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Tissue-Specific and Hormonal Regulation of Human Prostate-Specific Glandular Kallikrein†

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ABSTRACT: Kallikreins are involved in the posttranslational processing of a number of specific polypeptide precursors. Previously, human glandular kallikrein (hGK-1) mRNA has been identified in the prostate; however, the hGK-1 protein has not been identified and characterized. Therefore, its physiologic function in the prostate is not known. In this study, we have isolated a full-length hGK-1 cDNA from a human adenocarcinoma cell line, LNCaP. In vitro translation experiments demonstrated that the molecular size of the hGK-1 protein generated from this cDNA is similar to that of prostate-specific antigen (PSA), a protein which is produced exclusively in the prostate. In situ hybridization with a hGK-1-specific oligonucleotide probe (77 bases), which can differentiate hGK-1 mRNA from PSA mRNA, demonstrated the hGK-1 mRNA to be located in the prostate epithelium. The hGK-1 mRNA was colocalized with PSA mRNA in prostatic epithelia. Moreover, in situ hybridization studies revealed that the level of hGK-1 mRNA in human benign prostatic hyperplasia tissues is approximately half that of PSA mRNA. Furthermore, we have demonstrated that hGK-1 mRNA is under androgenic regulation in LNCaP cells. Time course analysis revealed that hGK-1 mRNA levels increased significantly at 5 h of mibolerone treatment and reached maximal levels by 9 h. In addition, hGK-1 mRNA levels were increased by dihydrotestosterone, but not by dexamethasone or diethylstilbestrol treatments. Flutamide, a nonmetabolized anti-androgen, repressed the androgenic effects. These studies suggest that expression of hGK-1 mRNA is regulated by androgen via the androgen receptor.

The glandular kallikreins are a subgroup of serine proteases which are involved in the posttranslational processing of specific polypeptide precursors to their biologically active forms (Clements, 1989). The rodent kallikrein gene family consists of at least 25 genes (Clements, 1989; Mason et al., 1983; Wines et al., 1989). However, the human kallikrein gene family is much smaller, consisting of three well-described members (Clements, 1989; Schedlich et al., 1987): prostate-specific antigen (PSA),[1] glandular kallikrein (hGK-1), and pancreatic/renal kallikrein (hPRK).

Deduced amino acid sequences indicate that hGK-1 may be a trypsin-like serine protease (Schedlich et al., 1987; Morris, 1989) whereas PSA is a chymotrypsin-like serine protease (Lilja, 1983; Watt et al., 1986). Therefore, these two genes may have different physiological functions. The cDNA and genomic sequences for PSA and hPRK have been reported (Lundwal & Lilja, 1987; Lundwall, 1989; Fukushima et al., 1985; Evans et al., 1988), but only the genomic sequence for hGK-1 is available (Schedlich et al., 1987). The DNA sequence homology between hGK-1 and PSA (exon regions) is 80%, whereas the homology between hGK-1 and hPRK is 65% (Schedlich et al., 1987; Morris, 1989). The amino acid sequence homology of hGK-1 with PSA and hPRK is 78% and 57%, respectively (Schedlich et al., 1987; Morris, 1989). The similarities of gene structure and deduced amino acid sequences of these human kallikreins suggest that their evolution may involve the same ancestral gene. Moreover, both hGK-1 and PSA are expressed only in the human prostate (Morris, 1989; Chapdelaine et al., 1988). Expression of hPRK is limited to the pancreas, submandibular gland, kidney, and other nonprostate tissues (Morris, 1989). Interestingly, the hGK-1 gene is located about 12 kbp downstream from the PSA gene in a head-to-tail fashion on chromosome 19 (Riegman et al., 1989). Thus, the relationship between hGK-1 and PSA gene expression is very intriguing, especially with respect to their

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[1] Abbreviations: hGK-1, human glandular kallikrein; PSA, prostate-specific antigen; hPRK, pancreatic/renal kallikrein(s); SDS, sodium dodecyl sulfate; BPH, benign prostatic hyperplasia; EDTA, ethylenediaminetetraacetic acid; PAGE, polyacrylamide gel electrophoresis.

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evolution and functional properties.

Tremendous interest has been generated in PSA because of the important role it plays as a marker to monitor therapy for prostate carcinoma (Guinan et al., 1987; Lange et al., 1989; Stamey et al., 1989; Kallian et al., 1986). PSA is expressed specifically in the prostate epithelium as determined by both immunohistochemical staining (Nadji et al., 1981; Stein et al., 1984) and in situ hybridization techniques (Qiu et al., 1990). Although the hGK-1 protein has not been identified and characterized, Northern blot analysis has revealed that hGK-1 mRNA is expressed only in prostate tissue (Morris, 1989; Chapdelaine et al., 1988). One of our goals was to determine whether the hGK-1 mRNA was also synthesized in epithelial cells of the prostate.

