Kallikrein 4 Is a Secreted Protein

To the editor: In the April 1, 2004 issue of Cancer Research, Xi et al. (1) reported that kallikrein 4 (KLK4) is predominantly a nuclear protein. They state that the KLK4 gene has only four exons when actually it has five (the first exon is noncoding; ref. 2), and they state that the first coding exon is not physiologically significant and is not present in the vast majority of KLK4 transcripts. We would like to raise several concerns about the experimental evidence used to support these conclusions.

The authors performed routine reverse transcription-PCR reactions that paired a common 3′-PCR primer with one of six different 5′-primers. Using total RNA from LNCaP cells as substrate, the authors discovered that the 5′-primers annealing to the first coding exon did not amplify KLK4 mRNA as efficiently as 5′-primers specific for the second coding exon. From this, they concluded that: “The putative first (coding) exon of KLK4, if present is at extremely insignificant low levels compared with downstream sequences.” No other analyses of the KLK4 transcripts were performed. Perhaps nicking of the mRNA during isolation and/or failure of the reverse transcriptase to extend to the 5′-end of the KLK4 transcripts can best explain these results.

On the basis of their reverse transcription-PCR results, the authors’ appear to demonstrate that coding exon 2 is at the 5′-end of their KLK4 mRNA. This is not possible because the 5′-end of coding exon 2 contains a consensus splice junction and not the required transcription initiation site. However, even if we assume that coding exon 2 does contain a valid transcription initiation site, the first ATG (Met50) is located midway through the exon (Fig. 1). Initiation at the Met50 codon deletes the signal peptide (Met1-Val24), propeptide (S25-Q30), and the NH2 terminus of the active enzyme (I32-W49). The KLK4 antibody the authors used to demonstrate nuclear localization was raised against the NH2-terminal peptide QIINGEDCSPHSQPW (Q31-W45). This antibody would not have detected a protein translated from transcripts starting with coding exon 2. This is a major inconsistency. The authors used this antibody for their Western blot analyses to demonstrate nuclear localization. This antibody did not detect KLK4 in the nucleus. Cross-reaction of the anti-peptide KLK4 antibody with an unidentified nuclear protein. They state that the first coding exon is not physiologically significant and is not present in the vast majority of KLK4 transcripts. We hope your readers will not accept as unchallenged the conclusion that KLK4 is the first member of the KLK family that is intracellularly localized.

Unfortunately, the authors were apparently unaware of the published data with the enamel matrix serine proteinase 1 designation. We hope your readers will not accept as unchallenged the conclusion that KLK4 is the first member of the KLK family that is intracellularly localized.

References
In Response: We have reported that human KLK4 is the first member of the kallikrein family that is intracellular in prostate cancer cells based on molecular, cellular, and biochemical data (1). Simmer and Bartlett raised several questions regarding both the methodology and the conclusions of our study. These concerns are largely based on previous studies that used bioinformatic methods to deduce the 5'-end of the KL4 mRNA, as well as studies done on nonhuman forms of KLK4 and the protein that they encode. Our conclusions, however, are based on direct experimental evidence and specifically on human KLK4 and the protein it encodes, hK4. This evidence is described below in detail.

To explain the lack of PCR amplification of sequences containing exon 1 of KLK4 in our experiments, Simmer and Bartlett propose technical difficulties, such as nicking or failure of extension by reverse transcriptase. This is extremely unlikely for the following reasons, which were in large part included in the current article (1) and a previous report (2) also from our laboratory:

1. KLK4 mRNA is one of the most abundant mRNA species in prostate cancer cells (our unpublished results). To suggest that nicking will occur in exactly the same region for all or majority of the KLK4 mRNA species (at the junction of exon 1 and exon 2) is not warranted. To our knowledge, there is no example of sequence-specific nicking of mRNA molecules.

2. In contrast to what Simmer and Bartlett suggest, we have carried out various additional studies on the characterization of KLK4 5'-end, some of which have previously been published. These included extensive 5'-rapid amplification of cDNA ends analysis and primer extension analyses, which did not yield any sequences containing exon 1 (2). Furthermore, we have exhaustively screened for human KLK4 in at least two independent cDNA libraries that are enriched for 5'-ends (SMART libraries, Clontech) and did not find any clones containing the proposed exon 1. In fact, to date, we are not aware of any report on the cloning of KLK4 containing the proposed exon 1, from any species, by non-PCR screening of cDNA libraries.

3. PCR amplification was used by others (3) also to find that KLK4 exon 1 sequences can be amplified from human prostate tissue. Almost identical and consistent with our data, it required these investigators 43 PCR cycles to obtain amplification, suggesting that these are very rare and unlikely to be physiologically significant.

4. As noted in our article and consistent with the data provided therein, in the human expressed sequence tag database, there are no expressed sequence tags for KLK4 that contain exon 1 sequences (none of 20), whereas one readily finds expressed sequence tags for the same region for other KLKs, e.g., KLK3 or PSA (at least 20 entries with parts or all of exon 1-specific sequences present), a known secreted protein in prostate of the KLK family that has the corresponding exon 1 represented in its mRNA.

