Identification of Human Male Germ Cell-associated Kinase, a Kinase Transcriptionally Activated by Androgen in Prostate Cancer Cells*

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Androgen is involved in both normal development and malignant transformation of prostate cells. The signal transduction pathways associated with these processes are not well understood. Using a novel kinase display approach, we have identified a protein kinase, human male germ cell-associated kinase (hMAK), which is transcriptionally induced by the androgenic hormone 5α-dihydrotestosterone (DHT). The kinetics of induction is rapid and dose-dependent, and the induction is not blocked by cycloheximide treatment. Real time reverse transcription-PCR studies demonstrated a 50-fold induction of hMAK by 10 nM DHT at 24 h post-stimulation. The expression levels of hMAK in prostate cancer cell lines are in general higher than those of normal prostate epithelial cells. A reverse transcription-PCR product encompassing the entire hMAK open reading frame was isolated. The results from sequencing analysis showed that the hMAK protein is 623 amino acids in length and contains a kinase catalytic domain at its N terminus, followed by a proline/glutamine-rich domain. The catalytic domain of this kinase contains sequence motifs related to both the cyclin-dependent kinase and the mitogen-activated protein kinase families. When expressed in COS1 cells, hMAK is kinase-active as demonstrated by autophosphorylation and phosphorylation of exogenous substrate and is localized in the nucleus. A 3.7-kilobase pair promoter of the hMAK locus was isolated from a human genomic DNA bacterial artificial chromosome clone and was shown to be activated by DHT. This activation can be blocked by an anti-androgen drug bicalutamide (Casodex), implicating the involvement of androgen receptor in this process. Taken together, these data suggest that hMAK is a protein kinase targeted by androgen that may participate in androgen-mediated signaling in prostate cancer cells.

Androgen and AR† play crucial roles in prostate development as well as in its malignant transformation (1, 2). Normal prostate epithelial cells depend on androgen for growth and differentiation (2, 3). Prostate cancer cells begin with androgen-dependent growth but later undergo a transition to an androgen-independent state (4, 5). Anti-androgen therapy helps control the early phase of the disease but is not curative, because of the emergence of androgen-independent tumor cells (6). Understanding the mechanisms of androgen action and the development of androgen insensitivity is key to the improvement of current therapies for prostate cancer. Interestingly, even at the androgen-independent state, a significant fraction of prostate cancer cells express AR, raising the possibility that androgen independence, at least in part, is caused by constitutive activation of AR in the absence of ligand (7). Alternatively, it is conceivable that signals downstream from androgen are constitutively activated, bypassing the need for androgen and AR. In either case, it is important to understand the fundamentals of AR-mediated signaling.

The detailed mechanism whereby AR transmits growth signals is a subject of intensive investigation. As a transcription factor, AR can participate in the direct activation or repression of key regulators involved in growth and cell cycle progression (8). It has been shown that androgen can induce the expression level of cell cycle promoting genes such as CDK2 and CDK4 and can down-regulate the cell cycle inhibitor p16, thus allowing prostate cells to enter the S phase (9). It has also been demonstrated that CDK inhibitors p21 and p27 can be modulated by androgenic hormone, and androgen independence is found to correlate with the sustained repression of these CDK inhibitors (10). A number of androgen-induced genes have been reported, including AlbZIP (11), p21 (12), neutral endopeptidase (13), NKK3.1 (14), kallikrein (15), PART-1 (16), prostate (17), protein (18), fatty acid synthase (19), and prostate-specific antigen (20). Some of these genes contain androgen response elements (AREs) in their promoter sequences and are direct transcriptional targets of AR (12, 15, 20). The relationship of these direct targets to growth is less clear, and some of them may be involved in differentiation, a physiological function of...
androgen in the development of the prostate. How activated AR mediates proliferative signals remains largely unclear. It was reported that androgen treatment results in the activation of Src and MAPK activity, as well as their associated signaling pathways (21, 22). The search for the direct target genes of AR, which may participate in these kinase signal cascades, should advance our understanding of how androgen induces cell growth. It will also help to uncover potential targets for the therapeutic intervention of prostate cancer during the transition from the androgen-dependent state to the androgen-independent state.

With this in mind, we began to look for protein kinases that are direct transcriptional targets of AR. We took advantage of a kinase display approach previously developed in our lab, which allows the profiling of tyrosine kinases and serine/threonine kinases by a single RT-PCR reaction (23). When this approach was applied to the study of prostate cancer cells, we uncovered a novel human serine/threonine kinase, hMAK (23). This kinase shares significant homology with rat MAK, a rat serine/threonine kinase originally discovered as a male germ cell-associated kinase (24). One intriguing feature of rat MAK is its highly restricted expression pattern in testis and at specific stages of spermatogenesis. Together with its closely related kinases MRK (MAK-related kinase) and MAPK/MAK/MRK-overlapping kinase (MOK), MAK forms a new subfamily of serine/threonine kinases with closest homology to CDK and MAPK family of kinases (25). Other than the structural and expression properties of these kinases, very little is known about their roles in cellular processes.