Androgens and the androgen receptors play an important role in the development and secretory properties of the prostate. Although the androgen receptor genes have recently been cloned (Chang et al., 1988; He et al., 1990; Lubahn et al., 1988; Tilley et al., 1989; Young et al., 1990), only a few genes in the human prostate (e.g., epidermal growth factor receptor, androgen receptor, prostatic acid phosphatase) have been described to be regulated by androgens (Schuurmans et al., 1988; Quarmby et al., 1990; Schulz et al., 1985). The expression of PSA protein has been correlated with fluctuating androgen levels during male development (Goldfarb et al., 1986). We have demonstrated recently that PSA mRNA is under androgenic regulation in a human prostate tumor cell line, LNCaP (Young et al., 1991). In addition to introns and exons, the 5′-flanking region of hGK-1 is highly homologous to that of PSA, suggesting that the expression of these two genes may be, at least in part, under the same control mechanisms including androgenic regulation.

Materials and Methods

Cloning and in Vitro Expression of hGK-1 cDNA. Five micrograms of total RNA isolated from LNCaP cells was used for synthesizing a first-strand cDNA library with an $(dT)_{18}$ primer in a 50-μL reaction solution according to instructions from BRL. Ten microliters of the above cDNA library was used in a polymerase chain reaction (PCR) to obtain hGK-1 cDNA. The two oligonucleotides used as primers in this reaction were as follows: 5′-TGTGTCAGCATGTGGGA-3′ (primer 1), which corresponds to the 5′-untranslated region of the first exon in hGK-1 (Schedlich et al., 1987), and 5′-CGTCGACTCTAGATTTTTTTTTTTT-3′ (primer 2), which was used to hybridize with poly(A) tails of the second-strand cDNA generated by primer 1 in the first round of the PCR. The reaction was performed with 30 cycles of denaturation (1 min at 92 °C), annealing (2 min at 56 °C), and polymerization (3 min at 72 °C). The amplified cDNA was then fractionated and purified by agarose gel electrophoresis. The purified cDNA was phosphorylated with T4 polynucleotide kinase and ligated into dephosphorylated pGEM7Zf(+) plasmid at the *Sma*I site with T4 ligase. The ligated DNA was transformed into *Escherichia coli* HB101 competent cells. Sequences of the selected plasmid clones were determined by a DNA polymerase chain termination technique according to Promega's instructions. Oligonucleotides (17–21-mers), which contained promoter regions for either Sp6 or T7 RNA polymerase as well as internal regions of hGK-1 cDNA, were used as primers for sequencing.

Constructs consisting of hGK-1 or PSA cDNA in pGEM7Zf(+) were linearized with the restriction enzyme *Xba*I or *Hin*dIII, respectively. Capped hGK-1 and PSA RNAs were obtained from the respective linearized plasmids with T7 or Sp6 RNA polymerase in the presence of 5 mM GpppG. After being treated with RNase-free DNase 1, the hGK-1 and PSA RNAs were translated with a micrococcal nuclease treated rabbit reticulocyte lysate in the presence of [^{35}S]methionine (10 mCi/mL; 1200 Ci/mmol, Amersham) according to instructions from Promega. Equal volumes (15 μL) of the translation product were incubated in the absence or presence of 1.8 μL of canine pancreatic microsomal membranes according to instructions from Promega and subjected to SDS–polyacrylamide gel electrophoresis (Young et al., 1988). Protein markers from Amersham were run in parallel lanes for determination of molecular weight. An autoradiograph was obtained after exposing the dry gel with Kodak X-ray film for 24 h.

Preparation of Probe. Oligonucleotides (77 bases) corresponding to different regions in exon 5 of the PSA and hGK-1 genes (nucleotides 1159–1235 and 728–805 relative to the respective initiation codons) were synthesized at the Mayo Molecular Biology Core Facility; 20-base oligonucleotides corresponding to the 3′ end of the 77-base oligonucleotides were also synthesized. The 77-mer and 20-mer oligonucleotides from the same gene were mixed at a molar ratio of 1:30, and an asymmetrical polymerase chain reaction amplification procedure was used to produce antisense oligonucleotide probes (Young et al., 1991). Briefly, the amplification reaction (50 μL) contains 30 pmol of the 20-mer oligonucleotide, 1 pmol of the 77-mer oligonucleotide, 0.04 mM each of dATP, dGTP, and dTTP, 1× GeneAmp PCR buffer (Perkin-Elmer), 1 unit of AmpliTaq DNA polymerase (Perkin-Elmer), and 125 μCi of [α-^{32}P]dCTP (~3000 Ci/mmol) or [α-^{35}S]dCTP (>1000 Ci/mmol). The reaction was performed with 12–15 cycles of denaturation (1 min at 92 °C), annealing (2 min at 56 °C), and polymerization (3 min at 72 °C). [^{32}P]- or [^{35}S]dCTP was incorporated into the newly synthesized probes.

