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In situ hybridization of mRNA for the gastric H^+, K^+-ATPase in rat oxyntic mucosa

Krister Bamberg [b], Sven Nylander [a], Kerstin G. Helander [b], Lennart G. Lundberg [a], George Sachs [b], Herbert F. Helander [a,b,*]

[a] *Cell Biology, Astra Hässle AB, S-431 83 Mölndal, Sweden*
[b] *UCLA and CURE Digestive Disease Center, VA WLA Medical Center, Los Angeles, CA, USA*

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Abstract

The H^+, K^+-ATPase member of the phosphorylating ion motive ATPases is composed of two subunits, a large α-subunit composed of about 1030 amino acids and a smaller β-subunit consisting of about 290 amino acids. By biochemical and immunological methods both subunits have been found in high abundance in the gastric parietal cell. In the present study in situ hybridization was used for localizing and comparing concentrations of the mRNA for the two subunits in the gastric epithelium. For this purpose ^{3}H-labelled probes were preferred. Hybridization was detected only in the parietal cells. The older parietal cells in the bottom of the mucosa gave a weaker hybridization signal than the younger parietal cells closer to the surface. The margin of experimental ulcers, where the parietal cells are of low differentiation, showed very weak, if any, hybridization. The differences observed in hybridization densities may reflect differences in mRNA synthesis or stability. It is conceivable that older parietal cells, as well as parietal cells of low differentiation, produce relatively small amounts of H^+, K^+-ATPase.

Key words: Parietal cell; Gastric mucosa; Gastric ulcer; In situ hybridization; ATPase, H^+/K^+-

1. Introduction

The gastric H^+,K^+-ATPase is an α,β heterodimer that is a member of the phosphorylating class of ion motive ATPases [1–5]. The enzyme is responsible for the final step of acid secretion acting as an H^+ for K^+ exchange pump [6]. Immunocytochemistry using a polyclonal antibody localized the pump to the parietal cell (7). With monoclonal antibodies specific for the α-subunit or the β-subunit it has been shown that these subunits are located in the mammalian parietal cell [1,5,8,9].

Whereas immunocytochemical techniques are a sensitive method for detection and localization of proteins in tissues, it is more difficult to derive quantitative conclusions. In contrast, in situ hybridization, while less sensitive, has the potential of quantitating the level of mRNA present in a given cell type.

Both ^{3}H and ^{35}S were used to radioactively label the RNA probes. They were able to detect the presence of the mRNA's, but ^{3}H-labelled probes were preferred for the quantitative conclusions because of their more distinct cellular localization. The data demonstrate the expression of both subunits in the parietal cell and lower levels of the mRNA in the older parietal cells found in the deeper levels of the gastric glands. In addition, mRNA expression was much diminished, or even absent, in parietal cells in the margin of an experimental gastric ulcer.

2. Materials and methods

Construction of probes

Fragments of the cDNA for the α-subunit of the rat gastric H^+, K^+-ATPase [10] were cloned into a pGEM4 modified plasmid vector kindly supplied by BM Stålbom. Templates for in vitro transcription were made using the polymerase chain reaction (PCR). The fragment between nt 527 and 788 was amplified using a

* Corresponding author: H.F. Helander, Cell Biology, Astra Hässle AB, S-431 83 Mölndal. Sweden. Fax: +46 317763705.

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pair of primers synthesized against either end of the region and purified on OPC columns. One of the primers contained the T7 promotor on the 5′ end, so that the amplified fragments could be used directly for in vitro transcription.

PCR was carried out using standard conditions (94°C, 65°C, 72°C, 1 min each) with a lower annealing temperature for the first 4 cycles (53°C), followed by 34 cycles under the standard annealing conditions of 65°C. The fragments were separated using 2% agarose gel electrophoresis and a standard DNA lane. The standard lane and the edge of the lane containing the fragments were stained with ethidium bromide, and the fragments cut out of the unstained gel. The DNA was extracted by freezing and grinding. They were further purified using phenol/chisam extraction and ethanol precipitation.

Synthesis of the sense and antisense probes for the α-subunit was carried out by in vitro transcription using 300 ng of template and ^{35}S-CTP. Labelling efficiency was around 70%, resulting in a specific activity of $1.7 \cdot 10^9$ cpm/μg. The DNA template was removed by treating with RNase-free DNase, and the probe was further extracted with Tris-EDTA saturated phenol/chisam, suspended in 10 mM Tris-EDTA with 20 mM DTT and stored at −80°C until used.

