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Clinica Chimica Acta 337 (2003) 115–126

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Steroid hormone regulation of the human kallikrein 10 (KLK10) gene in cancer cell lines and functional characterization of the KLK10 gene promoter

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Received 20 March 2003; received in revised form 29 July 2003; accepted 31 July 2003

Abstract

Background: The human kallikrein 10 (KLK10) gene is a new member of the human tissue kallikrein gene family. It encodes for a secreted serine protease (hK10) with predicted trypsin-like enzymatic activity. KLK10 is highly expressed in the sex organs and its expression level changes in malignancy. *Methods*: To determine the role of steroid hormones in KLK10 gene expression, we investigated its modulation by 17β-estradiol, 5α-dihydrotestosterone, norgestrel, dexamethasone and aldosterone, at both the transcription and translation level, in a panel of cancer cell lines. After steroid hormone stimulation, the change of KLK10 mRNA was monitored with reverse transcriptase polymerase chain reaction and hK10 protein levels in the culture supernatant were quantified with an hK10-specific immunoassay. The presence of hormone response elements in the KLK10 gene promoter was examined with the chloramphenicol acetyltransferase reporter gene system. *Results*: The KLK10 expression was mainly up-regulated by estrogens, androgens and progestins, and to a lesser extent by dexamethasone and aldosterone in the breast cancer cell lines BT-474, MCF-7 and T-47D, both at the mRNA and protein levels. The effect of stimulation of these steroids on KLK10 expression varied among the cell lines. Estrogens, androgens and progestins were most potent in the BT-474, T-47D and MCF-7 cells, respectively. The up-regulation effect of estrogens, androgens, and progestins on KLK10 expression can be blocked by their antagonists ICI-182, 780, RU-56,187, and mifepristone, respectively. Time course studies showed that hK10 protein started to increase 1 day after steroid hormone stimulation and this increase persisted for 7 days. These data suggest that steroid hormones up-regulate KLK10 gene expression through direct interaction between hormone-receptor complexes and their cognate hormone response elements. To search for hormone response elements, we functionally characterized the KLK10 promoter by placing it upstream of the chloramphenicol acetyltransferase reporter gene. We found that KLK10 promoter activity did not rely on the presence of functional estrogen and androgen receptors. Also, the presence of functional estrogen and androgen receptors did not increase its constitutive activity. We suggest that the hormone response elements that mediate the transcriptional regulation of KLK10 are unlikely to locate in the KLK10 promoter. *Conclusions*: Estrogens, androgens and progestins modulate KLK10 expression through

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0009-8981/$ - see front matter
doi:10.1016/j.cccn.2003.07.008

their own receptors but this regulation is not mediated by steroid hormone response elements in the promoter of the KLK10 gene.

Keywords: Serine proteases; Human kallikrein; Steroid hormone

1. Introduction

The human tissue kallikrein gene family is a group of homologous genes encoding for serine proteases and tandemly localized on chromosome 19q13.4. It is now clear that this gene family is composed of 15 members, known as human kallikreins 1 to 15 (KLK1 to KLK15). These genes participate in diverse physiological processes, such as maintenance of vascular tone and permeability, cleavage of growth factors, neural plasticity and skin shedding [1].

The human kallikrein 10 gene (KLK10; also known as the normal epithelial cell-specific 1 gene, NES1) is a member of the human tissue kallikrein gene family. It was cloned by subtractive hybridization, based on its down regulation in a radiation-transformed breast epithelial cell line compared to its normal counterpart [2]. The length of the KLK10 gene is about 5.5 kb and consists of six exons (one untranslated) and five introns [3]. It encodes for a secreted serine protease of 276 amino acids and a 30-kDa molecular weight. KLK10 is predicted to have trypsin-like enzymatic activity [2]. However, its physiological substrates and functions are still not known. Some experimental evidence suggests that it may function as a tumor suppressor. KLK10 expression has been found to be down-regulated in a number of breast and prostate cancer cell lines as well as in testicular carcinoma [2,4,5]. KLK10 overexpression can suppress tumor formation in nude mice [4]. KLK10 is widely expressed in many tissues, such as salivary gland, ovary, breast, prostate and testis. hK10 protein is also detectable in various biological fluids, including milk, seminal plasma, amniotic fluid, cerebrospinal fluid and serum [2,6].