Tissue Collection and Preparation. Fresh surgical specimens from the prostate, pancreas, colon, kidney, testis, and lung were used in these studies. Tissue specimens were fixed with a 4% paraformaldehyde and phosphate-buffered saline (PBS) solution (154 mM NaCl, 8 mM KH_2PO_4, and 15 mM Na_2HPO_4, pH 7.4) and incubated at 4 °C for 24 h. Each specimen was washed twice for 30 min in PBS at room temperature, dehydrated in graded ethanol solutions (70% for 40 min, 80% for 40 min, 95% twice for 30 min each, and 100% twice for 30 min each), and paraffin-embedded. A series of 5-μm tissue sections were cut and placed on glass slides coated with 0.5% gelatin and 0.5% chromium potassium sulfate. Slides were dried for 48 h at 42 °C and stored at 4 °C until ready for use.

In Situ Hybridization. Tissue sections were baked at 42 °C for 24 h. The slides were deparaffinized and dehydrated in three xylene and three 100% ethanol washes for 3 min each. The slides were prehybridized for 2 h at 40 °C in a solution containing 50% formamide, 50 mM NaH_2PO_4, 0.6 M NaCl, 5 mM EDTA, 0.05% yeast tRNA, 10% dextran sulfate, 10 mM dithiothreitol (DTT), and 0.05% sonicated denatured salmon sperm DNA and 10× Denhardt's solution [0.2% bovine serum albumin, 0.2% ficoll, and 0.2% poly(vinyl pyrrolidone)]. The slides were rinsed in 100% ethanol and air-dried. A wax pencil was used to outline each tissue section (approximately 18 × 18 mm area). Each of the outlined areas was covered with 20 μL of the prehybridization solution containing denatured ^{35}S-labeled kallikrein DNA probe at a concentration of 1×10^6 cpm/20 μL. A RNase-free 18 × 18 mm glass coverslip was placed over each outlined area and sealed with rubber cement. Hybridization was performed at 45 °C for

14 h. After hybridization, the coverslips were removed, and the slides were washed in a series of increasing stringencies of SSC solutions containing 0.5 mM dithiothreitol, 0.05% sodium pyrophosphate, and 0.05% sodium thiosulfate. The wash conditions were as follows: 1× SSC (150 mM NaCl and 15 mM sodium citrate, pH 7.0) and 0.2% SDS (sodium dodecyl sulfate) at 45 °C for 1 h, 0.5× SSC at 37 °C for 1 h, and 0.25× SSC at room temperature for 20 h. A duplicate set of adjacent tissue sections were prepared in the same manner, but probed with a ^{35}S-labeled cDNA from a 1-kb DNA ladder which served as a nonspecific control. An RNA specificity control was included by pretreating adjacent tissue sections for 30 min at 37 °C with RNase A (100 μg/mL) and RNase T (10 μg/mL) prior to probing with the ^{35}S-labeled kallikrein DNA.

Northern Blot Analysis. LNCaP cells (5 × 10^4 cells/mL) were propagated in RPMI-1640 medium with 5% serum and 2 mM glutamine until cultures reached 80% confluency. Cells were then incubated for 20 h in RPMI-1640 with 2% charcoal-stripped serum and 1 mM glutamine prior to the addition of one of the following: 3 nM mibolerone, 10 nM dihydrotestosterone in the absence or presence of 20 μM hydroxyflutamide, 50 nM diethylstilbestrol, 50 nM dexamethasone. Cells were harvested at the indicated times, and total RNA was extracted by the guanidinium isothiocyanate method (Chomczynski & Sacchi, 1987). Equal amounts of RNA (about 30 μg/lane) were fractionated in the presence of ethidium bromide by denaturing gel electrophoresis and transferred to a Zeta probe membrane (Kroczek & Siebert, 1990). The amount of RNA used was quantified by both spectrophotometric assay at 260 nm and ethidium bromide staining of the Zeta probe membrane or the gel itself. Zeta probe membranes were hybridized using a ^{32}P-labeled hGK-1 probe (g-2) and washed under the same conditions as described according to the methods of Young et al. (1991).

Results

A full-length hGK-1 cDNA (~1.5 kb) was isolated from LNCaP cells using the polymerase chain reaction technique as described under Materials and Methods. Identical sequences were obtained from several independent cDNA clones which are in general agreement with the reported genomic sequence. Noted differences include nucleotides 591 (G → T), 645 (G → A), and 695 (C → A) (relative to the initiation codon). The difference at nucleotide 695 resulted in an amino acid substitution at 232 (threonine → asparagine). Therefore, these changes appear to be insignificant. The genomic sequence (Schedlich et al., 1987) suggested that a polyadenylation signal was located about 30 bp downstream of the stop codon of the hGK-1 transcript. However, our cDNA, which contained the entire 3′-untranslated region and thus more sequence information, revealed another polyadenylation signal located at 600 bp downstream of the stop codon and 30 bp upstream of the poly(A) tail. This latter polyadenylation signal may be novel as evidenced in the Northern blot analysis in this and other studies (Morris, 1989; Chapdelaine et al., 1988) which revealed a single 1.6-kb transcript in the prostate.