In this study, we reported the cloning of the hMAK gene in its entirety. Structurally, hMAK carries a kinase domain at its N terminus, followed by a proline/glutamine-rich domain. It also contains a canonical sequence for nuclear transport or nuclear localization signal (NLS). Significantly, we have shown by kinase display and real time RT-PCR approaches that hMAK is transcriptionally activated by treating human prostate cancer LNCaP cells with DHT. The promoter for hMAK was then isolated and shown to be transactivated by androgen-dependent prostate cancer LNCaP cells with DHT. The promoter for hMAK was initially recovered from the kinase profile study of human prostate tissue from the random cDNA library (26).Primers corresponding to the 5’- and 3’-termini of hMAK sequences, obtained by rapid amplification of cDNA ends (RACE) approach, were used to amplify the hMAK coding sequences from reverse transcribed pooled human testis RNA (CLONTECH, Palo Alto, CA) with the High Fidelity PCR System (Roche Molecular Biochemicals). PCR products containing the full-length hMAK coding region were then cloned into pCR TOPO TA Cloning kit (Invitrogen) and sequenced using an ABI cycle sequencing system.

Construction of Mammalian Expression Plasmids Containing Full-length hMAK—The full-length coding region of hMAK was subcloned into the mammalian expression vector pEF-1 (Invitrogen), which utilizes an Efi-I promoter to drive the expression of the inserted gene. A 12-amino acid HA epitope tag (MYYYPDYDVPDYAS) or an 11-amino acid T7 epitope tag (MASMTGGGQMG) was introduced in-frame at the N terminus of hMAK, and the reading frames were verified by automated DNA sequencing.

Site-directed Mutagenesis—To make a kinase-dead hMAK (hMAK-KR) expression construct, a nucleotide point mutation was introduced into the pEF-1 mammalian expression vectors containing the epitope-tagged hMAK coding region using the QuickChange kit (Stratagene, Cedar Creek, TX). This substitution mutation will result in the replacement of the invariant lysine located in the ATP-binding pocket of hMAK kinase domain with an arginine residue and make hMAK catalytically inactive (dead). The primer sequences used in making the hMAK-KR mutant are as follows: sense, 5′-GTGGCCATCAGAAGGATGAAGAGAAAGT-3′; antisense, 5′-CTCCTCATCTTCGTAGTGCCAC-CAGCTCGCCGATTTC-3′. The mutation site was underlined. The resultant point mutation was verified by automated DNA sequencing.

Growth of prostate cancer cell lines in culture—Human prostate cell lines LNCaP-FGC (LNCaP), DU145, and PC-3 were obtained from the American Type Culture Collection (Manassas, VA). LNCaP is an androgen-dependent prostate cancer cell line expressing AR and prostate-specific antigen and was maintained in RPMI 1640 medium supplemented with 10% fetal bovine serum (FBS) from Invitrogen. DU145 and PC-3 are androgen-independent prostate cancer cell lines that do not express AR or prostate-specific antigen and were cultured in RPMI 1640 medium supplemented with 10% FBS. DU145 is from a brain metastasis, and PC-3 is from a bone metastasis.

Kinase Display Profile Study—Total RNA samples were isolated from human prostate cell lines by Trizol reagent (Invitrogen). These samples were used to study kinase expression by kinase display approach developed earlier in our lab (23). Following reverse transcription using kinase family RT primers, PCR amplification was performed using the highly redundant degenerate primers recognizing the invariable motifs of subdomains VII–XI of kinase catalytic domains. As a modification of this approach, the 5′-ends of the sense primers were doubly labeled with biotin-11dUTP (Amersham) by polydeoxy nucleotide kinase (Roche Molecular Biochemicals) (27–29). PCR amplicons were isolated by agarose gel electrophoresis, quantitated for radioactivity, and then divided into aliquots of equal counts. Aliquots of the radioactive RT-PCR products were digested with different restriction endonucleases and separated on a DNA sequencing gel. The resolved 5′-end-labeled restriction fragments were analyzed by comparison with our kinase restriction fragment data base and quantitated using the Molecular Imager FX phosphor image detection system (Bio-Rad).

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**EXPERIMENTAL PROCEDURES**

**Reagents**—DHT (Sigma) was dissolved in highly refined ethanol (Gold Shield Chemical Co., Hayward, CA) at 1 mM (10−3 M) concentration as stock solution. Serial 10-fold dilutions were made from this stock solution to prepare DHT solutions of different concentrations, which were diluted 1:1000 to make the final concentrations of DHT needed to be used for the differential induction results, and the resultant point mutation was verified by automated DNA sequencing.