For the synthesis of the antisense and sense probes against the β-subunit, the 5′ overhangs of the BstEII-BstEII fragment (997 nt) from a gastric H^+,K^+-ATPase β-subunit cDNA clone [11] (kindly provided by Dr. G. Shull) were filled in with Klenow polymerase (BRL, Gaithersburg, MD) and then the fragment was ligated into the *Sma*I site of the BluescriptSK$^-$ (Stratagene, La Jolla, CA) using T4 ligase. Antisense RNA was synthesized from linearized plasmid by T7 RNA polymerase. Sense RNA was transcribed by the T3 polymerase using manufacturer's (Promega, Madison, WI) protocol. 5,6^{3}H-UTP or ^{35}S-UTP was incorporated as the label. Following treatment with DNase I, the RNA probes were subjected to mild alkaline hydrolysis in a 0.1 M Na_2CO_3/$NaHCO_3$ buffer at 60°C for 20 min. The reaction was stopped by addition of 10% acetic acid and the RNA was precipitated. This treatment yielded RNA fragments of about 300 bases in length as determined by agarose gel electrophoresis.

Tissue preparation

Non-starved, adult, male rats were sacrificed and parts of the stomach from the corpus region were rapidly removed and put into 4% formaldehyde in 0.1 M phosphate buffer (pH 7.2) at room temperature (RT). Tissue containing the ulcer region as well as adjacent undamaged mucosa was obtained from a rat in which an experimental ulcer had been produced 3 days before death. For this purpose, the animal was anesthetized, the abdomen was opened and a limited area of the serosa of the gastric corpus was exposed to 80% acetic acid for one minute. Following rinsing with physiological saline, the abdomen was closed and the rat returned to the cage. This procedure results in a standardized wound in the oxyntic mucosa, which heals within 2–3 weeks [12].

After 2 h of fixation, the specimens were rinsed in the buffer, dehydrated through ethanol, cleared in xylene and paraffin embedded. Five μm thick sections were picked up on glass slides (Probe-on Plus, Fisher, Pittsburg, PA). The sections were cleared by two changes of xylene and hydrated through decreasing concentrations of ethanol. Other pieces of formaldehyde fixed gastric corpus were instead frozen, and 9 μm cryostat sections were used; there were no obvious differences in the results of the hybridizations when comparing paraffin sections with cryostat sections.

The sections were then treated with 0.02 M HCl for 10 min, rinsed with phosphate-buffered saline (PBS) and then placed in 0.01% Triton X-100 in PBS for 1 min. After rinsing they were digested in proteinase K (1 μg/ml, Sigma, St. Louis, MO) in 50 mM Tris, 5 mM EDTA (pH 8.0) for 5–30 min at 37°C. Following rinsing in PBS, the sections were placed in 2 mg/ml glycine in PBS for 6 min at RT and post-fixed briefly in 4% formaldehyde. The sections were rinsed and then acetylated for 10 min in a fresh solution of 0.25% acetic anhydride in 0.1 M triethanoleamine (pH 8.0). After a water rinse, the sections were allowed to dry at RT.

Hybridization

The tissue sections were next incubated in prehybridization, hybdridization and post-hybridization solutions. The prehybridization solution consisted of 50% formamide, 5 × SSC (1 × SSC is 150 mM NaCl, 15 mM Na citrate), 10% dextran sulphate, 2 × Denhardt's solution (1 × is 0.02% Ficoll, 0.02% polyvinyl pyrrolidine, 0.02% bovine serum albumin), 0.5% SDS, 100 mM DTT (but 1 mM when ^{3}H-labelled probes were used) and heat denatured salmon sperm DNA (100 μg/ml). The tissue sections were exposed to this solution for 2 h at 37°C. The prehybridization solution was removed and fresh prehybridization mix containing heat denatured probe was added (50 000 cpm/μl); 25 μl of the mixture was used per slide. The hybridization for the α-subunit mRNA was carried out at 50°C for 18 h; the β-subunit was hydridized at 37°C for 15 h. The sections incubated with the α probe were rinsed in 1 × SSC for 30 min at 40°C, followed by RNase digestion (RNase A 100 μg/ml) for 30 min at 37°C. Rinsing was carried out in 1 × SSC at 40°C for 2 × 5 min, 0.1 × SSC at 40°C for 30 min and finally in 0.1 × SSC at RT for 30 min. in the case of the β probes, the slides were first rinsed in 4 × SSC and then treated with pancreatic RNase (50 μg/ml) in SSC for 30 min

at 37°C. After rinsing, the sections were treated with 2 × SSC for 2 h at RT followed by 0.1 × SSC for 30 min at 55°C.