The putative protein for hGK-1 has never been isolated from human tissues. Therefore, one of the hGK-1 cDNA clones was transcribed and translated in vitro in order to characterize the protein product. The pGEM7Zf(+) plasmid containing hGK-1 cDNA was linearized with *Xba*I restriction enzyme and amplified by T7 RNA polymerase in the presence of GpppG in order to obtain capped hGK-1 RNA, and a rabbit reticulocyte lysate was used to synthesize the hGK-1 prepropolypeptide from hGK-1 RNA. As shown in Figure 1, lane

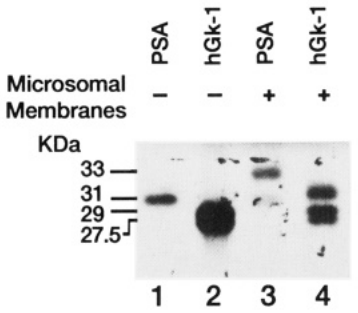

FIGURE 1: In vitro translation of hGK-1 and PSA. Capped RNA was synthesized from linearized plasmids containing either hGK-1 or PSA cDNA. These RNAs were used for in vitro translation in a rabbit reticulocyte system in the presence of [^{35}S]methionine. The labeled translation products for hGK-1 (lanes 2 and 4) or PSA (lanes 1 and 3) were further incubated either in the absence (lanes 1 and 2) or in the presence (lanes 3 and 4) of canine pancreatic microsomal membranes and subjected to SDS–polyacrylamide gel electrophoresis. Detailed procedures are described under Materials and Methods. The autoradiograph was obtained by exposing the dried polyacrylamide gel to Kodak X-ray film for 24 h. "+" or "–" represents the presence or absence of membranes, respectively. Molecular masses were calculated from the migration of radiolabeled bands relative to molecular mass standards.

2, the in vitro synthesized ^{35}S-labeled hGK-1 peptide was resolved as a doublet of approximately 29 kDa. For comparison, the PSA prepropolypeptide (Figure 1, lane 1) was also synthesized and resolved as a single band of 31 kDa. The production of doublet peptides from a single species of in vitro transcribed hGK-1 RNA could be due to either proteolytic cleavage or translation from an internal initiation codon. However, this latter possibility is less likely because the use of any other in-frame or out-of-frame ATG's would generate a much smaller polypeptide. Therefore, the 29-kDa polypeptide is most probably synthesized from the authentic initiation codon as predicted from the hGK-1 cDNA.

We next tested the ability of microsomal membranes to modify the in vitro synthesized hGK-1 peptide posttranslationally. After being treated with canine pancreatic microsomal membranes, which contain posttranslation processing enzymes, the apparent molecular size of hGK-1 was increased (Figure 1, lane 4), suggesting that posttranslational modification of the peptide had taken place. A similar increase in size of the PSA peptide was observed (Figure 1, lane 3). Interestingly, only a single polypeptide for hGK-1 (Figure 1, lane 4) was generated from the doublet proteins by microsomal membrane treatment. It is possible that both polypeptides were modified to an indistinguishable size. Alternatively, one of the polypeptides may have been changed to 31 kDa while the other was degraded. However, this treatment markedly reduced the amount of hGK-1 polypeptides recovered in the SDS–polyacrylamide gel (Figure 1, lane 4 vs lane 2). Thus, the hypothesis of proteolytic degradation of the hGK-1 peptide is substantiated by these results.

Recent studies (Morris, 1989; Chapdelaine et al., 1988) have indicated that both hGK-1 and PSA mRNAs are expressed in human prostate at relatively high levels. However, the hGK-1 and PSA transcripts have a high degree of homology (>82%). In order to obtain unequivocal quantitative and qualitative measurements of both transcripts, we developed gene-specific nucleotide probes and conditions under which these transcripts would not cross-hybridize (Young et al., 1991). These probes, designated as g-2 (hGK-1 specific) and p-2 (PSA specific), were used for the following studies.

Expression of hGK-1 mRNA had not been localized previously to a particular cell type within the prostate. Therefore,

