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A distinct carbonic anhydrase in the mucus of the colon of humans and other mammals

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Summary

We have collected gastrointestinal, mainly colonic, mucus from humans, guinea pigs, rats, and normal and carbonic anhydrase II (CAII)-deficient mice. In the mucus of all species, substantial CA activity was present. Using antibodies against human CA isoforms we found that the human mucus CA differs from cytosolic CAI and CAII, membrane-bound CAIV, and the secreted CAVI of saliva. The high sensitivity of mucus CA to acetazolamide rules out its identity with cytosolic CAIII. Partial sequences obtained from the purified human mucus CA show similarity, but not identity, with human CAI, and clear differences from the other known CAs. Additional evidence concerning the CA isoform present in mucus was obtained for the mucus CA of other species and was derived from: (1) the mucus of CAII-deficient mice, whose high CA activity confirms that it is not CAII that is responsible; (2) the inhibitory effect of iodide, which shows that mucus CA from mice, guinea pig and humans does not have the high anion sensitivity of CAI; (3) the inactivating effect of 0.2% SDS on guinea pig, mouse and human mucus CA, ruling out the SDS-resistant CAIV; and (4) the partitioning of guinea-pig mucus CA into the water phase in Triton X114 phase separation experiments, which also argues against its identity with membrane-bound CAs, such as CAIV. A comparison of colonic mucus CA activity in normal and germ-free rats indicates that the mucus CA is not of bacterial origin but is produced by the gastrointestinal tissues. We conclude (from its immunoreactivity, from iodide inhibition and from partial amino acid sequences) that mucus CA of human origin probably represents an isozyme, which is specific for mucus and is not identical with the known CA isozymes. The results obtained for mucus CA of other species collectively point in the same direction.

Key words: human, guinea pig, rat, mouse, gastrointestinal mucus, carbonic anhydrase, colon, stomach.

Introduction

In a previous study, we used a mass spectrometric technique to measure intracellular CA activity and membrane bicarbonate permeability of intact guinea pig colonic epithelium (Böllert et al., 1997). In addition, this technique responds very sensitively to the presence of extracellular CA activity. Indeed, in these measurements we observed an extremely high extracellular CA activity on the apical side of the colonic epithelia. While this could, in principle, have been due to a membrane-bound CAIV associated with the apical epithelial membrane, we noticed that the bulk of this extracellular activity could easily be removed by rinsing the epithelial surface with saline. This prompted us to look for CA activity in the colonic mucus as reported in the present study.

Materials and methods

Materials

Nitrocellulose membranes (Parablot NCL, 0.45 μm pore size) were from Macherey & Nagel (Düren, Germany). For ultrafiltration membranes type PM10 from Amicon/Millipore (Eschborn, Germany) were used. Acetazolamide was from Lederle (Wolfratshausen, Germany). A 10 kDa protein ladder for calibration of SDS-PAGE was obtained from Life Technologies (Eggenstein, Germany). Enhanced chemiluminescence (ECL) system (RPN 2106) was from Amersham Life Science (Arlington Heights, IL, USA). Antibodies were those of S. Parkkila, Oulu, Finland (CAVI, Parkkila et al., 1991), or were gifts from N. D. Carter, London, UK (CAI) and W. S. Sly, St Louis, USA (CAIV), or were purchased from The Binding Site Ltd, Birmingham, UK

(CAII). All other chemicals were purchased from Sigma (Munich, Germany), Merck (Darmstadt, Germany), Pharmacia (Uppsala, Sweden) or Serva (Heidelberg, Germany).

Animals

Guinea pigs

Male guinea pigs (*Cavia aperea f. porcellus* Bohlken 1961, body weight 550–700 g) from Pietsch (Hohlenberg, Germany) were used. Maintenance and treatment of the animals was as described previously (Engelhardt et al., 1994). After decapitation, between 8.00–9.00 a.m., the abdomen was opened and the large intestine removed, cut into the different segments (caecum, proximal colon and distal colon) and rinsed with 25 mmol l^{-1} Tris-SO_4 pH 8.7 to remove luminal contents. Mucus samples were obtained from the second rinse of the intestinal segments with the same buffer solution. This washing containing diluted mucus was microscopically free of epithelial cells. After the second washing, the pieces of intestine were rinsed several more times before they were used to prepare tissue homogenates. For the latter purpose, to 1 g of tissue we added 3 ml buffer (20 mmol l^{-1} Tris-HCl, 250 mmol l^{-1} sucrose, 2 mmol l^{-1} EDTA, 5 mmol l^{-1} DTT, 0.2 mmol l^{-1} PMSF, pH 7.4) and then homogenized the suspension with an Ultra Turrax 3×30 s on ice at maximal speed. Subsequently, the homogenate was centrifuged at 4°C for 1 h at 100,000 ***g*** to obtain the supernatant.