Many human tissue kallikrein genes, including KLK2 and KLK3, have long been known to be regulated by steroid hormones [1]. The up-regulation of both KLK2 and KLK3 by androgens has been demonstrated in a number of in vitro and in vivo studies [7–9]. In contrast, KLK1 is found to be primarily up-regulated by estrogens, based on its differential expression in the menstrual cycle [10]. Steroid hormones exert their effect by binding to their cognate hormone receptors. Upon binding to the receptor, the hormone-receptor complex translocates into the nucleus and modulates gene transcription through the following mechanisms: direct pathway and cross-talk with other transcription factors (indirect pathway) [11]. In the direct pathway, the hormone-receptor complex directly binds to the cognate DNA sequences, termed steroid hormone response elements (HREs), usually in the promoter region of the regulated genes, recruits coactivators/corepressors and interacts with the general transcription machinery to modulate gene transcription. Androgen receptor (AR), progesterone receptor (PR) and glucocorticoid receptor (GR) share a very similar consensus DNA-binding sequence, whereas, the estrogen receptor has a quite different one [12,13]. In the cross-talk pathway, the hormone-receptor complex does not bind to the promoters of the targeted genes. Instead, it interacts with other transcription factors that can modulate gene expression through protein–protein interactions or bridging molecules. In this case, no cognate HRE is present in the promoters of the targeted genes [14]. In light of these theories, the promoters of KLK2 and KLK3 genes have been extensively studied. Three regions harboring androgen response elements (AREs), including AREI, AREII and enhancer AREs, have been identified within the 5 kb KLK3 promoter, through functional studies in prostate cancer cell lines and transgenic mouse models [9,15–18]. Similarly, two AREs have also been found in the KLK2 promoter [19–21]. It is believed that these AREs are primarily responsible for the transcriptional up-regulation of KLK2 and KLK3 genes by androgens. Several sequence motifs similar to the consensus estrogen response elements (EREs) were also found in the KLK1 promoter [22]. However, these predicted sequences have not been functionally tested. As a result, the mechanism underlying the transcriptional

up-regulation of KLK1 by estrogens is still not known.

Whether and how KLK10, a new member of the human kallikrein gene family, is regulated by steroid hormones has not as yet been determined. Previously, we found that KLK10 was up-regulated by androgens, estrogens and progestins at the mRNA level in the breast cancer cell line BT-474 [23]. In this study, we further investigated the regulation of KLK10 by steroid hormones, including estrogens, androgens, norgestrel, dexamethasone, and aldosterone, at the protein level. The KLK10 gene promoter was also functionally characterized.

2. Materials and methods

2.1. Chemicals

17β-Estradiol, 5α-dihydrotestosterone, dexamethasone, aldosterone and norgestrel were from Sigma, St. Louis, MO.

2.2. Cell lines

The breast cancer cell lines BT-474, MCF-7, T-47D, BT-20 and ZR-75-1, the ovarian cancer cell lines HTB-75 and OVCAR-3, the prostate cancer cell line LNCaP, and the human embryonic kidney cell line (HEK) were all from the American Type Culture Collection (ATCC), Rockville, MD. The ovarian cancer cell line BG-1 was a gift form Dr. H. Rocheford, Montpellier, France and the MFM-223 breast carcinoma cell line was a gift from Dr. R. Hackenberg, Marburg, Germany.

2.3. Stimulation of the cancer cell lines with steroid hormones

Cancer cell lines were cultured in RPMI media (Gibco BRL, Gaithersburg, MD) supplemented with glutamine (200 mmol/l), bovine insulin (10 mg/l), fetal bovine serum (10%), antibiotics and antimycotics in plastic flasks, at 37 °C, 5% CO_2, to near confluency. The cells were then aliquoted into 24-well tissue culture plates and cultured to 50% confluency. One day before the experiments, the culture media were changed into phenol red-free media containing 10% charcoal-stripped fetal bovine serum. To carry out the stimulation experiments, various steroid hormones dissolved in 100% ethanol were added into the culture media, at a final concentration of 10^{-8} mol/l. Cells stimulated with 100% ethanol were included as controls. The cells were maintained for another 24 h, then harvested for mRNA extraction. For hK10 protein measurements, cells were seeded in 75 cm^2 flasks and steroid stimulation was performed as described above. Culture supernatant was collected 7 days later.

2.4. Time course experiments

The BT-474, MCF-7 and T-47D cells were cultured and stimulated as described above. Cells were harvested for mRNA extraction at 1, 2, 4, 8, 24, 36 and 48 h after hormone stimulation. For hK10 protein measurements, culture supernatants were collected 1, 3, 5 and 7 days after steroid hormone stimulation.

2.5. Blocking experiments

The BT-474, MCF-7 and T-47D cells were cultured as described in the stimulation experiments. To block the steroid hormone receptors, antagonists for different steroid hormones were added into the culture media at a final concentration of 10^{-6} mol/l (100 times higher than the concentration of the stimulating steroids) and incubated for1 h. Subsequently, different steroid hormones were added into the culture media at a final concentration of 10^{-8} mol/l. Cells were maintained for 7 days and the culture supernatant was harvested for hK10 measurement.

2.6. Total RNA extraction and reverse transcriptase-polymerase chain reaction

Total RNA was extracted from the cell lysates using Trizol reagent (Invitrogen Canada, Burlington, ON) following the manufacturer's instructions. Total RNA concentration was determined spectrophotometrically. Two micrograms of total RNA was transcribed into cDNA with OligodT primer in a reaction mixture of 20 μl using the Superscript™ preamplification system (Invitrogen Canada). PCR was performed for KLK10, PSA, actin and PS2 with the primers and conditions described elsewhere [23].