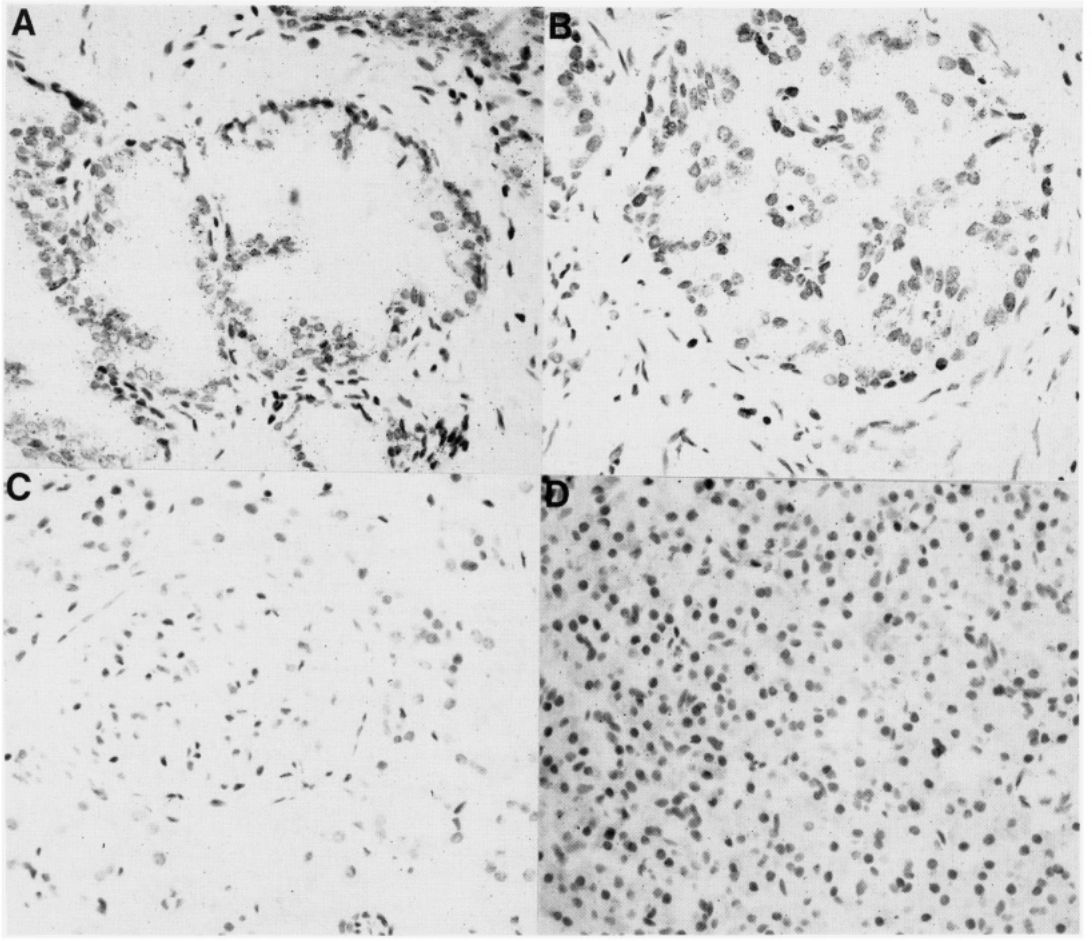

FIGURE 2: Localization of hGK-1 mRNA in human prostate by in situ hybridization. Tissue was fixed and cut into 5-μm sections as described under Materials and Methods. Each tissue section (1.5 cm^2) was incubated with ^{35}S-labeled g-2 probe (1×10^6 cpm/25 μL). Hybridization was performed at 45 °C for 16 h. Panels A and B show BPH and prostatic cancer tissues, respectively. Panels C and D are kidney and pancreatic tissue sections, respectively.

we used the in situ hybridization technique with a ^{35}S-labeled hGK-1-specific probe to localize hGK-1 mRNA in human prostate. hGK-1 mRNA was detected in epithelium of both normal and benign prostatic hyperplasia (BPH) prostate tissue (Figure 2A) as well as in prostatic cancerous tissues (Figure 2B). The exposed sliver granules were located within the acinar cells and appeared to be in a higher concentration within the perinuclear region. There was very little background activity within the prostatic stromal tissue.

Since another kallikrein, hPRK, is expressed at high levels in kidney and pancreas (Morris, 1989; Fukushima et al., 1985; Evans et al., 1988), it was important to determine whether our hGK-1 probe would cross-hybridize with mRNA in these tissues. It can be seen that neither kidney (Figure 2C) nor pancreas (Figure 2D) exhibited appreciable hybridizable activity. Thus, these data confirm the specificity of the hGK-1 probe. Other tissues such as colon, breast, testis, and lung exhibited no hybridizing reactivity (data not shown), which supports the concept that hGK-1 is prostate-specific. When prostate tissue specimens were pretreated with RNases A and T before hybridization, there was no specific binding to the glandular epithelium (data not shown).

In order to compare the relative levels of mRNA for hGK-1 and PSA in human prostate tissues, an IBAS 2000 image analyzer was utilized to measure the hybridization signal in situ (Qiu et al., 1990). In this study, the levels of PSA and hGK-1 mRNAs were compared within adjacent sections of BPH tissues from six patients. As shown in Table I, the ratio of grains counted for hGK-1 mRNA to that for PSA mRNA in BPH tissues varied between 0.3 and 0.6. The average level of hGK-1 mRNA is approximately half that of PSA in prostatic epithelia (Table I). However, if one considers that the specific radioactivity in one molecule of hGK-1 probe is less than that for the PSA probe, the expression of hGK-1 mRNA may be underestimated.