Mice

Normal and CAII-deficient mice (*Mus domesticus* L.) were bred in cooperation with the central animal facility of the Medical School. Breeding was started with two pairs of mice (C57 BL/6J – Car 2°) from the Jackson Laboratory (Bar Harbor, USA) as first described elsewhere (Lewis et al., 1988). Characterization of the genotype of the animals was achieved by measuring carbonic anhydrase (CA) activity in red cell lysate. Normal animals have an activity of about 60,000–80,000 units (in undiluted red cells), whereas the homozygous CAII-deficient animals have an activity of only 500–1000 units. Heterozygous animals have activities of 30,000–50,000 units. This allows one to distinguish clearly between the three groups on the basis of CA activity measurements.

Mice were anesthetized with ether and killed by neck dissection. Mucus samples were obtained as described for guinea pigs, with buffer solution containing 5 mmol l^{-1} imidazole pH 7.4, from the second rinsing of the intestinal segments. After repeated rinsing of these segments, the tissues were minced, diluted 1:4 with ice-cold buffer solution, and homogenized three times for 30 s with an Ultra Turrax. After centrifugation at 4°C and 100,000 ***g*** for 1 h we obtained the cytosolic supernatant of the pieces of large intestine. Samples of saliva of mice were obtained by rinsing the mouth of the animals with buffer solution.

Rats

We used female Wistar rats (*Rattus norvegicus albinos* Berkhaut 1769) purchased from Charles River (Sulzfeld, Germany). Germ-free rats were bred in the central animal facility of the Medical School. Animals were anesthetized and killed as described above and mucus samples and saliva were also obtained as described for mice

Rabbits

Chinchilla bastards (*Chinchilla lanigera* Molina 1782) from Charles River (Sulzfeld, Germany) were used for immunization to raise antibodies against CAI and CAII from guinea pigs.

Human material

Mucus samples of human gastric and colonic mucosa were taken during endoscopies performed for diagnostic purposes by the Dept of Hepatology and Gastroenterology, Medical School in Hannover. Colonic mucus was obtained from the solution used to rinse the large intestine prior to endoscopy. It was not possible, therefore, to distinguish between mucus from the proximal and the distal colon. The large intestine was rinsed with Oralav (Braun, Melsungen Germany) until no stool could be seen macroscopically. The solution from the rinse following thereafter was collected. The samples were concentrated by ultrafiltration using a membrane type PM10. After concentrating the samples about 10-fold, they were dialyzed against 5 mmol l^{-1} imidazole, pH 7.4, using dialysis tubing (Sigma Lot 26H1022) before carrying out measurements. The temperature during concentration and dialysis was 4°C.

Methods

Purification of cytosolic carbonic anhydrases

Purification of CA from lysed red cells and colonic mucus of guinea pigs was carried out by affinity chromatography. For the purification of CAI and CAII, first a chloroform–ethanol-extraction of the lysed red cells was performed as described by Bernstein and Schraer (1972). The fraction containing CA was applied to an affinity column and washing as well as elution steps were carried out as described by Whitney (1974). To purify the CAI fraction, we performed a re-chromatography step as described by Whitney because this fraction was contaminated with CAII, whereas the CAII fraction was highly pure after the first affinity chromatography step. The purified fractions of CAI and CAII were concentrated about 10-fold by ultrafiltration using a membrane type PM10. After concentrating, the samples were dialyzed against 25 mmol l^{-1} Tris-SO_4 pH 7.4.

The diluted mucus from guinea pigs was purified and concentrated in two filtration steps. Initially, a membrane with a pore diameter of 0.45 μm was used to remove particles from the mucus samples. The filtrate was concentrated about 50-fold using a membrane type PM10. Finally, the sample was dialysed against 25 mmol l^{-1} Tris-SO_4 pH 8.7 before it was applied to the affinity column. Washing steps were carried out as described by Whitney (1974). The mucus CA was eluted with 7.5 mmol l^{-1} sodium azide pH 5.7. Concentration and dialysis were performed as described for CAI and CAII.