2.7. Quantification of hK10 in cell culture supernatants

The concentration of hK10 was measured with an hK10-specific immunoassay developed in our laboratory [6,24]. In brief, 96-well polystyrene plates were first coated with 500 ng/well of hK10 monoclonal antibody (code B14). After overnight incubation, the plates were washed. One-hundred microliters of culture supernatant or standards and 100 μl of another biotinylated hK10 monoclonal antibody (code 5D3) (50 ng of antibody per well) were then sequentially added into each well. The plates were incubated for 2 h with gentle shaking at room temperature, and washed. Subsequently, alkaline phosphatase-conjugated strepadvidin was added, incubated for 30 min, and washed. Finally, diflunisal phosphate (DFP) and terbium-based detection was performed. Fluorescence was measured with a time-resolved fluorometer, the Cyberfluor 615 Immunoanalyzer (MDS Nordion, Kanata, ON, Canada) as described elsewhere [25]. The calibration and data reduction were performed automatically.

2.8. Construction of the KLK10 promoter-CAT reporter gene plasmid

A PAC clone derived from the kallikrein gene locus, containing the KLK10 gene and its 5′ flanking region, was isolated from a genomic library as previously described [3]. Using this PAC as a template, the 2.5-kb full-length KLK10 promoter was obtained by PCR with primers 5′-CGTTCTAGAGATCCAATGGCT-3′ (forward) and 5′-AGGGGATCTAGACGTGCCTCT-3′ (reverse). This fragment was then cloned into the promoterless pCAT-basic vector (Promega, Madison, WI) upstream of the chloramphenicol acetyltransferase (CAT) gene using standard molecular biology methods. The construct was verified by DNA sequencing.

2.9. Control plasmids containing functional AREs and EREs

The plasmids containing functional AREs and EREs were available in-house. They were used in all transfection experiments as positive controls. In the ARE plasmid, the PSA promoter, including ARE I, ARE II and the enhancer AREs were cloned in front of the CAT reporter gene in the pCAT-basic vector [15]. Similarly, in the ERE plasmid, four copies of the consensus ERE sequence (5′ CAGGTCAGAGTGACCTG 3′) were inserted upstream of a minimal promoter (derived from the *Xenopus* vitellogenin B1 gene), to direct the expression of the CAT reporter gene [26].

2.10. DNA transfection and reporter gene assays

The day before transfection, the HEK, T-47D, LNCaP, ZR-75-1, and BG-1 cells were plated in phenol red-free RPMI or DMEM media containing 10% charcoal-stripped fetal bovine serum at a density so that they were 80–90% confluent on the day of transfection. Ten micrograms of plasmid containing the KLK10 promoter, or ARE, or ERE, or empty vector pCAT-basic, along with 0.5 μg pRSV-β-gal control plasmid (Promega) were transfected into the cells with the Lipofectamine™ 2000 reagent (Invitrogen Canada) following the manufacturer's recommendations. Six hours after transfection, steroid hormone stimulation was performed as described above. Forty-eight hours after the transfection, cell monolayers were washed with phosphate buffer saline and lysed with the lysis buffer from the CAT ELISA kit (Boehringer Mannheim, Germany). This kit was subsequently used to measure the CAT concentrations in the cell lysates following the manufacturer's instructions. The β-gal activities in the cell lysates were quantified with the *o*-nitrophenyl β-D-galactopyranoside (ONPG) substrate.

3. Results

3.1. Differential regulation of KLK10 expression by steroid hormones in various cancer cell lines

Previously, we have demonstrated by RT-PCR, that KLK10 expression was up-regulated mainly by estrogens, and to a lesser extent by androgens and progestins in the breast cancer cell line BT-474 [23]. In order to examine whether the hormonal regulation of KLK10 is cell-line specific, in the current study, we extended our investigation to a panel of other cancer cell lines that contain different amounts of steroid hormone receptors (Table 1). The regulation of

Table 1
Cancer cell lines included in the study and their levels of ER, AR and PR

Cell line	Receptor levels[a]		
	ER	AR	PR
Breast cancer cell line			
BT-474	+	+	+
MCF-7	++	+	+
T-47D	+	+	+++
ZR-75-1	+	++	+
BT-20	–	–	–
MFM-223	–	–	+++
Ovarian cancer cell line			
BG-1	+++	+	+++
Caov-3	+	unknown	+
OVCAR-3	+	+	+
Prostate cancer cell line			
LNCaP	–	++++	–

[a] Data of receptor levels are from the following references: breast cancer cell lines, Ref. [8]; ovarian cancer cell lines, Refs. [39–43]; prostate cancer cell line LNCaP, Ref. [44].

KLK10 expression by steroid hormones was examined at both the mRNA and protein level. The steroid hormones tested included 17β-estradiol, 5α-dihydrotestosterone (DHT), norgestrel, dexamethasone, and aldosterone.

The regulation of KLK10 by these steroids was first investigated at the mRNA level with RT-PCR. Since alcohol was used to dissolve all steroids and it had no effect on KLK10 expression, alcohol-treated cells were used as controls [23]. Upon steroid hormone stimulation, KLK10 expression was not affected in the hormone receptor-negative cell line BT-20. Among the receptor-positive cell lines, only the breast cancer cell lines BT-474, MCF-7 and T-47D showed increased KLK10 expression upon stimulation (Fig. 1A). The potency of each steroid hormone varies among the cell lines. In the BT-474 cells, the KLK10 gene is mainly up-regulated by 17β-estradiol, and to a lesser extend by DHT. Norgestrel, dexamethasone and aldosterone have no significant effect. These results are in accord with our previous observations [23]. In the MCF-7 cells, all steroid hormones can up-regulate KLK10 gene expression. Norgestrel appears to be the most potent stimulant. In the T-47D cells, DHT up-regulated KLK10 gene expression more prominently than the other tested steroids.