**Human Prostate Cell Cultures**—Primary cultures of normal human prostate epithelial cells (PrEC) were purchased from Clonetics (Walkersville, MD) and cultured in serum-free prostate epithelial cell growth medium following the vendor’s directions. The prostate carcinoma cell line LNCaP-FGC (LNCaP), DU145, and PC-3 were obtained from the American Type Culture Collection (Manassas, VA). LNCaP is an androgen-dependent prostate cancer cell line expressing AR and prostate-specific antigen and was maintained in RPMI 1640 medium supplemented with 10% fetal bovine serum (FBS) from Invitrogen. 22Rv1 is an AR-positive prostate cancer cell line derived from a relapsed xenograft, kindly provided by Dr. Thomas G. Pretlow and Dr. James W. Jacobberger (26), and was cultured in RPMI 1640 medium supplemented with 10% FBS. Although its growth no longer requires androgen, the 22Rv1 cell line is still androgen-responsive. DU145 and PC-3 are androgen-independent prostate cancer cell lines that do not respond to androgen.
samples were isolated. Reverse transcription reactions were carried out using oligo(dT)12-18 primers, and the cDNA was amplified separately by PCR either for hMAK or for β-actin as described above. Forty cycles were programmed for real-time quantitative RT-PCR to analyze these samples on an iCycler iQ Real-Time PCR Detection System (Bio-Rad). A high-performance SYBR Green 1 (FMC, Rockland, ME) was used for double strand DNA detection. The iCycler iQ system allows kinetic measurements of a PCR process and reports data in real time.

Screening Human Genomic DNA BAC Library for Clones Containing hMAK Gene—Easy-to-Screen™ DNA pools (Incyte Genomics, St. Louis, MO) were used to quickly identify hMAK positive clones from a human genomic DNA BAC library. The primer set used for PCR screening is unique to the 5'-untranslated region of hMAK mRNA. The primer sequences are as follows: sense, 5'-TTAGGTGGTTTCCTGCGATTCT-CTTC-3', and antisense, 5'-TACTCTTGCCCATGACGACACTC-3'. Once positive clones were identified, individual BAC clones were purchased from Incyte Genomics and then amplified.

Construction of Luciferase Reporter Driven by hMAK Promoter—Using one of the BAC clones containing the hMAK gene (described above) as a template, a 3.7-kbp DNA fragment containing the hMAK promoter was obtained by PCR amplification. Sequences of the primers used in PCR amplification are: sense, 5'-TTAACGTTGAGGTGGG-GTAGTG-3', and antisense, 5'-TAGGATGGAATGCGCCAGG-3'. The 3.7-kbp fragment containing the hMAK promoter was cloned into the pcRII-TOPO vector as described above and was then subcloned into the firefly luciferase reporter vector pG5-Lucic-Basic (Promega, Madison, WI) between the Kpn1 and XhoI sites. This 3.7-kbp fragment of the hMAK promoter region encompasses the transcription start site. The 5'- and 3'-ends of this fragment are 3919 and 179 bp upstream of the hMAK translation initiation codon, respectively.

Transient Transfection Assay and Western Blotting Analysis—Cos1 cells were transiently transfected with the expression plasmids containing T7-tagged hMAK using FuGENE 6™ reagent (Roche Molecular Biochemicals) according to the manufacturer’s instructions. To examine the expression of hMAK by Western blotting, cell lysates containing 25 μg of total protein were analyzed by SDS-PAGE on a 10% Tris-glycine gel. For immunodetection, the separated proteins were electrophoretically transferred onto a polyvinylidene difluoride membrane (Millipore, San Jose, CA) using a semi-dry transfer apparatus (Bio-Rad) according to the manufacturer’s protocol. The membranes were blocked in 5% nonfat dry milk in Tris-buffered saline with Tween 20 (TBST) for 1 h at room temperature and incubated in mouse monoclonal antibody to T7 epitope tag (Novagen, Madison, WI) at 1 μg/ml in TBST plus 5% nonfat dry milk, followed by horseradish peroxidase-conjugated goat anti-mouse secondary antibody (Jackson Immunoresearch Laboratories, Inc., West Grove, PA) at a dilution of 1:10,000 (50 ng/ml). Detection was performed using the SuperSignal chemiluminescent system (Pierce) according to the vendor’s directions.

Dual Luciferase Assay—LNCaP cells were seeded in 24-well plates at 1 × 10^4/well and incubated at 37 °C with 5% CO2 for 24 h. The firefly luciferase and the renilla luciferase plasmid pRL-SV40 (Promega) were transiently transfected into LNCaP cells using the Lipofectin® reagent following the manufacturer’s protocol (Invitrogen). The next day, the cells were fed with fresh medium containing 1% CDS plus DHT. 1 day after hormone treatment, the cells were fed with fresh medium containing 1% CDS plus DHT and/or B1 at different concentrations. The cells were lysed with 1 ml of kinase assay buffer following the manufacturer’s protocol using an EG & G Berthold LB96V microplate luminometer (PerkinElmer-Wallac, Inc., Gaithersburg, MD). At least three independent experiments were performed in each setting.