Production of polyclonal antibodies

To obtain polyclonal antibodies against CAI and CAII of guinea pig, rabbits were immunized with the purified enzyme fractions. For the immunization about 50 µg protein in complete Freund's adjuvant were injected. The booster injection followed 4 weeks later in incomplete Freund's adjuvant. The antisera were obtained from a blood sample 2 weeks after the booster injection. Before immunization, 5 ml blood were taken to obtain pre-immune serum.

Protein determination

Determination of protein concentration was carried out by the method of Lowry et al. (1951) modified by Peterson (1977) using the protein assay kit from Sigma (Procedure No. P 5656). The samples were solubilized with sodium deoxycholate, and protein was precipitated with trichloroacetic acid before the assay.

Measurement of carbonic anhydrase activity

CA activity was determined according to the micromethod of Maren (1960) as modified by Bruns and Gros (1991). The principle of this method is to determine the (acid) change of pH caused by CO_2 hydration in the reaction vessel following the addition of alkaline barbital buffer to the CO_2-saturated assay volume. The pH change is visualized by the pH indicator phenol red. The time needed after addition of barbital buffer to reach the pH at which the indicator turns from red to yellow is measured at 0°C. In the presence of CA this time is reduced. One enzyme unit is defined as the final enzyme concentration in the assay volume that halves the uncatalyzed reaction time. Specific CA activity is obtained by dividing the CA units of a sample by its protein concentration.

Measurements with the CA inhibitors acetazolamide and KI were carried out by adding the inhibitor into the reaction vessel and allowing 2 min incubation of sample and inhibitor at 0°C before the barbital was added. To determine the inhibitory effect of SDS, samples were preincubated for 30 min at room temperature with a final concentration of 0.2% (w/v) SDS before being added into the reaction vessel where an identical final SDS concentration was established.

Triton X-114 phase separation

The phase separation with Triton X-114 was carried out by a method similar to that of Bordier (1981): equal volumes of ice-cold Triton X-114 solution (2.2% (w/v) Triton X-114, 300 mmol l^{-1} NaCl, 20 mmol l^{-1} Tris-HCl, pH 7.4) and sample were mixed in an Eppendorf test tube. The mixture was shaken, incubated for 4 min at 31°C and centrifuged for 3 min at 2000 ***g*** at room temperature in an Eppendorf centrifuge (type 5414, Eppendorf, Germany). After centrifugation the supernatant (aqueous phase No. 1) was removed and mixed again with Triton X-114 solution (4.5% Triton X-114, 150 mmol l^{-1} NaCl, 10 mmol l^{-1} Tris-HCl pH 7.4) to a final Triton concentration of 0.85% (w/v) before the procedure was repeated. After removing the new supernatant (aqueous phase No. 2), the two sediments (Triton phases) were combined. Determination of total volumes and CA activities was carried out for each phase and used to estimate the distribution of the total amount of CA activity between the two phases.

Immunoblotting

After SDS polyacrylamide gel electrophoresis (separating gel: 15%, stacking gel: 5%), proteins were electrophoretically transferred to nitrocellulose using a semi-dry blotting system at 0.8 mA cm^{-2} for 1.5 h. The blocking of non-specific binding was carried out with BSA (15 g 500 ml^{-1} in PBS/Tween). For the immunostaining, polyclonal antibodies against CAI, CAII and CAVI were used at a dilution of 1:500 and those against CAIV at 1:1000. Secondary antibodies were labeled with peroxidase and used in same dilution as the primary antibody. Detection was carried out using DAB as substrate for peroxidase (CAI, CAII, CAVI) or the ECL system according to the manufacturer's recommendations (CAIV).

Sequence analysis

Isolated human mucus CA was sequenced as follows. After treatment with trypsin (0.036 g l^{-1}) the cleavage products were separated by µHPLC, fractions were sequenced by Edman degradation and mass spectra were obtained by MALDI-TOFMS analysis.