We then examined the effect of steroid hormones on KLK10 expression at the protein level. Upon steroid hormone stimulation, as expected from the mRNA data described above, only the BT-474, MCF-7 and T-47D showed increased hK10 protein production (Fig. 1B). In the BT-474 cells, 17β-estradiol and DHT were the strongest up-regulating steroids for hK10 protein production, inducing at least 50- and 20-fold increase in hK10 protein level, respectively. Norgestrel displayed a weak stimulation effect and dexamethasone and aldosterone had no significant induction of hK10 production. In the MCF-7 cells, the effect of steroid hormones is different. Norgestrel was the most potent stimulant and increased hK10 protein levels in the supernatant by about 7-fold. The rank of potency was norgestrel>DHT>dexamethasone>17β-estradiol and aldosterone. Similarly, all steroid hormones exhibited a stimulation effect on hK10 production in the T-47D cells. However, the rank of potency was different, being DHT>dexamethasone>aldosteone>17β-estradiol and norgestrel. The strongest stimulant DHT increased hK10 protein levels in the supernatant by about 8-fold. These results are consistent with the RT-PCR data presented above, suggesting that the up-regulation of hK10 expression by steroid hormones is concordant at the mRNA and protein level.

3.2. The effect of steroid hormone antagonists on steroid hormone-induced hK10 protein production

To further examine whether the steroid hormones tested up-regulate hK10 production through their own receptors, we performed blocking experiments, in which antagonists of estrogens (ICI-182,780), androgens (RU-56,187) and progestins (mifepristone) were used to block their cognate receptors prior to adding the steroid hormones. These antagonists, when added alone, at 10^{-6}–10^{-9} mol/l concentration, had no effect on hK10 protein production (data not shown). When they were incubated in mixtures with different steroid hormones, they exhibited a different degree of blocking effect. The results obtained from the breast cancer cell lines BT-474, MCF-7 and T-47D are presented in Table 2. As expected, the androgen antagonist RU-56,187 could block most of DHT activity, but not 17β-estradiol and norgestrel. Mifepristone, a progesterone and androgen antagonist, could block effectively hK10 production induced by