Immunoprecipitations and in Vitro Kinase Assays—In vitro kinase assay was conducted on COS1 cells cultivated in 100-mm tissue culture dishes and transiently transfected either with empty expression vector as a control or with expression plasmid containing T7 epitope-tagged full-length wild type or KR mutant hMAK, respectively. T7-tagged hMAK proteins were immunoprecipitated from equal amounts of cell lysates by incubation with anti-T7 monoclonal antibody and capture with protein A-coupled Sepharose beads. The beads were washed three times with RIPA lysis buffer (Upstate Biotechnology, Inc., Lake Placid, NY) and then rinsed twice with kinase buffer containing 20 μM of kinase assay buffer containing 20 μM HEPES, pH 7.5, 10 mM MgCl₂, and 1 mM dithiothreitol. Kinases captured in the immune complex were incubated with 20 μl of kinase assay buffer containing 20 μM HEPES, pH 7.5, 10 mM MgCl₂, 10 mM MnCl₂, 1 mM dithiothreitol, 2 μM ATP, 10 μM ATP, 1 μM of exogenous substrates such as myelin basic protein and supplemented with 10 μCi of [γ-32P]ATP. After incubating at room temperature for 30 min, the reactions were terminated by adding 20 μl of 2× SDS-PAGE sample buffer and then loaded onto a 15% SDS-polyacrylamide gel for electrophoresis. The gel was exposed to x-ray films at ~70 °C using an intensifying screen.

RESULTS

Identification of an Androgen-inducible Kinase by a Novel Kinase Display Approach—AR is known to be a transcription factor, and its action is mediated through the activation of target genes. There is considerable evidence indicating that androgen treatment activates various kinase-signaling pathways (21, 22). We hypothesized that there exist kinases that are transcriptionally activated by AR. In an attempt to identify such kinases, we took advantage of the kinase profile approach developed earlier in our lab, based on the use of a set of degenerate primers that recognize nearly all tyrosine kinases and a subset of serine/threonine kinases (23). We have now modified this approach by radioactively labeling the 5'-end of the sense primer, followed by restriction endonuclease digestions of the radioactive RT-PCR products and subsequent gel analysis of the 5'-end-labeled fragments. Based upon the sizes of the labeled fragments, individual kinases can be “read” directly from the electropherogram. By digesting with multiple enzymes and displaying all of the radioactive labeled bands in a single gel, the expression profiles of kinases can be effectively identified (27–29). This approach, based on direct radioactive labeling, does not depend on hybridizations and is highly sensitive. It is also highly quantitative for low abundance genes such as certain protein kinases. This kinase display approach has been fruitfully used by us and by other labs to obtain the tyrosine kinase expression profiles of tumor samples, to identify overexpressed kinases, and to discover novel kinases (23, 27–33). Relying on its high sensitivity, we have applied this approach for the detection of androgen-inducible kinases. Human androgen-dependent LNCaP prostate cancer cells were chosen for this study. Total RNA samples were collected from LNCaP cells with or without DHT treatment. RT-PCR was conducted using the degenerate primers recognizing the invariable motifs of the kinase domain (the corresponding regions of hMAK recognized by the primers are shown in Fig. 2), followed by digestion with a number of restriction enzymes including Msei, BstYI, and BglII. Because only the 5'-end of the sense primer is labeled, each kinase is represented by only one band, i.e. the 5'-end-labeled digestion fragment. We were particularly interested in bands that vary in intensity after androgen treatment. As shown in Fig. 1, most of the bands do not show variation upon DHT treatment, indicating that most of the kinases are not transcriptionally modulated by androgen. One kinase was represented by a 31-bp band on the Msei digestion showing a 6.2-fold increase (based on phosphor imaging analysis) in LNCaP cells after stimulation with 10 nM DHT for 24 h compared with the basal expression level without DHT treatment. Similarly, in either BstYI or BglII digestion (these two enzymes recognize identical core sequences), a 100-bp band increased in intensity by ~6.5- and 6.4-fold, respectively, fol-
Molecular Cloning and Sequencing Analysis of hMAK

To this end, we designed primers based on the sequences immediately flanking the putative full-length RT-PCR product revealed a full-length hMAK coding region. The coding region of hMAK was obtained from reverse transcripted pooled human testis RNA and used later for expression analysis (see below). We chose testis RNA as the source for obtaining hMAK RT-PCR product because of its abundant expression in this tissue. Sequencing of the RT-PCR product revealed a full-length hMAK coding region 623 amino acids in length, which can be divided into three domains, an N-terminal 284-amino acid protein kinase catalytic domain, a central proline/glutamine-rich domain, and a C-terminal domain. Analysis of the deduced amino acid sequence of hMAK revealed that it shares about 90% and 89% homology with 84% and 83% identity to rat and mouse orthologues, respectively (Fig. 3); both were initially identified by their cross-hybridization with Ros tyrosine kinase and showed predominant expression in testis (24, 36). The N-terminal catalytic domain of hMAK is almost identical to that of the rat and mouse orthologues. A 64-amino acid stretch downstream from the kinase domain of hMAK (residues 306–369) has a high proline and glutamine content (48%) (Fig. 2) and shares high homology with the rat (73%) and mouse (78%) orthologues (Fig. 3). The C-terminal domain further downstream of the proline/glutamine-rich region of hMAK shares some sequence homology with the C-terminal non-kinase domain of the closely related human MRK at certain amino acid residue stretches but has no significant homology to any other known human protein and is much less conserved among different species.