µHPLC separation of tryptic cleavage products

Prior to the chromatographic separation of 20 µl of sample solution, 125 µl of the incubation mixture were lyophilized and dissolved in 25 µl 0.06% (v/v) aqueous TFA. Chromatography was carried out at 30°C (215 nm) using a Reprosil-Pur C18-AQ column (250 mm×1 mm i.d.; 3 µm; A. Maisch, Ammerbuch, Germany) and a rising acetonitrile gradient from 10 to 60% (v/v) eluent B within 50 min (20 µl min^{-1}) collecting fractions of 1 min each. The mobile phase comprised eluent A (H_2O, 0.06% v/v TFA) and eluent B (ACN, 0.05% v/v TFA). The HPLC system consisted of a solvent delivery system 140B, a programmable absorbance detector 785A and an oven-injector 112A from Applied Biosystems (Weiterstadt, Germany). UV absorbance was monitored by a Chromjet Integrator (Spectra-Pysics, Fremont, CA, USA).

MALDI-TOFMS analysis

MALDI mass spectra were obtained on a Voyager DE Pro mass spectrometer (Applied Biosystems, Weiterstadt, Germany) in the positive linear operation mode. The matrix comprised α-cyano-4-hydroxycinnamic acid mixed with L-fucose (2.5 mg ml^{-1} each, both Sigma-Aldrich, Steinheim, Germany) dissolved in a 50% (v/v) mixture of ACN/0.1% (v/v) aqueous TFA. Equal volumes of 1 µl of the sample solution and of the matrix were mixed on a stainless steel multiple sample tray according to the dried droplet technique. Data acquisition and analysis were performed using the Voyager control software and Data Explorer version 4.0 software supplied by the manufacturer.

Table 1. *Specific CA-activity [U ml mg^{-1} protein] in the mucus of the gastrointestinal tract of different species*

	Mouse	Rat	Guinea pig	Human
Saliva	63±25 (*N*=5)	112 (*N*=1)	–	1.3 (*N*=1)
Gastric mucus	–	–	–	2.3±1.6 (*N*=6)
Caecum mucus	30±12 (*N*=7)	67±7 (*N*=3)	47±31 (*N*=18)	–
Proximal colon mucus	20±9 (*N*=6)	52±5 (*N*=3)	185±123 (*N*=18)	6.7 (*N*=2)
Distal colon mucus	10±9 (*N*=7)	42±21 (*N*=3)	77±68 (*N*=17)	–

Values are means ± S.D. *N* is the number of samples studied, each CA activity measurement is in triplicate.

Amino acid sequence analysis

N-terminal sequencing was performed on a Procise 494 sequencer (Applied Biosystems, Weiterstadt, Germany) by Edman degradation with on-line detection of phenylthiohydantoin-amino acid using the standard protocol recommended by the manufacturer. Sequence search and identification was performed using the MS-Edman 2.2.1. software from ProteinProspector 3.2.1.

Results

As shown in Table 1, high CA activities are found in the mucus of colon and caecum of all species studied and in saliva of humans, mice and rats. In addition, CA activity was detected in human gastric mucus. Saliva contains the only known secreted isoenzyme of carbonic anhydrase, CA VI (Fernley et al., 1979). Parkkila et al. (1997) also found some CAVI also in gastric juice as well as in gastric mucosa using western blots. They suggested that it represents CA swallowed with saliva because CAVI is not expressed in gastric epithelium. The existence of CA activity elsewhere in the mucus of the gastrointestinal tract, such as colon and caecum, has not been reported before.

To investigate whether the mucus CA in the large intestine is a soluble or a membrane-bound isoenzyme, phase separation experiments with Triton X-114 were carried out (Fig. 1). Soluble proteins like CAI and CAII from red cell lysate partition into the water phase in this experiment (left-hand column), whereas membrane-bound CA from heart sarcolemmal vesicles partitions into the Triton phase (right-hand column in Fig. 1; data from Bruns and Gros, 1992). Figure 1 shows the results for mucus samples from caecum and colon of guinea-pigs. As in red cell lysate, <10% of CA activity is found in the Triton >80% is found in the water phase. These results show that mucus CA of the large intestine is a soluble isoenzyme, indicating that it cannot be identical with membrane-bound CAIV.

Table 2 shows the sensitivity of mucus CA towards SDS. The membrane-bound CA IV is the only CA isoform that is known to resist SDS as is exemplified for sarcoplasmic reticulum (SR) vesicles from rat skeletal muscles in the last line of Table 2. Mucus CA of gastric mucosa and of colon and caecum as well as CA of red cell lysate are fully inhibited after incubation with 0.2% SDS. Like the phase separation experiments, these results argue against the existence of a membrane-bound CAIV in the mucus of the gastrointestinal tract.