Autoradiography

The sections were dehydrated through ethanol and allowed to air-dry. The slides were dipped in a Kodak NTB2 emulsion diluted with equal volume water at 45°C and allowed to dry. Exposure was in the dark at 4°C for 1–2 weeks when ^{35}S was used, and 1–3 months for the ^{3}H-labelled probes. After development and fixation, the sections were stained with hematoxylin and eosin and mounted with Permount. The parietal cells were readily identified by their eosinophilic staining properties; the silver grains overlying the cells did not interfere with the identification.

Analysis of silver grain distribution was done in sections comprising the entire thickness of the oxyntic mucosa from two rats: one was hybridized with a ^{35}S-labelled antisense probe against the α-subunit of H^+,K^+-ATPase and the other with a ^{3}H-labelled antisense probe against the β-subunit. The number of silver grains was counted in randomly chosen parietal cells showing their nucleus in the section. Photomicroscopy was carried out using a dark-field condenser.

3. Results

It was found that antisense riboprobes for both the α- and β-subunits of the H^+,K^+-ATPase hybridized selectively on the parietal cell (Fig. 1A,C), and no qualitative differences were observed when comparing the hybridization of the two types of probes. Very few parietal cells were seen without silver grain density exceeding that of the background, and outside of the parietal cells no selective hybridization was observed. Sense probes showed no selectivity for the parietal cell or any other structure (Fig. 1B). Comparison between different antisense probes showed more exact localization over the parietal cells with the ^{3}H-labelled probes (Fig. 1C) than with ^{35}S-labelled probes (Figs. 1A and