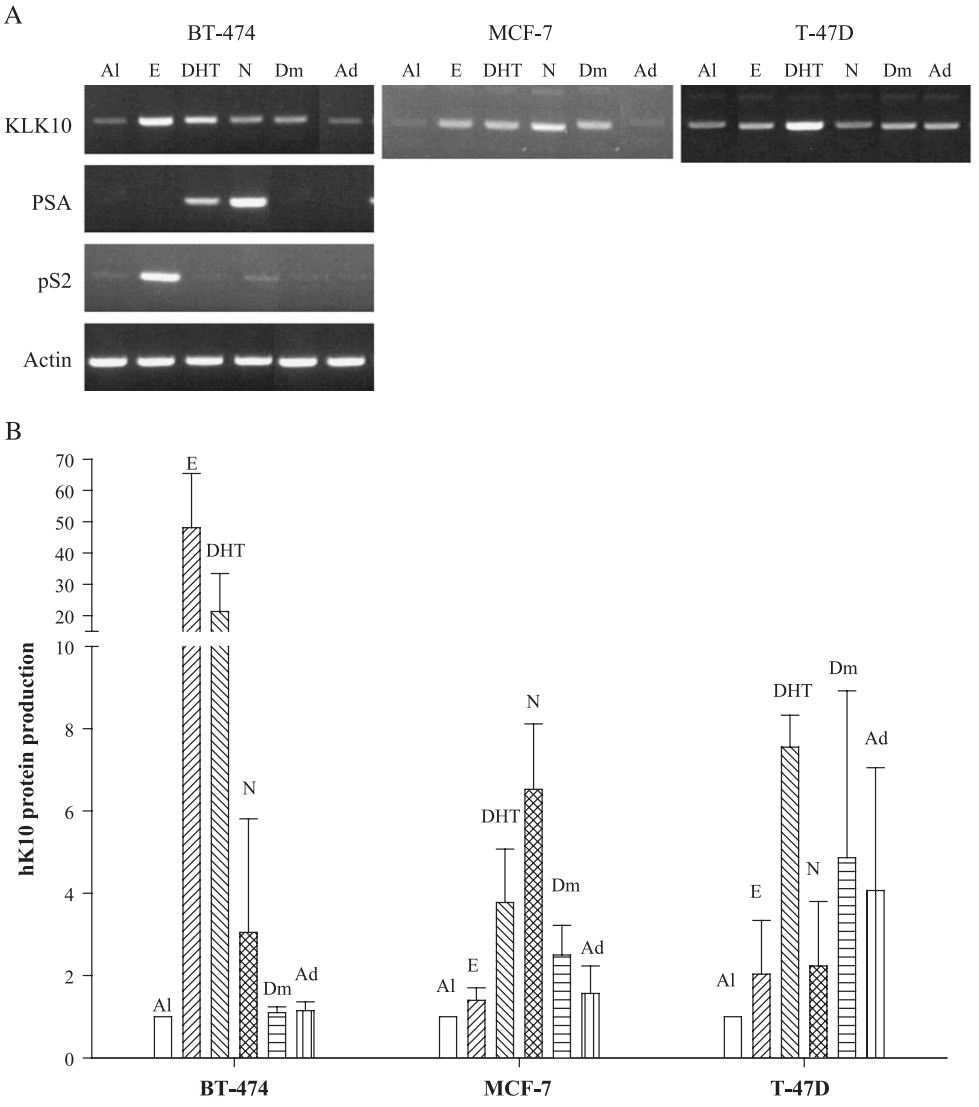

Fig. 1. Differential regulation of KLK10 gene expression by steroid hormones at the transcription level, as shown by RT-PCR (A) and translation level (B) in the breast cancer cell lines BT-474, MCF-7 and T-47D. In panel A, the PSA gene (up-regulated by androgens and progestins) and pS2 gene (up-regulated by estrogens) and actin gene were used as controls. In panel B, hK10 protein levels were quantified with an ELISA method [6] and expressed as fold induction over the hK10 protein level produced by the alcohol-treated cells. Al, ethanol; E, 17β-estradiol; DHT, 5α-dihydrotestersterone; N, norgestrel; Dm, dexamethasone; Ad, aldosterone.

norgestrel and DHT. ICI-182,780, being an estrogen antagonist, almost completely blocked the hK10 production induced by 17β-estradiol. It also significantly reduced expression of hK10, induced by DHT and norgestrel. These data indicate that 17β-estradiol, DHT and norgestrel, up-regulate hK10 protein production by binding to their cognate receptors.

3.3. Kinetics of steroid hormone-regulated KLK10 expression

To study the kinetics of KLK10 gene expression after steroid hormone stimulation, time course experiments were conducted. BT-474, MCF-7 and T-47D cells were stimulated with 17β-estradiol and DHT at a

Table 2
Blocking of steroid hormone-induced hK10 protein production by steroid hormone antagonists

Stimulating hormone	Percentage of blocking on hK10 production (%)[a]		
	RU-56,187	ICI-182,780	Mifepristone
17β-Estradiol	0	65–99	0
DHT	66–87	51–73	62–87
Norgestrel	0	64–65	84–85

[a] Data presented are percentage ranges obtained from three experiments in the breast cancer cell lines BT-474, MCF-7 and T-47D.

concentration of 10^{-8} mol/l and cells were harvested at different time points. Previously, we have shown that upon steroid hormone stimulation, the kinetics of KLK10 gene expression in BT-474 cells are similar to those of pS2 and PSA genes and that steroid hormone stimulation results in a steady accumulation of the KLK10 mRNA over 24–48 h in BT-474 cells [23]. When the same experiments were repeated in the MCF-7 and T-47D cells, similar results were obtained (data not shown). To further investigate whether this increase of KLK10 mRNA could lead to the elevation of secreted hK10 protein level, the kinetics of hK10 protein production upon steroid hormone stimulation were also examined. As shown in Fig. 2, hK10 protein level in the culture supernatant starts to increase 1 day after stimulation and continues to accumulate over 5–7 days.

3.4. Organization of the KLK10 promoter

The data presented above clearly demonstrate that the KLK10 gene is up-regulated mainly by estrogens, androgens and progestins in breast cancer cell lines at both the transcriptional (mRNA) and translational (protein) level. The rapid increase of hK10 protein level in response to steroid hormone stimulation indicates that steroid hormones up-regulate KLK10 expression through the direct interaction between the hormone-receptor complexes and HREs. Therefore, we decided to functionally characterize the KLK10 gene promoter, to determine whether it contains any HREs. A schematic diagram illustrating the organization of the KLK10 gene promoter in the kallikrein locus is shown in Fig. 3. The KLK10 gene resides downstream of the KLK11 gene. The sequence between the polyadenylation site "AATAAA" of the KLK11 gene and the transcription start site of the KLK10 gene is considered to include the full-length KLK10 gene promoter. Since its homologous gene, PSA (KLK3), is known to contain AREs in its promoter, we first investigated whether there was sequence similarity between the PSA and KLK10 gene promoters. A homology comparison between the full-length KLK10 gene promoter and the 5-kb PSA gene promoter (Genbank accession number U37672), which contains the ARE I, ARE II and enhancer AREs, was performed with the BLAST2 algorithm. No significant homology was found (data not shown). We then functionally characterized the KLK10 gene promoter. Two primers were designed to amplify and clone the full-length KLK10 promoter sequence by PCR. The forward primer lies 40 bp downstream of the polyadenylation site of the KLK11 gene and the reverse primer is in the untranslated exon I (Fig. 3). Thus, the obtained sequence includes the full-length KLK10 promoter and 37 bp from exon I. This sequence was then placed upstream of the chloramphenicol acetyltransferase (CAT) reporter gene.