To investigate whether the mucus CA in the large intestine is identical to one of the known cytosolic isoenzymes, CAI or CAII, we carried out experiments with CAII-deficient mice. Results are shown in Table 3: CAII-deficient mice show a large reduction of CA activity in red cell lysate from about 71,000 units for wild-type mice to about 900 units. This can be explained by the lack of the high-activity isoform CAII. The remaining CA activity is likely to be mostly due to CAI; the

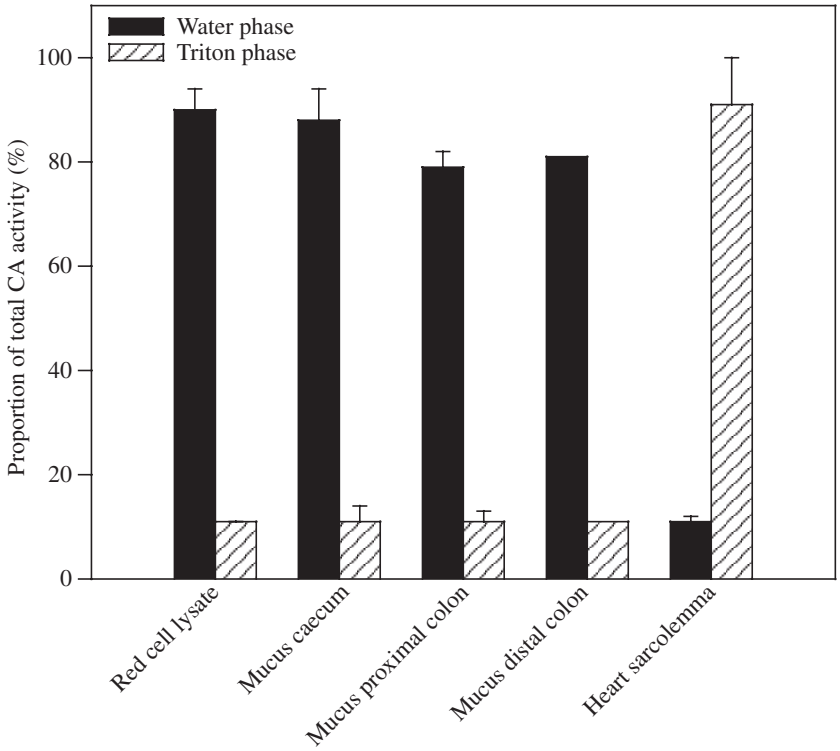

Fig. 1. Distribution of CA activity after phase separation with Triton X-114. The values shown on the *y* axis are means ± S.D., from three experiments each for mucus of caecum and proximal colon from the guinea pig, two experiments for distal colon from the guinea pig, and four experiments for human red cell lysate and rabbit heart sarcolemmal vesicles. Values for heart sarcolemmal vesicles are from Bruns and Gros (1992).

Table 2. *Sensitivity of mucus-CA for 0.2% SDS % inhibition*

	Guinea pig	Mouse	Human	Rat
Gastric mucus	–	–	100 (*N*=3)	–
Caecum mucus	99±2 (*N*=3)	85±15 (*N*=3)	–	–
Proximal colon mucus	95±5 (*N*=3)	100 (*N*=3)	100 (*N*=3)	–
Distal colon mucus	98±3 (*N*=3)	100 (*N*=3)	–	–
Red cell lysate	–	100 (*N*=3)	100 (*N*=5)	–
SR-vesicles	–	–	–	6 (*N*=2)

All values represent % inhibition after incubation with 0.2% SDS and are means ± S.D. 'Mouse' refers to normal wild-type animals. SR is sarcoplasmic reticulum from rat skeletal muscle.

Table 3. *CA activity and iodide inhibition in wild-type and CAII-deficient mice*