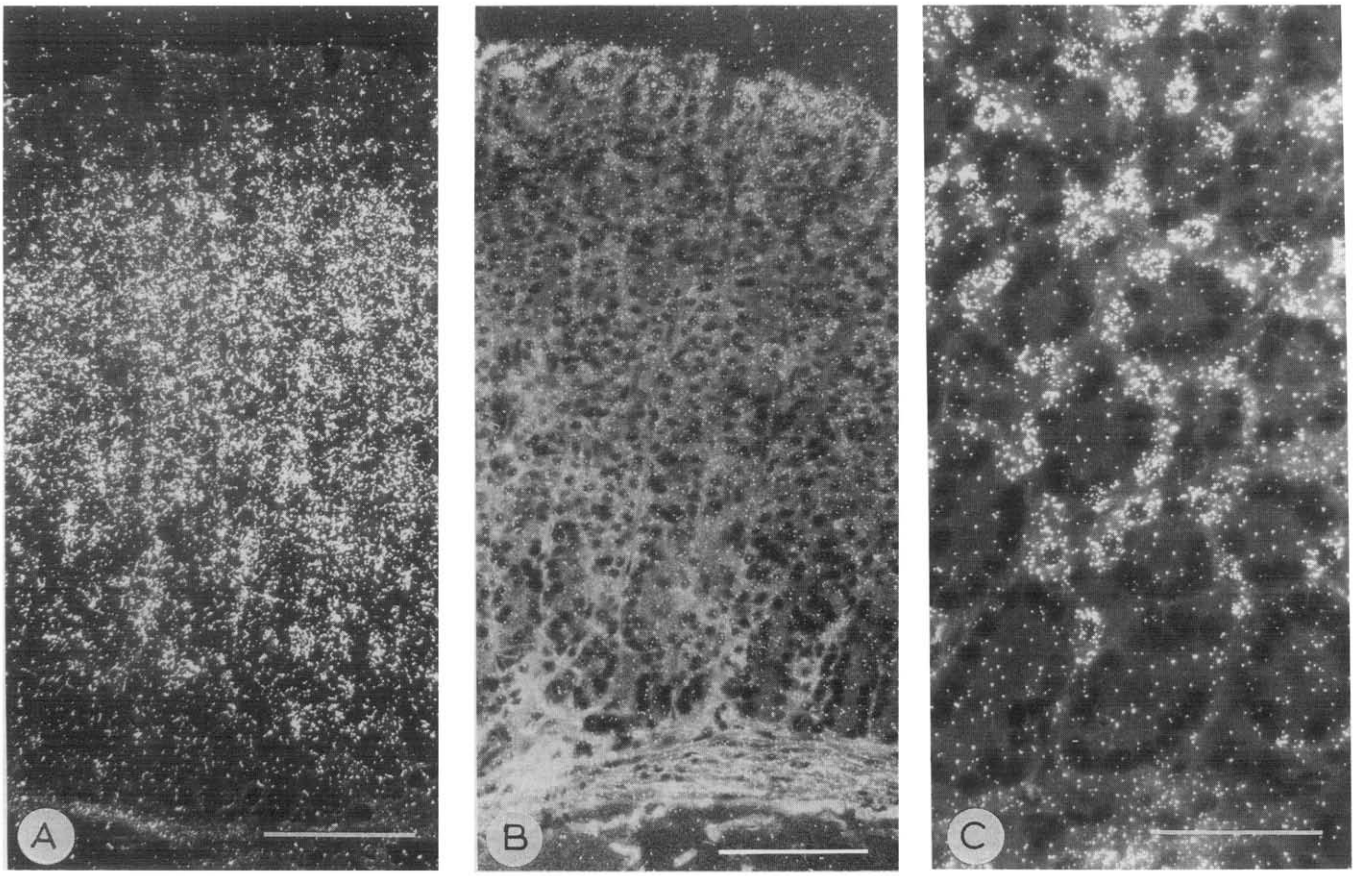

Fig. 1(A) In situ hybridization with the ^{35}S-labelled antisense α probe. Labelling is highest in the parietal cell rich region of the oxyntic mucosa. This section is typical of eight examined. Exposure time 11 days. Cryostat section, ×200. Bar = 100 μm. (B) In situ hybridization using ^{35}S-labelled sense α probe. No selective hybridization is found. Exposure time 11 days. Paraffin section, ×200. Bar = 100 μm. (C) A higher magnification of in situ hybridization with the ^{3}H-labelled β antisense probe. With this higher resolution probe it is possible to state that labelling is confined to the parietal cells. It is also possible to count the number of silver grains over each cell. Exposure time 88 days. Paraffin section, ×450. Bar = 50 μm.

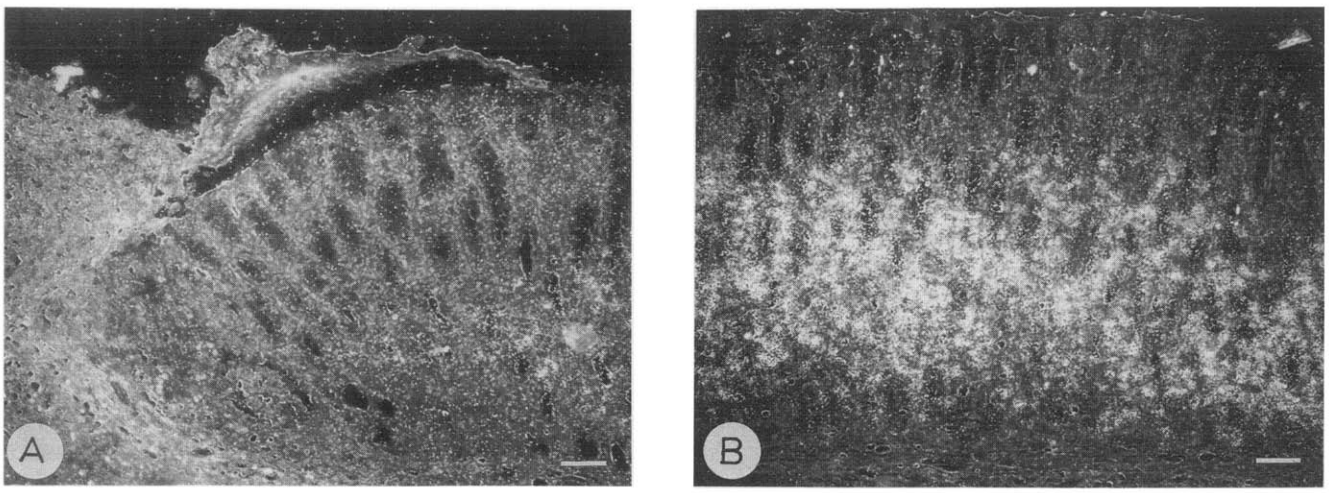

Fig. 2. Hybridization of section through an ulcer in the oxyntic mucosa using ^{35}S-labelled antisense probe for the β-subunit. It can be seen that the ulcer margin (A) shows very little hybridization whereas at 1.5 mm from the margin (B) normal signal is observed in the parietal cells. Paraffin section, ×60. Bar = 100 μm.