3.5. Full-length KLK10 promoter activity in different cancer cell lines with various estrogen and androgen receptor levels

To investigate the role of ER and AR in KLK10 gene expression, the full-length KLK10 promoter activity was investigated in various cell lines that

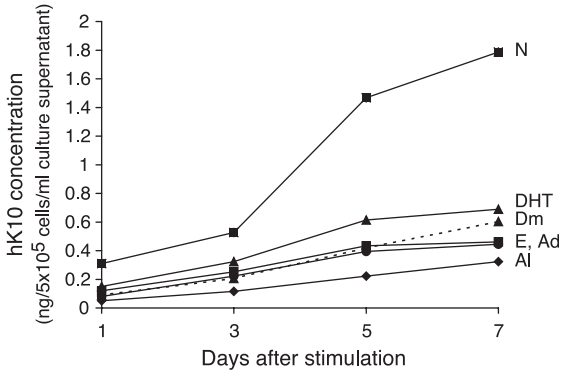

Fig. 2. Kinetics of hK10 protein production induced by steroid hormones in the breast cancer cell line MCF-7. The hK10 protein was quantified in the tissue culture supernatant by ELISA [6]. Al, ethanol; E, 17β-estradiol; DHT, 5α-dihydrotestersterone; N, norgestrel; Dm, dexamethasone; Ad, aldosterone.

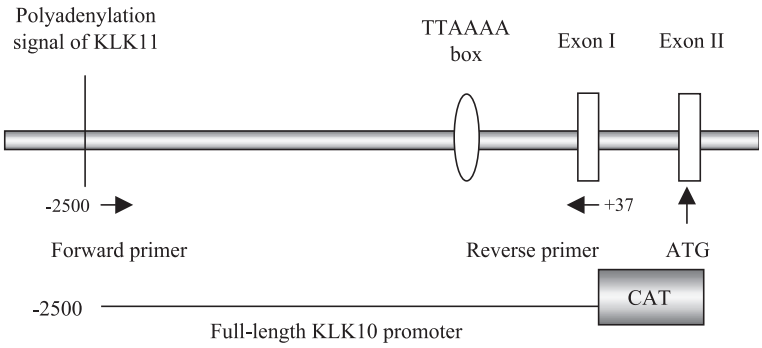

Fig. 3. Schematic diagram showing the organization of the KLK10 gene promoter in the kallikrein locus and the construction of the KLK10 promoter-CAT reporter gene plasmids. Exons I and II of the KLK10 gene are represented with boxes. The oval shape box denotes the TATA box variant "TTAAAA" box. The first base pair of the cDNA sequence (Genbank accession No.: AF024605) was assigned as "+1". A forward primer located in exon I (+37) and a reverse primer located 40 bp downstream of the KLK11 polyadenylation site were designed to obtain the full-length KLK10 promoter by PCR.

have different amounts of steroid hormone receptors. Plasmids containing functional AREs (the PSA promoter) and EREs (ERE control plasmid) were included as positive controls in all transfections. We first studied KLK10 promoter activity in the human embryonic kidney cells (HEK), which lack both ER and AR. In the HEK cells, the PSA promoter and ERE control plasmids had no significantly higher activity than the promoterless pCAT-basic vector. Presumably, this lack of activity is due to the absence of active ER and AR. In contrast, in this cell line, the KLK10 promoter is very active and its activity is up to 35-fold higher than that of the pCAT-basic vector (Fig. 4). These data indicate that unlike the PSA promoter, the KLK10 promoter does not rely on the presence of ER and AR to be active.

We next examined whether the presence of active ER and PR could increase the KLK10 promoter activity in various cancer cell lines with different amounts of ER and AR. The breast cancer cell line ZR-75-1 and the ovarian cancer cell line BG-1 were included as ER-positive cell lines. We obtained similar results in the ZR-75-1 and BG-1 cells. Representative data obtained with BG-1 cells are presented in Fig. 5A. It is shown that the KLK10 promoter has very low activity (2- to 4-fold increase over the pCAT-basic vector). The low KLK10 promoter activity in the breast/ovarian cancer cells in comparison to the HEK cells may be attributed to lower transfection efficiency or different transcription factor profiles in these cells. Upon 17β-estradiol challenge, the hK10 promoter activity does not significantly increase. However, although the ERE control plasmid is not active without 17β-estradiol treatment, its activity is

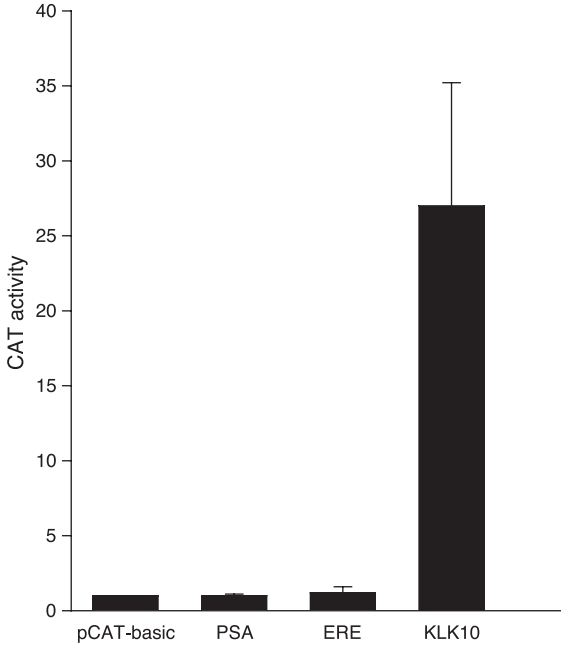

Fig. 4. Full-length KLK10 promoter activity in the human embryonic kidney (HEK) cells. The pCAT-basic, PSA and ERE are control plasmids. CAT activity is presented as fold induction over the pCAT-basic vector. Note significant KLK10 promoter activity in the HEK cells without any stimulation or presence of steroid hormone receptors.