5. The reverse transcription-PCR was performed by eight different primer pairs (six in the current study and two in a previous report (2)) and therefore the data are very robust.

6. In contrast to what is pointed out by Simmer and Bartlett, our analysis did not only include routine reverse transcription-PCR reactions, but quantitative reverse transcription-PCR analysis as well, noted as “data not shown” in our article and inspected during the review process by the reviewers.

7. Others have independently arrived at similar conclusions as us (e.g., Nathalie Martin, INSERM, Paris, France, personal communication).

In summary, our analysis of KLK4 mRNA 5'-end is very detailed, robust, and consistent with all of the other data we have on hK4.

The second issue that was raised is related to the specificity of the antiserum that we have raised and used in this study. This antiserum, an anti-peptide antiserum that was affinity purified before use, has been extensively characterized as described in the article, and these data were also provided as “data not shown” during the review process to the journal. First, the specificity of this antiserum was verified by ectopic expression and peptide competition experiments by Western blot analysis (Fig. 1), as well as immunohistochemistry (data not shown). The specific peptide successfully competed the binding of the antibody to hK4 that is ectopically expressed in Cos cells, in LNCaP whole cell extracts, or in human prostate tissue. Second, hK4 antiserum does not recognize the close family members KLK2 and KLK3 in prostate cancer cells, which are abundant proteins in this cell type. Third, the accumulation of the protein that is recognized by our antiserum in LNCaP cells is regulated by androgens and, to a weaker extent, by estrogen and progesterone, consistent with the Northern blot data (e.g., ref. 2), indicating that our antiserum is specific to hK4. In addition, this antiserum recognizes a protein predominantly expressed in the basal cell compartment of prostate epithelium (data not shown), consistent with the mRNA in situ hybridization data (1). Lastly, the tagged versions of hK4, recognizable by specific antisera against the tags, when ectopically expressed, have predominantly nuclear localization, similar to native hK4 in LNCaP cells (1) and ovarian carcinoma cells (data not shown). In summary, these data are very strong evidence that our antiserum is specific to hK4.

Given the robustness of the reverse transcription-PCR experiments and the specificity of the antiserum, the question arises as to what residue is the translation start point for hK4. Because there are no methionines within or upstream of the peptide that we used to raise the antiserum in KLK4 mRNA, these data suggest that KLK4 translation may be achieved without an AUG and initiator tRNA. It is one of the most common beliefs among molecular biologists that an AUG and an initiator tRNA is required for initiation of protein synthesis, but this is not the case (4). The current data suggest that an alternative translation initiation may be used in the case of KLK4. Additional work is required in this regard. In summary, contrary to what was suggested, there is no inconsistency in the data presented in terms of the location of the peptide used for immunization and the data obtained with this antiserum.

The next question is related to the size of hK4 in human prostate. Simmer and Bartlett point to studies by Obiezu et al. (3) who used an antiserum that they generated and size exclusion gel chromatography to estimate the size of hK4 in prostate cancer specimens or amniotic fluid and arrived at a figure of ~30 kDa. In contrast, we have used Western blot analysis on LNCaP cells, which, at least in part, may be the source of this discrepancy. Furthermore, using three independent polyclonal antisera specific for hK4 and Western blot analysis, Myers and Clements (5) and Dong et al. (6) found hK4 to be ~40 kDa in prostate, endometrial, and uterine cancer cell lines. This is not too different from our observations. It is also not clear as to why the antiserum used by Obiezu et al. (3) does not recognize hK4 in ~50% of the prostate cancer samples, where it is detected in 100% of the samples in our analysis (1), as well as of others (7). We also do not know the basis for the nuclear localization that we (Xi et al., unpublished data) observe as opposed to the cytoplasmic staining suggested...
by findings of the Obiezu group (8), although the figure provided therein (in black and white) does not allow conclusive evaluation of the nature of the staining; in fact, there appears to be prominent nuclear staining, but whether this is specific to hK4 or simply due to the counterstain cannot be ascertained. Clearly, these reagents, developed in different laboratories, should be tested side by side for specificity, as well as on the same biological samples with the same methodology, to conclusively examine the source of these apparent differences/discrepancies.

As for the references, when summarizing the original cloning reports on human KLK4, we regret that one of the references on PRSS17 involving Simmer et al. was deleted from our list, although the name was included in the text. We are certainly familiar with the work of Simmer and others on the nonhuman forms of KLK4. However, because our study focused only on human KLK4 and prostate cancer, which is a uniquely human disease, and given the space limitations of the journal, only the relevant studies could be included as references.

We hope that these points clarify the current status of our understanding of KLK4 in humans based on experimental evidence. Clearly, future work should focus on possible species- and tissue-specific differences in structure, expression, and function of KLK4, facilitated by free reagent exchange between the different groups and their testing, and not by theoretical/computational argumentation or comparison of different experimental paradigms, tissues, or species.

Fahri Saatcioglu
Department of Molecular Biosciences
University of Oslo
Oslo, Norway

References
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James P. Simmer and John D. Bartlett

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