Phylogenetic Tree and Structure of hMAK—A BLAST search against the GenBank™ protein data base was performed using the kinase catalytic domain of hMAK as the query sequence. By comparing the hMAK kinase domain with the catalytic domain sequences of related kinases obtained through BLAST search, a phylogenetic tree was generated using the neighbor joining method (Fig. 4A). The kinase domain of hMAK was found to share high homology with those of two other human serine/threonine kinases, human MRK (95% homology) (37) and human MAPK/MAK/MRK-overlapping kinase (MOK) (63% homology) (25). In addition to the rat and mouse orthologues, homologues of hMAK were also identified from other species (Fig. 4A), including CG4588 of Drosophila melanogaster (47% homology), M04C9 of Caenorhabditis elegans (53% homology), MHK of Arabidopsis thaliana (43% homology) (38), Ptk1 of Schizosaccharomyces pombe (42% homology), and a meiosis induction protein kinase IME2 of Saccharomyces cerevisiae (37% homology) (39). BLAST search results also revealed that the N-terminal kinase catalytic domain of hMAK shares significant homology with those of the CDK and MAP protein kinase family members (Fig. 4A). The kinase domain of hMAK has a TY motif (amino acids 14 and 15) in its ATP-binding pocket, similar to the conserved threonine (Thr14) and tyrosine (Tyr15) motif found in CDK1 (Fig. 4B). Phosphorylation of this site by Wee-1 kinase inactivates CDK1 and dephosphorylation by cdc25 phosphatase activates it (40). Another characteristic feature of hMAK is the presence of a TDY (amino acids 157–159) motif in its ATP-binding pocket, similar to the conserved threonine (Thr14) and tyrosine (Tyr15) motif found in CDK1 (Fig. 4B). Phosphorylation of this site by Wee-1 kinase inactivates CDK1 and dephosphorylation by cdc25 phosphatase activates it (40). Another characteristic feature of hMAK is the presence of a TDY (amino acids 157–159) motif in the activation loop of its kinase catalytic domain, which is similar to the TEF motif in ERK1, the TEP motif in JNK1, and the TGY motif in p38 (Fig. 4B). It has been shown that in the cases of ERK1, JNK1, and p38, phosphorylation of the threonine and tyrosine residues in this motif by upstream kinases such as MEK1 and MK4 results in their activation (41). The existence of such motifs in hMAK sequence suggests the potential regulatory mechanisms of its catalytic activity.

The Dose Response and Kinetics of Androgen-induced hMAK mRNA Expression—The initial discovery of hMAK as an androgen-induced kinase was based on a kinase display approach using degenerate primers. We wished to use hMAK gene-specific primers in conjunction with real time RT-PCR to independently confirm this observation. LNCaP cells were cultured
FIG. 2. Nucleotide and deduced amino acid sequences of hMAK. The composite nucleotide sequence determined from overlapping hMAK rapid amplification of cDNA ends (RACE) clones is depicted. The nucleotides and deduced amino acids are numbered on both left and right with the amino acid numbers in parenthesis. In-frame stop codons are in bold and underlined. The primer sequences used in the kinase display analysis of hMAK are marked with horizontal arrows. The asterisk indicates that the 5’-end of the sense primer was radioactively labeled. The MseI, BstYI, and BglII restriction enzyme cutting sites applied in kinase display for hMAK are labeled and marked by vertical arrows, with the enzyme recognition sequences underlined. The 12 nearly invariant amino acid residues characteristic of protein kinases are present in hMAK (in bold and underlined). The N-terminal kinase catalytic domain (Kinase Domain) and the proline/glutamine-rich domain (P/Q rich Domain) are boxed and labeled. The putative NLS is underlined.

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The amplicons of H9252 were analyzed in the ethidium bromide-stained agarose gel (Fig. 5) to determine the expected migration pattern of a 433-bp hMAK RT-PCR product. The authenticity of the amplified products was verified by the comparison of their migration patterns with those of the standard reference samples. In subsequent studies to other prostate cells harboring AR, using the same conditions that yielded quantitative results in the real-time RT-PCR experiment (Fig. 5), the cells cultured in androgen-free medium were treated with 10 nM DHT and harvested at 24 h post-stimulation. LNCaP cells served as a positive control, and the androgen inducibility of hMAK was reproduced in this experiment (Fig. 7, lanes 1 and 2). 22Rv1, an AR-positive cell line derived from a relapsed prostate cancer xenograft, does not express a basal level of hMAK even in the absence of androgen. However, it is noteworthy that both LNCaP and 22Rv1 express a basal level of hMAK even in the absence of androgen.

We also studied the induction kinetics of hMAK expression by 10 nM DHT in LNCaP cells. Total RNA was extracted from LNCaP cells continuously exposed to DHT for 6–72 h. The expression levels of hMAK mRNA were determined by RT-PCR using the same conditions as in the real-time RT-PCR described above. As shown in Fig. 6A, up-regulation of hMAK mRNA expression was dose-dependent and time-dependent. The expression levels of hMAK mRNA were reduced at 48 and 72 h after DHT treatment, which reveals the transient nature of androgen-induced hMAK expression in LNCaP cells. To address whether the induction of hMAK expression requires protein synthesis, a protein synthesis inhibitor cycloheximide was included in another experiment. As shown in Fig. 6B, the DHT-induced hMAK expression is insensitive to the cycloheximide treatment (compare lanes 1–3 to lanes 4–6), implicating the direct participation of AR or other co-factors in this induction.