Table I: Comparison of hGK-1 and PSA mRNA Levels in Adjacent Human Prostate Tissue Sections by in Situ Hybridization Analysis[a]

prostate tissues[b]	hGK-1 (grains 10^{-4} μm^{-2})	PSA (grains 10^{-4} μm^{-2})	hGK-1/PSA
1	50 ± 9[c]	82 ± 27	0.6
2	38 ± 20	92 ± 31	0.4
3	34 ± 9	70 ± 32	0.5
4	59 ± 16	111 ± 71	0.5
5	79 ± 15	185 ± 39	0.4
6	31 ± 20	92 ± 36	0.3
mean	49 ± 22[d]	105 ± 55[d]	0.5

[a] As described under Materials and Methods. [b] Adjacent tissue sections obtained from prostate containing normal and BPH tissues in six patients. [c] (±) indicates the range of one standard deviation. [d] $p < 0.005$ as determined by paired analysis.

In order to evaluate whether hGK-1 is under androgenic control, we tested the effects of mibolerone, a nonmetabolizable synthetic androgen, on hGK-1 mRNA in LNCaP cells. After 20 h of steroid depletion, cells were fed on charcoal-striped serum either in the absence or in the presence of 3 nM mibolerone. RNA was extracted at various time intervals and fractionated on a formaldehyde–agarose gel for Northern blot analysis. In the absence of androgen, expression of hGK-1

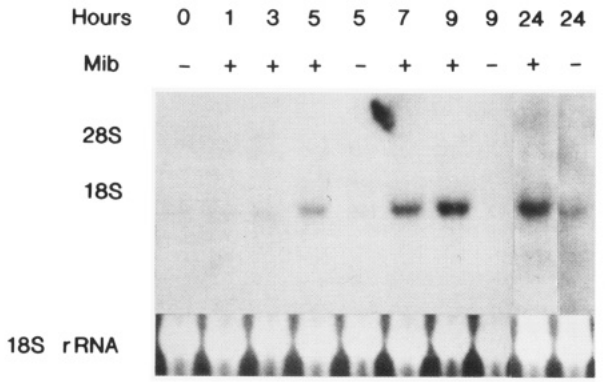

FIGURE 3: Kinetics of the androgenic response of hGK-1 mRNA in LNCaP cells. LNCaP cells were incubated either without (−) or with (+) 3 nM mibolerone for 1, 3, 6, 9, and 24 h. Total RNA was extracted, and equal amounts (30 μg) were applied to each lane of a formaldehyde–agarose gel. Following electrophoresis, RNA was transferred onto a Zeta probe membrane which was hybridized with a ^{32}P-labeled hGK-1 probe (g-2) as described under Materials and Methods. The autoradiograph was obtained after a 40-h exposure at −72 °C. Ethidium bromide staining of 18S rRNA on the same Zeta probe membrane was used as internal control for normalization of total RNA.

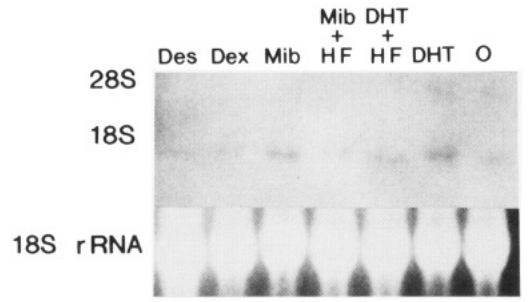

FIGURE 4: Steroid specificity of hGK-1 mRNA induction in LNCaP cells. LNCaP cells were incubated either without (0) or with 50 nM dexamethasone (Dex), 50 nM diethylstilbestrol (Des), 3 nM mibolerone (Mib), 3 nM mibolerone plus 2000 nM hydroxyflutamide (Mib + HF), 10 nM dihydrotestosterone (DHT), or 10 nM DHT plus 2000 nM hydroxyflutamide (DHT + HF) for 9 h prior to RNA extraction. Northern blot analysis was similar to that described in the legend to Figure 3.

mRNA remained low throughout the 24-h period (Figure 3). However, within 5 h after addition of androgen, hGK-1 mRNA levels increased, and maximum levels were achieved after 9 h. Thus, hGK-1 mRNA appears to be under androgenic regulation. Moreover, increased levels of hGK-1 mRNA were also observed with dihydrotestosterone treatment, and the effects of both androgens were reduced in the presence of a 200-fold excess of hydroxyflutamide, an anti-androgen (Figure 4). However, neither diethylstilbestrol nor dexamethasone affected significantly the levels of hGK-1 mRNA (Figure 4). These results suggest that the androgenic effect on hGK-1 mRNA in LNCaP cells may be via the androgen receptor.

Discussion

Although the first descriptions of hGK-1 were based on genomic DNA sequence (Schedlich et al., 1987), Northern blot (Morris, 1989), and primer extension (Chapdelaine et al., 1988) analyses, the complete characterization of the hGK-1 transcript and protein has remained elusive. The sequence of the hGK-1 transcript isolated from LNCaP cells is in agreement with the reported gene sequence with some minor differences. These alterations may represent either sequence polymorphism or mutations in the cell line used for isolating the hGK-1 mRNA. One of these nucleotide changes actually caused an amino acid substitution at residue 232. However, this amino acid may not be critical for the function of the hGK-1 protein, since it is not among those key amino acids important for substrate recognition or catalytic activity (Loor et al., 1989).