2B). The counting of silver grains over parietal cells was therefore much easier and the results presumably more accurate with the ^{3}H-labelled probes.

Comparing the levels of hybridization with the ^{3}H-labelled probe for the α-subunit mRNA, the parietal cells in the upper two thirds gave a significantly higher number of silver grains (28 per cell ± 1.7, $n = 18$, n is number of cells counted) than the parietal cells in the bottom one third of the epithelium (14 ± 1.6, $n = 7$). For the sections hybridized with the ^{35}S-labelled probe for the α-subunit the corresponding figures were 9.6 ± 1.1 vs. 4.0 ± 0.7 ($n = 19$). Thus, for both subunits there appeared to be a 2-fold greater quantity of mRNA in younger parietal cells.

In Fig. 2, it can be seen that the parietal cells at the ulcer margin were only weakly labelled – or even unlabelled – with the β antisense probe, whereas more distant parietal cells were labelled normally.

4. Discussion

Using in situ hybridization technology, the ontogeny of the α subunit has been investigated in rats at different stages of development. It was possible to show the presence of the mRNA for H^+,K^+-ATPase at 1 week after birth [13], with the densest distribution in the midportion of the gastric glands; more precise cellular localization was not attempted. It may be noted that the more sensitive antibody techniques are able to detect the presence of this subunit already at 2 days before birth [9].

In this study, we have extended the application of in situ hybridization to the cellular level, with the results that this method is able to localize the mRNA for both subunits of the H^+,K^+-ATPase to only the parietal cell of the stomach, in agreement with conclusions reached using antibodies.

Immunohistochemical labelling of tissues is difficult to quantitate. In contrast, the radioactive methods often employed for in situ hybridization lend themselves readily to counting of the number of silver grains. In the case of ^{35}S probes, the higher energy of the isotope makes exact localization somewhat difficult, although the sensitivity is increased allowing for shorter exposure of the emulsion. The lower energy of ^{3}H provides a more precise localization, but exposure of the autoradiograms is prolonged.

Our data appear to indicate that there is about 2-fold more mRNA for both subunits of H^+,K^+-ATPase in the parietal cells from the superficial layers of the mucosa ('neck parietal cells') than in those from the bottom layers ('base parietal cells'). If this finding is not due to an unrecognized methodological error, it is interesting in view of the fact that the parietal cells are produced from the progenitor cells in the neck region of the mucosa and most of them migrate downward in the epithelium [14]; hence the base parietal cells are older than the neck parietal cells.

The differences in mRNA hybridization signals between the two types of parietal cells may indicate differences in mRNA turnover, but some morphological differences should also be taking into account: Using enzyme histochemical methods Coulton and Firth [15] demonstrated lower activities of several enzymes, including H^+,K^+-ATPase, in the base parietal

cells than in the neck parietal cells. These features do not necessarily reflect a general decline of the metabolic capacities of the parietal cells with increasing age, since succinic dehydrogenase activity was higher in the older base parietal cells [15]. By electron microscopy, the base parietal cells have a smaller secretory surface (i.e., the surface facing the gland lumen or the secretory canaliculi), a finding which prompted the hypothesis that the base parietal cells should produce less HCl than the neck parietal cells [16]. Our present observations are consistent with this hypothesis.

The acetic acid ulcer of the gastric mucosa is often used in experimental ulcer research, but it must be recalled that important biological differences exist between this type of ulcer and peptic ulcers in patients.

Three to ten days after the induction of an acetic acid ulcer in the rat corpus mucosa, the parietal cells in the ulcer margin display less mitochondria and tubulovesicles than normal, and there are few, if any, secretory canaliculi [12]. These findings were interpreted as signs of low differentiation, an impression which is strengthened by the apparent loss of mRNA for the β-subunit for H^+,K^+-ATPase observed in the present study.

Functionally, these parietal cells have lost their capacity to secrete acid [12]. It remains to determine whether the parietal cells of the ulcer margin are dedifferentiated as a result of the trauma, or if they represent newly formed cells arising from proliferating epithelium.

In conclusion, our results show the presence of mRNA for both subunits of H^+,K^+-ATPase in the parietal cells of the rat oxyntic mucosa. The levels of mRNA for both the α- and the β-subunits appear to be lower in the parietal cells near the bottom of the glands than in those closer to the mucosal surface. In the margin of an ulcer induced by acetic acid, the levels of mRNA for H^+,K^+-ATPase were very low.

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