In an additional experiment (Fig. 6B, lanes 7–9), DHT was added to LNCaP cells cultured in regular 10% FBS-RPMI 1640 medium, and the induction of hMAK expression was again observed, despite the presence of endogenous androgen in the medium. The results suggest that the observed hMAK induction by androgen is not peculiar to the charcoal/dextran-stripped fetal bovine serum.

**hMAK Expression in Prostate Cancer Cells and Its Inducibility by Androgen**—To determine whether the androgen inducibility of hMAK is a general phenomenon, we extended our studies to other prostate cell lines harboring AR, using the same conditions that yielded quantitative results in the real-time RT-PCR experiment (Fig. 5). The cells cultured in androgen-free medium were treated with 10 nM DHT and harvested at 24 h post-stimulation. LNCaP cells served as a positive control, and the androgen inducibility of hMAK was reproduced in this experiment (Fig. 7, lanes 1 and 2). 22Rv1, an AR-positive cell line derived from a relapsed prostate cancer xenograft, does not require androgen for growth but is responsive to androgen (26).

PrEC are normal prostate epithelial cells, which express much lower level of AR (data not shown). As shown in Fig. 7 (Fig. 7, lanes 4–9), DHT was added to LNCaP cells cultured in charcoal/dextran-stripped fetal bovine serum.

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**FIG. 4.** Comparison of the kinase domain of hMAK with the catalytic domains of related kinases. 

**A**. Phylogenetic analysis of the catalytic domain sequences of selected protein kinases related to hMAK. A phylogenetic tree was constructed by comparing the protein kinase domain sequences of hMAK with those of related kinases, including members of CDK and MAPK family, using the neighbor joining method. Branch length along the horizontal axis indicates evolutionary distance with nodes reflecting predicted gene duplication events. The sequence data were obtained from the results of a BLAST homology search using the kinase domain of hMAK as the query sequence.

**B**. Alignment of the catalytic domain sequences of hMAK, CDK1, ERK1, JNK1, and p38. The sequences were aligned by ClustalW algorithm. The identical and chemically conserved amino acid residues within the aligned kinases are indicated by dark and light shading, respectively. The conserved TY motif in the CDK kinase family members and the TXY motif in the MAP kinase family members are labeled.
cells but were significantly higher than that of the untreated PrEC. Consistent with our hypothesis, the levels of hMAK expression in these cell lines were not inducible by DHT. These results suggest that in addition to androgen, there are other factors in prostate cancer cells that govern the expression of hMAK and that the increased expression of hMAK may play a role in prostate carcinogenesis to an advanced state.

hMAK Promoter Is Responsive to Androgen—Having established that hMAK is an androgen-inducible gene and given that AR is a transcription factor, we wished to determine whether the inducibility is indeed at the transcriptional level. To explore this possibility, we proceeded to clone the hMAK promoter region. By PCR screening of human genomic DNA BAC libraries with a set of primers unique to the hMAK mRNA 5'-untranslated sequences, we have identified three BAC genomic clones containing the hMAK gene. One of the BAC clones was used to isolate a 3.7-kbp fragment of the hMAK promoter, followed by sequencing verification of the entire region. This promoter region, encompassing the transcriptional start site and containing several putative ARE (see “Discussion”), was then cloned into a reporter vector carrying the firefly luciferase gene. Reporter analysis was performed by transient transfection of LNCaP cells with the plasmid, followed by luciferase activity assay of the cell lysate. This promoter fragment was found to be responsive to androgen stimulation (Fig. 8), as demonstrated by a 9.9-fold increase in luciferase activity after 10 nM DHT treatment for 24 h.

To further test whether AR was involved in the transactivation of the hMAK promoter by androgen treatment, BI, a non-steroidal anti-androgen clinically used for prostate cancer treatment, was applied in the luciferase reporter assay during DHT treatment. The data demonstrated that BI, at either 5 or 10 μM, could block the induction of hMAK promoter activity by DHT (Fig. 8). Furthermore, by overexpressing wild type AR in LNCaP cells, this blockade of DHT inducibility could be attenuated (Fig. 8). Taken together, these data strongly suggest that

![Fig. 5. Real time RT-PCR kinetic study of hMAK expression in response to DHT treatment. A, a 433-bp fragment of hMAK cDNA was amplified from reverse transcribed RNA samples of LNCaP cells with or without DHT treatment. Relative fluorescence intensity versus cycle number is plotted for each sample to permit simple visualization of the exponential phase of PCR amplification. B, data of hMAK was normalized by comparison with the amplification of a 304-bp β-actin cDNA. Induction folds of hMAK expression levels of DHT-treated samples over untreated ones are shown. C, representative agarose gel shows the PCR products of hMAK (upper panel) and β-actin (lower panel). MW lane, molecular mass markers (100-bp ladder).](image)
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AR is involved in the transactivation of hMAK and validate our original hypothesis that hMAK is an androgen-inducible serine/threonine kinase.

