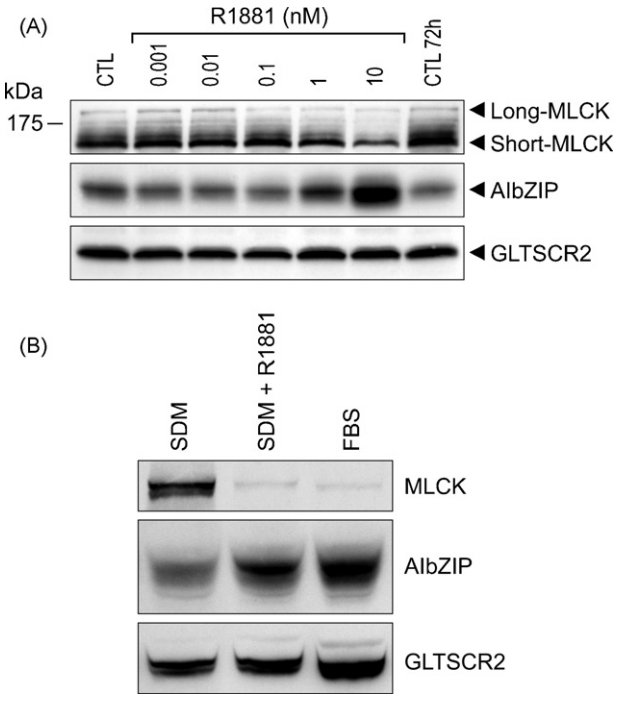

Fig. 5. Androgen regulates MLCK protein levels in LNCaP cells. (A) LNCaP cells were cultured for 6 days in hormone-depleted medium and then treated with R1881 (0.001–10 nM) for 72 h. (B) LNCaP cells were cultured in steroid-depleted medium (FBSA), FBSA containing R1881 for 4 days, or standard medium (FBS). Immunoblotting was performed on whole cell extracts and MLCK protein (150 kDa) was detected using the K36 mouse monoclonal MLCK antibody. The androgen-regulated AIbZIP protein served as an indicator of AR activation whereas the nucleolar protein GLTSCR2 served as the loading control.

standard medium, steroid-depleted medium and steroid-depleted medium supplemented with R1881. Briefly, cells were incubated 6 days in 0.25% hormone-depleted fetal bovine serum and then 4 days with or without R1881 (10 nM). The short MLCK isoforms were detected only in steroid-depleted LNCaP cells but not in R1881-treated cells or in cells cultured in standard medium (Fig. 5B). Again, MLCK and AIbZIP protein levels were regulated in an opposite manner.

4. Discussion

The results presented here indicate that MLCK is a novel target of androgen action in prostate cells. Ribonuclease protection assays and immunoblotting experiments revealed that both the long (non-muscle) and short (smooth muscle) isoforms of MLCK are expressed in LNCaP cells and that both are down-regulated by androgens.

The mammalian myosin light chain kinases are encoded by two distinct genes. One of these genes (MYLK2) is localized to chromosome 20q13.31 and is exclusively responsible for the expression of skeletal muscle MLCK. The other gene (MYLK1) is located on chromosome 3q21 and is responsible for the expression of long MLCK (also known as non-muscle-MLCK; 214 kDa), short MLCK (alternatively named smooth muscle-MLCK; 150 kDa) and telokin/KRP (C-terminal part of MLCK) [16]. Both variants (long and short) share an actin binding domain, a myosin light chain binding domain, a catalytic domain and a regulatory domain which includes an inhibitory segment and a calmodulin binding domain. In addition, the long MLCK variant also carries an exclusive amino terminal extension that includes SH2/SH3-binding domains, six Ig modules and two additional actin-binding motifs [10]. Five different variants of the long MLCK isoform have been reported (isoforms 1, 2, 3a, 3b and 4). The differences between these isoforms are mainly created by alternative mRNA splicing involving exons 11 and 30 [12].

MLCK activity is intimately linked to cytosolic Ca^{2+} concentration. The elevation of intracellular Ca^{2+} levels allows the activation of MLCK through a conformational change produced by the interaction of Ca^{2+}/calmodulin. This complex alters the tridimensional organization of the protein and thus favors the transfer of phosphate from ATP to myosin regulatory light chain (MLC). The phosphorylation of MLC at Ser19 induces myosin-II filament assembly and regulates its motor activity [1]. Numerous cellular activities like motility, adhesion and secretion are mediated by the myosin-II protein. Consequently, MLCK regulation has a major incidence on the initiation of these processes. Beyond tight regulation of MLCK by Ca^{2+}, some protein kinases are also able to modulate its influence over myosin-II activation. For example, Ras/mitogen-activated protein (MAP) kinases (ERK1 and ERK2) phosphorylate MLCK and enhance its activity [11]. MLCK activity is also known to be regulated by at least one member of the family of p21-activated kinases (PAKs). These kinases are the downstream effectors of the Rho family of guanosine triphosphatases (Rho, Rac, Cdc42). The GTP-bound forms of Rho proteins interact with the regulatory domain of PAKs to induce PAK activity and/or regulate PAK localization [4]. Phosphorylation of MLCK by PAK1 inhibits MLCK activity [21]. In this regard, it is of interest to note that RAC1 and PAK4 mRNA levels increased slightly in R1881-treated LNCaP cells (data not shown). This could suggest that androgens exert a dual effect on MLCK by inhibiting MLCK expression and up-regulating the expression of MLCK inhibitors. However, this is purely speculative as these microarray data have not been validated and the effect of PAK4 on MLCK has not been demonstrated. No other genes that are functionally linked to MLCK were regulated by R1881 in LNCaP cells.

The consequences of MLCK down-regulation by androgens in prostate cells are not entirely clear. Previous studies attributed pro-survival as well as pro-apoptotic functions to MLCK. Thus, cells treated with pharmacological inhibitors of MLCK (ML-7 or ML-9) showed a decrease in MLC phosphorylation and an increase in the incidence of cell death [2,5]. Gu and co-workers also demonstrated that ML-7 action, alone or in synergistic combination with etoposide, can induce apoptosis in mouse mammary (Mm5mt) and rat prostate (Mat-Ly-Lu or MLL) cancer cell lines in vitro as well as reduce, in vivo, the growth of mammary tumours in mice and prostate tumours in rats [5]. On the other hand, overexpression of dominant negative MLC, in MDCK epithelial cells has been reported to diminish the intracellular trafficking of TNFR-1 and its apoptotic-related signalling [9]. The fact that androgens stimulate LNCaP proliferation argues that MLCK might serve a pro-apoptotic function in these cells.

In conclusion, normal and cancerous prostatic growth is known to be stimulated by androgens. Uncovering the molecular and cellular changes associated with androgen action is certainly a key step towards a better comprehension of the disease. Herein, we have shown that androgens down-regulate the expression of myosin light chain kinase in LNCaP human cancer cell lines. The consequences of this regulation have not yet been elucidated, however we hypothesis that this could prevent the putative pro-apoptotic function of the kinase.

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