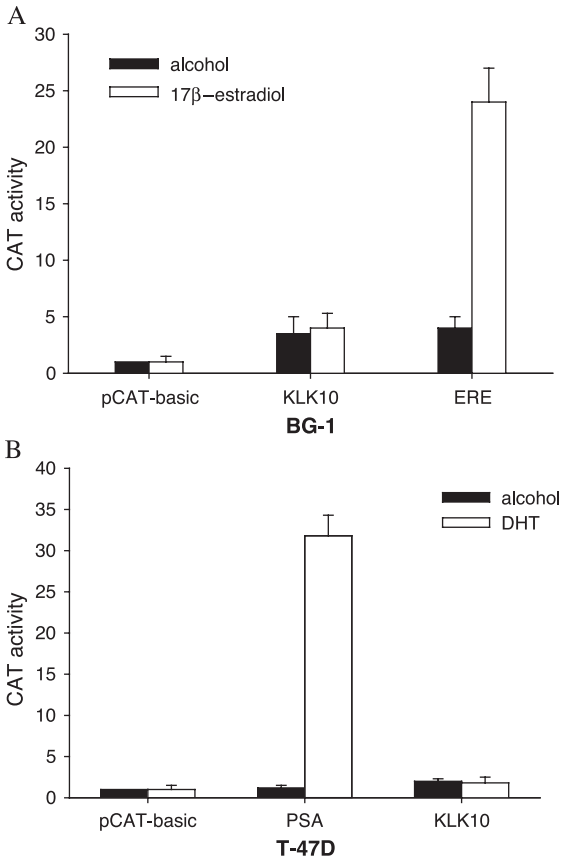

Fig. 5. Full-length KLK10 promoter activity in the presence of active ER and AR. (A) Full-length KLK10 promoter activity stimulated with alcohol and 17β-estradiol in the ovarian cancer cell line BG-1. (B) Full-length KLK10 promoter activity stimulated with alcohol and DHT in the breast cancer cell line T-47D. The plasmids p-CAT basic, PSA and ERE are controls. CAT activity is presented as fold induction over the pCAT-basic vector. Note absence of KLK10 promoter activity after stimulation.

dramatically increased (up to 28-fold) after stimulation with 17β-estradiol. Similar experiments were repeated in the AR-positive prostate cancer cell line LNCaP and the breast cancer cell line T-47D. Representative data for T-47D cells are shown in Fig. 5B. Clearly, the PSA promoter activity depends on the activation of AR by DHT. In sharp contrast, the KLK10 promoter activity is low and it does not increase upon DHT stimulation. These data suggest that the KLK10 promoter transcriptional activity is not affected by the presence of active ER or AR. We conclude that the KLK10 promoter sequence examined does not contain HREs that mediate the regulation of KLK10 gene expression by estrogens and androgens.

4. Discussion

In this investigation, we demonstrated that KLK10, a new member of the human tissue kallikrein gene family, is up-regulated by estrogens, androgens, progestins, glucocorticoids and minerolocorticoids at both the transcription and translation level. Through functional characterization of the KLK10 promoter, we have shown that the KLK10 gene promoter is unlikely to harbor HREs that mediate this hormonal regulation of KLK10.

The expression of KLK10 is up-regulated only in the receptor-positive breast cancer cell lines BT-474, MCF-7 and T-47D, but not in the receptor-negative cell line BT-20. These data suggest that the presence of functional ER, AR and PR is a prerequisite for the hormonal regulation of KLK10. However, although the other tested cancer cell lines also have functional ER, PR or AR (Table 1), the steroids 17β-estradiol, norgestrel and DHT did not show any effect on KLK10 expression. The data suggest that the presence of active steroid hormone receptors does not necessarily imply that KLK10 will be regulated by steroid hormones. Furthermore, the potency of the steroid hormones on KLK10 expression does not correlate with the levels of their cognate receptors in these cell lines. 17β-Estradiol, DHT and norgestrel are most potent in the BT-474, T-47D and MCF-7 cells, respectively (Fig. 1). However, the most abundant ER and PR concentrations are found in the MCF-7 and T-47D cells, respectively (Table 1). This lack of hormonal regulation in certain receptor-positive cell lines and the dissociation between steroid hormone potency and levels of their cognate receptors could be due to involvement of other cellular factors or the KLK10 gene itself. It is now clear that the function of steroid hormone receptors is modulated by steroid hormone receptor coactivators/corepressors, which act as bridging molecules between hormone-receptor complexes and the basal transcription machinery, to turn-on or suppress transcription [27,28]. Consequently, the concentration of steroid hormone receptors does not necessarily determine their functional activity. It has

been shown that the levels of a number of coactivators/corepressors vary significantly among different breast cancer cell lines [29]. Therefore, it is possible that different amounts of coactivators/corepressors could play an important role in the differential regulation of KLK10 by steroid hormones in the BT-474, MCF-7 and T-47D cells. In the receptor-positive ovarian cancer cell lines, the lack of hormonal regulation of KLK10 could be due to absence of critical coactivators/corepressors. Epigenetic modifications of the KLK10 gene may also interfere with steroid hormone regulation. It has been demonstrated that methylation of the KLK10 gene (exons 2–4) is a mechanism that could account for the down-regulation of KLK10 gene expression in some breast cancer cell lines, such as ZR-75-1 [30]. Given that the KLK10 gene is silenced by methylation in the ZR-75-1 cells, it is not surprising that the expression of the KLK10 gene is not induced by steroid hormones despite the fact that the ZR-75-1 cells are receptor-positive.