Kinase Activity of hMAK—We next asked whether hMAK is a bona fide protein kinase. For the lack of a specific antibody against hMAK to detect endogenous kinase activity, we used T7-tagged hMAK expressed in COS1 cells. The T7-tagged hMAK was immunoprecipitated and tested for its intrinsic as well as exogenous kinase activities in an in vitro kinase reaction. Fig. 9A shows that hMAK is properly expressed at the expected size (≈70 kDa for T7-tagged hMAK) as demonstrated by Western immunoblotting using the anti-T7 antibody. Fig. 9B reveals that hMAK is autophosphorylated and has the ability to phosphorylate the exogenous substrate myelin basic protein. The kinase activity was derived from hMAK but not an associated kinase, as demonstrated by the inclusion of a kinase-dead hMAK control (hMAK-KR). The highly conserved lysine residue located in the ATP binding pocket of hMAK kinase domain is required for its catalytic activity. The hMAK-KR mutant carrying a substitution of this lysine with an arginine residue was negative for autophosphorylation and exhibited only a basal level of myelin basic protein phosphorylation (Fig. 9B, hMAK-KR lane). These results demonstrated that hMAK is an active kinase. However, the kinase activity was relatively weak compared with other serine/threonine kinases such as CDK and MAPK, suggesting that hMAK may be tightly regulated and requires additional stimuli for full activity.

Subcellular Localization of hMAK—To study the subcellular localization of hMAK, we constructed a HA-tagged hMAK in the pEF-1 vector. This plasmid was transiently transfected into COS1 cells, and the subcellular location of hMAK proteins was examined using anti-HA monoclonal antibody. Indirect immunofluorescence staining results showed that hMAK is mainly confined to the nucleus when expressed in COS1 cells but not in the nucleolus (Fig. 10). Upon inspection of the deduced amino acid residues of hMAK, a putative NLS was found to be present in the hMAK sequence (KEKRRKK) (Fig. 2), suggesting that hMAK might have an intrinsic nuclear localization nature. Further experiments are needed to confirm whether this motif is an authentic nuclear localization signal.

DISCUSSION

While profiling protein kinases expressed in the prostate carcinoma xenograft CWR22, we discovered several new protein kinases including the human orthologue of rat and mouse male germ cell-associated kinase (rat and mouse MAK) (23). To the best of our knowledge, this was the first time that hMAK was found to be expressed in prostate cells. In this study, we proceeded to clone the hMAK gene and identified hMAK as a novel serine/threonine kinase that is transcriptionally activated by androgen and likely to be a direct target of AR. The discovery of hMAK as an androgen-inducible kinase was aided by a novel RT-PCR based “kinase display” approach we developed previously (27–29). This approach displays the kinase amphicons in a gel, and the identities of the kinases can be directly recognized by the sizes of their characteristic 5’-end restriction fragments. As such, no sequencing and cloning are required, making this approach highly effective and quantitative. For LNCaP cells with or without 10 nM DHT treatment, phosphor imaging analysis of the kinase display autoradiogram in three independent restriction endonuclease digestions consistently yielded greater than 6-fold of induction of hMAK expression after treating with androgen, attesting to the reproducibility of this approach. In agreement with the kinase display results, real time RT-PCR using gene-specific primers revealed an ∼9-fold induction of hMAK under similar conditions of androgen treatment. The results from these independ-
ent approaches provide strong evidence that hMAK is transcriptionally activated by androgenic hormone. Additional studies revealed that this induction was dose- and time-dependent and was not blocked by a protein synthesis inhibitor cycloheximide. A reduction of activation was seen at DHT doses higher than 100 nM and after prolonged exposure. These properties parallel the growth response of LNCaP cells to DHT, suggesting a physiological role of hMAK in the androgen signaling pathway (42, 43). Importantly, the androgen inducibility of hMAK was extended to two other AR-positive prostate cells (22Rv1 and PrEC), indicating that this was not a peculiarity of LNCaP cells. To establish that the activation was at the transcriptional level (as opposed to mRNA stability level), we isolated the promoter of hMAK and showed that the luciferase reporter gene linked to this promoter was androgen-inducible. The nonsteroidal anti-androgen BI could also block the inducibility, further implicating the involvement of AR in this process.

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**Fig. 8. Luciferase reporter analysis of hMAK promoter.** Luciferase reporters were transfected into LNCaP cells to examine their response to 10 nM DHT treatment. The cells were transfected with the promoter-less reporter pGL3-Basic or the reporter containing a 3.7-kbp hMAK promoter region (p3.7-Luc). All of the samples were co-transfected with a control reporter pRL-SV40, which serves as an internal control to normalize the experimental firefly luciferase readouts. 24 h after transfection, the cells were switched to medium with or without 10 nM DHT and cultured for another 24 h. The open bars represent the data of pGL3-Basic transfected samples, and the solid bars represent the data of p3.7-Luc transfected samples. For the samples labeled with BI, anti-androgen BI was added at indicated concentration when DHT was added to the culture to examine the effects of BI on DHT induced hMAK promoter activity. For samples labeled with wild type AR (wtAR), LNCaP cells were also co-transfected with vectors expressing wild type AR in addition to the p3.7-Luc and pRL-SV40 reporters. In all of the luciferase experiments, dual luciferase assay were performed 24 h after the treatment. The results are shown in induction fold of DHT-treated versus untreated samples and are the means with standard deviations of three independent experiments.