The molecular mass of hGK-1, on the basis of our SDS–polyacrylamide gel electrophoresis (PAGE) data, was approximately 29 kDa for the in vitro translated product in the absence of canine pancreatic microsomal membranes and 31 kDa in the presence of membranes, suggesting posttranslational modifications. The molecular weight of hGK-1 calculated from its deduced amino acid sequence is 28 644 for the prepropeptide and 26 133 for the mature peptide. Thus, these data would predict that hGK-1 is modified posttranslationally, which alters the migration of the protein on SDS–PAGE. Since both hGK-1 and PSA are glycoproteins, as judged by either biochemical assay (Lee et al., 1989) and/or the deduced amino acid sequence (Schedlich et al., 1987; Morris, 1989; Lundwall & Lilja, 1987; Lundwall, 1989), such changes would be consistent with predicted sites of glycosylation on the peptide. On the other hand, the number of carbohydrate residues on PSA is known. Therefore, it is easy to reconcile the observed migration of the mature PSA peptide on SDS–PAGE (33–35 kDa) with the calculated amino acid content (26 460 Da) plus carbohydrate content (7%). However, we cannot rule out other modifications, such as acetylation or phosphorylation. These results suggest that the physicochemical properties of the hGK-1 protein are slightly different from those of PSA.

Kallikrein gene products are known to exert either kininogenase (Clements, 1990; Margolius, 1989) or growth factor processing activities (Clements, 1990; Margolius, 1989). These modified polypeptides then bind to cell-surface receptors in order to stimulate a variety of cellular responses including secretion, communication, and proliferation (Margolius, 1989). The physiological function of hGK-1 in the prostate is unknown. Presumably, hGK-1 possesses trypsin-like proteolytic activity as deduced from its primary structure. Human prostate does produce a number of growth factors including epidermal growth factor (Schuurmans, 1988), transforming growth factor β (Derynck et al., 1987), and prostatic growth or inhibiting factor (Hierowski et al., 1987; Konig et al., 1987). It has been postulated that hGK-1 might be involved in the processing of such factors (Clements, 1990). Since the biological function of PSA is to liquefy the seminal fluid coagulum by digesting a seminal vesicle-specific antigen through its serine protease activity (Lee 1989), it is possible that hGK-1 exerts its function by acting on seminal plasma proteins as well. However, presently it is not even known whether hGK-1 is a secretory protein. The alteration in its molecular weight properties after addition of microsomal membranes suggests that posttranslational modifications might prepare the peptide for secretion.

Levels of hGK-1 mRNA have been estimated to be 10–20% of those found for PSA mRNA in prostate tissues by Northern blot analysis (Chapdelaine, 1988; Henttu et al., 1990). However, we found levels of hGK-1 mRNA to be at least half that of PSA mRNA in normal prostate or BPH tissues based on in situ hybridization analysis. The discrepancy between these values may lie in the nature of the probe and the detection system used. The probes used in the present study exhibited less than 35% homology between hGK-1 and PSA, in contrast to 65–73% homology for previous studies. Also we used probes of about twice the length of those used in

previous studies (Morris, 1989; Chapelaine et al., 1988; Henttu et al., 1990), thus presenting a greater surface for hybridization. We have demonstrated previously (Young et al., 1991) that these probes do not cross-react with their counterpart cDNA, whereas there was no demonstration of specificity of the probes used in previous studies (Morris, 1989; Chapdelaine et al., 1988; Henttu et al., 1990). Therefore, the conditions used for Northern blot analysis in previous studies may be in favor of binding of PSA probes to PSA mRNA but gave rise to low binding of the hGK-1 probe to hGK-1 mRNA. More importantly, in situ hybridization with adjacent tissue sections allowed direct measurement of PSA and hGK-1 mRNAs for comparison purposes. Previous studies (Chapdelaine et al., 1988; Henttu et al., 1990) did not control for the possible preferential isolation of one mRNA species over the other species in the purification of poly(A^+) mRNA, which was used in the Northern blot analyses. The aforementioned factors may account for a more accurate estimation for hGK-1 mRNA levels in the present study.

Androgens play a critical role in the developmental and secretory properties of the prostate. In this study, we have demonstrated that expression of the hGK-1 mRNA is under androgenic regulation. Examination of the 5′-flanking region of the hGK-1 gene revealed a steroid hormone response element (HRE)-like sequence located approximately 160 bp upstream from the cap site (Schedlich et al., 1987). The PSA gene also contains a HRE-like sequence at a similar location (Lundwall, 1989). Indeed, the putative HREs of hGK-1 and PSA genes are virtually identical, differing by one nucleotide only. Furthermore, sequence comparison between hGK-1 and PSA 5′-flanking regions revealed 80% homology in their proximal regions (−1 to −330 relative to the major cap site). Therefore, it is suggested that the expression of these two genes may share some common regulatory mechanisms.