Estrogens, androgens and progestins are all key modulators of KLK10 gene expression. These hormones, besides interacting with their own cognate receptors through high affinity binding, are known to be able to cross-bind to other receptors through low affinity association. It has been reported that estrogen (at a concentration of 10^{-7} mol/l) can also bind to AR [31]. This cross-interaction is thought to primarily account for the estrogen up-regulation of PSA gene expression in the breast cancer cell line BT-474 [8]. The regulation of KLK10 by steroid hormones, however, does not seem to involve cross-binding of steroid hormones to steroid hormone receptors. As we previously reported, steroid hormones up-regulate KLK10 gene expression in a dose-dependent manner, and this induction can occur at a concentration as low as 10^{-13} mol/l, which is 100-fold lower than the affinity constant of their association to their own receptors [23]. Furthermore, in the blocking experiments, it was shown that the up-regulation of hK10 by these steroid hormones could be blocked by their cognate antagonists (Table 2). The blocking of ICI-182, 780 on norgestrel action may be due to the fact that PR expression is up-regulated by estrogen and that ICI-182, 780 reduces PR expression. Our data indicate that estrogens, androgens and progestins up-regulate KLK10 expression through binding to their own receptors.

KLK10 is abundantly expressed in the sex organs, including breast, ovary, prostate and testis. It is reasonable to speculate that the expression of KLK10 in these organs is modulated by sex hormones. Our data obtained from the breast cancer cell lines support this notion. In ovarian cancer, hK10 protein level is greatly elevated [32]. Androgen and estrogen over-stimulation has been implicated with the pathogenesis of this malignancy [33,34]. Therefore, the overexpression of KLK10 in ovarian cancer may be due to sex hormone imbalance. However, as also indicated by our studies in the cancer cell lines, sex hormones may not be the only modulators of KLK10 gene expression in these organs. In breast cancer, we found that high hK10 levels negatively correlated with the levels of ER and PR [35], suggesting that other factors are likely implicated. Therefore, when interpreting KLK10 expression levels in the sex organs and steroid hormone-related diseases, a complicated interplay among the KLK10 gene, steroid hormone receptors, and other KLK10 modulators should be considered.

Our time course studies clearly showed that hK10 protein started to increase in rapid response to steroid hormone stimulation, indicating that steroid hormones up-regulate KLK10 gene expression very likely through the direct interaction between hormone-receptor complexes and their cognate hormone response elements. Using various ER and AR positive- and negative-cell lines and the CAT reporter gene system, we have shown that the full-length KLK10 gene promoter is active, regardless of ER and AR presence. Furthermore, presence of active ER and AR does not increase the constitutive activity of the KLK10 gene promoter. These features of the KLK10 promoter are very different from those of its homologous gene, PSA, in which promoter activity entirely depends on, and is dramatically induced by, the presence of active AR. The lack of hormonal response of the KLK10 promoter could be due to the following reasons. First, the full-length KLK10 promoter we examined is unlikely to contain any active AREs or EREs. Although most of the EREs and AREs are present in the promoters of the target genes, other regions, such as exons and the 3′ untranslated region, may also possibly harbor HREs. Recently, it has been reported that exons 2–4 of the KLK10 gene are involved in the modulation of KLK10 gene expression through DNA

methylation [30]. It is conceivable that these exons might also contain other transcription factor binding sites, including HREs. Second, it is possible that the HREs mediating the hormonal regulation of KLK10 are not within the KLK10 gene itself, but locate elsewhere within the KLK locus. Within the human kallikrein gene family, except for KLK2, KLK3 (PSA) and KLK4, which are all clearly up-regulated only by androgens, expression of all other KLK genes is modulated by multiple steroid hormones [1]. Interestingly, all these KLK genes are transcribed in the same direction (telomere to centromere) opposite to that of KLK2 and KLK3. Therefore, it is likely that most of the KLK genes are regulated by a common locus. This mode of regulation has been observed in many gene families, such as the β-globin family [36,37]. Also, this mechanism has been proposed to explain the tissue expression pattern of the rat kallikrein gene family. It has been speculated that a common control region upstream of the entire rKLK locus may confer the salivary gland expression of all the rKLK genes [38].

In summary, KLK10 is mainly up-regulated by estrogens, androgens, and progestins, and to a less extent, by dexamethasone and aldosterone at both the transcription and translation level. It is unlikely that the KLK10 promoter contains HREs that directly mediate this hormonal regulation. Other regions within the KLK10 gene and the human tissue kallikrein locus should be considered.

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