**Fig. 9. Characterization of hMAK kinase activities.** A, detection of hMAK protein expressed in COS1 cells. The cell lysates of COS1 cells transfected with expression vectors with or without T7-tagged hMAK were immunoblotted as described under “Experiment Procedures.” B, kinase assay of hMAK. Following immunoprecipitation of the same COS1 cell lysates, the immune complex was then subjected to an in vitro kinase assay using myelin basic protein (MBP) as exogenous substrate.

**Fig. 10. Subcellular localization of hMAK protein.** COS1 cells were transiently transfected with a control vector (left panels) or with an expression plasmid coding for HA-tagged hMAK (right panels). Indirect immunofluorescence study was performed with anti-HA antibody followed by fluorescein isothiocyanate (FITC)-conjugated anti-mouse IgG, and the cells were examined under a fluorescence microscope (original magnification, ×400).
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lization. Interestingly, we found that all of the prostate cancer cell lines examined express basal levels of hMAK. Although their levels are not as high as that of androgen-treated LNCaP cells, they are significantly higher than that of the normal PrEC. Although it is difficult to draw conclusions with only a small number of samples analyzed, the results are consistent with the notion that there are other inducers of hMAK, and the acquisition of these inducers may be one means to bypass androgen dependence.

We have positively identified the kinase activity of hMAK based on its autophosphorylation and its ability to phosphorylate myelin basic protein in an in vitro kinase reaction. The level of kinase activity is relatively low as compared with other related kinases. We interpret this to mean that hMAK requires other agonists for its full activation, the identities of which have yet to be established. The kinase domain of MAK shares structural characteristics with both CDKs and MAPKs. Most conspicuous is the presence of TY and TXY motifs in the kinase domain of MAK. The TY residues in CDKs are sites of phosphorylation by Weel (40), and the T/E/G/P/Y motifs in MAPKs are sites of phosphorylation by the MEK family of kinases (41). Both motifs are involved in the regulation of kinase catalytic activity. Experiments are in progress to identify the putative upstream kinase(s) involved in the activation of hMAK.

To our knowledge, hMAK is one of only two kinases that were transcriptionally regulated by AR. The other kinase is SPAK, a Ste20 kinase, which is also specifically expressed in testis and prostate (44). The induction kinetics of SPAK by androgen, however, is much longer with the maximum activation occurring only after 48–72 h. At present it is not clear whether SPAK is a direct transcriptional target of androgen or whether the promoter of SPAK contains ARE sequences. The maximum fold induction of SPAK by androgen (4.9- and 4.7-fold at 1 and 10 nm, respectively, of R1881, a synthetic androgen) is comparable with that of hMAK. It is tempting to speculate that the kinases such as hMAK and SPAK, whose expressions are controlled by androgen, may be involved in AR signaling.

There are at least two ways that hMAK can participate in AR signaling. First, hMAK may propagate the signal initiated by androgen and function as a downstream kinase. In this capacity, hMAK may be involved in phosphorylating critical substrates in cell proliferation and differentiation of prostate cells. The possibility that hMAK may require an additional agonist to fully activate its kinase activity suggests that hMAK may be an integrator of multiple signal pathways. A second possible role of hMAK is to modulate AR activities, either positively or negatively. The activities of AR may be modified through direct phosphorylation by hMAK. There are several precedents to support this notion. cAMP-dependent protein kinase, MAPK, and protein kinase B/AKT are among the kinases that have been shown to phosphorylate AR (17, 45, 46). At least in the case of MAPK and AKT, the phosphorylation sites on AR have been identified, and mutations of these sites dampen the transactivation activity of AR (47, 48). By contrast, a newly identified serine kinase PAK6 was found to associate with AR and repress the transactivation potential of AR without phosphorylation (49). It is possible that PAK6 may be involved in phosphorylation of co-activators or co-repressors associated with AR and thereby indirectly modulating AR activity. Experiments are in progress to study whether and how hMAK modulates AR activity. In this regard, it is noteworthy that we found hMAK localized in the nucleus. The nuclear localization of hMAK is consistent with the presence of a putative NLS (KEKRRK) in the hMAK sequence. Direct demonstration that this is a functional NLS will require mutational analysis of these basic residues.

In summary, we have identified a new human serine/threonine kinase that is transcriptionally activated by AR under physiological conditions. This kinase is the orthologue of rat and mouse MAK, which were expressed almost exclusively in testis and in an early stage of spermatogenesis (24, 36). The human counterpart is also expressed highly in testis. Here, we report that hMAK is expressed in prostate cancer cells. It has been shown that androgen treatment activates cellular kinases such as MAPK and Src (21, 22), but the intermediate kinase(s) that propagate these signals remain unclear. The fact that AR tightly controls the expression level of hMAK makes it a good candidate kinase to propagate AR-mediated signals. Alternatively, hMAK may modulate the activity of AR or its co-activators/repressors through phosphorylation and serve as a regulator of AR activity. In either case, its possible roles in prostate development as well as in prostate cancer progression merit further investigation.

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