In summary, we have demonstrated that the expression of hGK-1 mRNA is approximately half that of PSA mRNA when LNCaP cells are exposed to 3 nM mibolerone. This is consistent with the results obtained in tissue sections by in situ hybridization. Since our in vitro cell system was designed to study the maximal androgenic effect on PSA and hGK-1 expression with minimal influences from other factors, we conclude that androgens must be responsible for the differential expression of these two genes. These effects could be due to either differences in their putative HRE sequences, causing differential induction by the androgen receptor, or posttranscriptional factors, such as androgen-induced mRNA stabilizing factors. However, we found that hGK-1 mRNA has a lower level of basal expression than that of PSA mRNA in the absence of androgens. In addition, the distal regions (beyond −330 bp of the 5′-flanking regions in both genes) have less than 33% homology. This suggests that the expression of these two highly homologous genes may be regulated by different mechanisms.

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Registry No. Kallikrein, 9001-01-8; dihydrotestosterone, 521-18-6; mibolerone, 3704-09-4.

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Inhibition of NAD(P)H:(Quinone-Acceptor) Oxidoreductase by Cibacron Blue and Related Anthraquinone Dyes: A Structure-Activity Study†

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ABSTRACT: Cibacron Blue, a widely used ligand for affinity chromatography, is a potent inhibitor of NAD(P)H:(quinone-acceptor) oxidoreductase (EC 1.6.99.2) (quinone reductase). This property has been exploited to purify quinone reductase, to identify its nucleotide-binding site, and to obtain diffraction-grade crystals of this enzyme [Prochaska, H. J. (1988) *Arch. Biochem. Biophys. 267*, 529-538; Ysern, X., & Prochaska, H. J. (1989) *J. Biol. Chem. 264*, 7765-7767]. To define the structural region(s) of the dye responsible for its inhibitory potency, Cibacron Blue was synthesized and the dye, its synthetic intermediates, and some analogues of these intermediates were crystallized as novel trialkylamine or choline salts. These compounds were characterized by proton NMR and mass spectrometry, and their inhibitory potencies were measured. Only two of the four ring systems of the Cibacron Blue molecule are required for potent inhibition. Acid Blue 25 [1-amino-4-(phenylamino)anthraquinone-2-sulfonic acid] is an inhibitor (K_i = 22 nM) almost as potent as Cibacron Blue (K_i = 6.2 nM). However, removal of any of the three substituents on the anthraquinone ring of Acid Blue 25 markedly reduced inhibitory potency. These results are consistent with the proposal that Cibacron Blue is primarily a mimic for the ADP fragment of mono- and dinucleotides. The difference absorption spectrum of the Acid Blue 25-quinone reductase complex was very different from that of the complex with Cibacron Blue. In contrast to other compounds tested, Procion Blue M-3GS, the electrophilic dichlorotriazine precursor of Cibacron Blue, was an irreversible inhibitor of quinone reductase (K_D = 16 nM, k_3 = 0.03 min^{-1}), and the inactivation was blocked by Cibacron Blue, a monochlorotriazine.

Cibacron Blue is a potent inhibitor of many nucleotide-dependent dehydrogenases and kinases (Dean & Watson, 1979; Lowe & Pearson, 1984), including NAD(P)H:(quinone-acceptor) oxidoreductase (EC 1.6.99.2). QR[1] is a widely distributed flavoprotein that catalyzes obligatory two-electron reductions of quinones and protects cells against the toxicities of quinones (Benson et al., 1980; Ernster et al., 1987; Prochaska & Talalay, 1991). The observation that Cibacron Blue is a high-affinity ligand for QR is of interest for several reasons: (a) pure QR can be isolated in a single step from crude liver cytosols by Cibacron Blue-agarose chromatography (Prochaska, 1988; Sharkis & Swenson, 1989); (b) Cibacron Blue binds to the nucleotide folds of many proteins, and this property was used to identify correctly the glycine-rich consensus amino acid sequence involved in nucleotide binding to QR (Prochaska, 1988; Liu et al., 1989); and (c) Cibacron Blue cocrystallized with QR to provide crystals suitable for high-

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[1] Abbreviations: QR, quinone reductase, NAD(P)H:(quinone-acceptor) oxidoreductase (EC 1.6.99.2), also known as DT-diaphorase or menadione reductase; MTT, 3-(4,5-dimethylthiazo-2-yl)-2,5-diphenyltetrazolium bromide; FAB, fast atom bombardment mass spectrometry; CI/EI, chemical ionization/electron ionization mass spectrometry; C.I., *Colour Index*; bromaminic acid, 1-amino-4-bromoanthraquinone-2-sulfonic acid; ASSO, 1-amino-4-[(4-amino-3-sulfophenyl)amino]-anthraquinone-2-sulfonic acid; Acid Blue 25, 1-amino-4-(phenylamino)anthraquinone-2-sulfonic acid; PIC, paired ion chromatography; TLC, thin layer chromatography.

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