	Genotype	Specific CA activity (U ml mg^{-1} protein)	Inhibition with 7 mmol l^{-1} KI (%)
Red cell lysate	Wild type	71 100±10 600 (*N*=7)	44±4 (*N*=24)
	CAII-deficient	887±132 (*N*=6)	100±0 (*N*=9)
Cytosol caecum	Wild type	289±45 (*N*=7)	65±4 (*N*=7)
	CAII-deficient	96±34 (*N*=6)	100±0 (*N*=6)
Cytosol proximal colon	Wild type	186±35 (*N*=7)	61±6 (*N*=7)
	CAII-deficient	58±19 (*N*=6)	100±0 (*N*=6)
Cytosol distal colon	Wild type	195±36 (*N*=7)	54±6 (*N*=7)
	CAII-deficient	5±2 (*N*=6)	100±0 (*N*=6)
Mucus caecum	Wild type	30±12 (*N*=7)	50±7 (*N*=7)
	CAII-deficient	42±9 (*N*=6)	55±11 (*N*=6)
Mucus proximal colon	Wild type	20±9 (*N*=7)	35±10 (*N*=7)
	CAII-deficient	40±15 (*N*=6)	56±9 (*N*=5)
Mucus distal colon	Wild type	10±9 (*N*=7)	–
	CAII-deficient	21±16 (*N*=6)	33±9 (*N*=6)
Saliva	Wild type	63±25 (*N*=5)	46±13 (*N*=5)
	CAII-deficient	83±39 (*N*=5)	41±10 (*N*=5)

Values are means ± S.D.

latter conclusion derives from: (1) the expected very low contribution of CA III to intra-erythrocytic red cell CA activity (Carter et al., 1984); and (2) the (line 2 of Table 3) complete inhibition of the CA activity of red cells from CAII deficient mice by 7 mmol l^{-1} iodide, when in contrast iodide at the same concentration inhibits CA activity in wild-type mouse red cells by about 44% only. This finding tallies with the known difference in anion sensitivity of CAI and CAII (Maren et al., 1976).

In the cytosolic supernatant of the intestinal homogenates, we obtain results similar to those in red cell lysate. Compared with the wild-type mice, the cytosolic supernatant of intestinal segments of CAII-deficient mice shows a considerable reduction of specific CA activity. As in red cell lysate, 7 mmol l^{-1} iodide caused a complete inhibition of CA activity in the cytosolic supernatant of CAII-deficient mice, whereas inhibition in wild-type mice was only about 60%. By analogy to the red cell lysates, these data suggest that the intestinal epithelium of normal mice contains cytosolic CAII as well as CAI, while only CAI is expressed in the CAII-deficient mice. This agrees with early studies of Carter and Parsons (1970), which showed the existence of both CAI and CAII in the epithelial cells of the large intestine.

Conversely, Table 3 shows unaltered or even increased CA levels in the mucus of the CAII-deficient mice. This clearly indicates that the CA activity in the mucus of mice is not due to CAII. In addition, 7 mmol l^{-1} iodide could not completely inhibit the CA activity in the mucus of either CAII-deficient or normal mice. Iodide caused a similar fractional inhibition in the mucus of both genotypes by about 50%. Similarly, 7 mmol l^{-1} iodide inhibited the CA in colonic mucus samples from humans and guinea pig by about 40% only. The mucus isozyme therefore is only moderately anion-sensitive, which shows that mucus CA activity is not due to CAI.

Western blots with available anti-human CA isoform antibodies were carried out to characterize further the CA in the mucus of the gastrointestinal tract. Figure 2 shows the results for human samples. Identical amounts of CA activity were applied onto the lanes with colonic mucus and the corresponding control lanes with positive control samples,

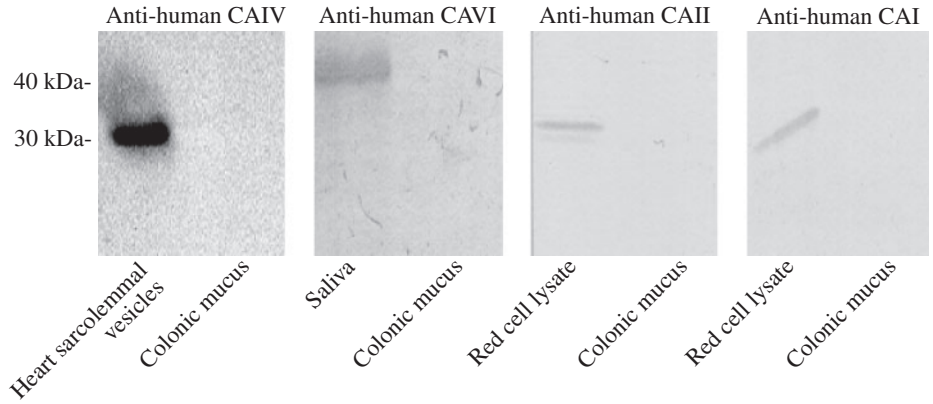

Fig. 2. Western blot analyses of human colonic mucus. For each analysis identical amounts of CA activity were applied for mucus samples and positive controls, respectively: 0.012 U ml^{-1} in the case of CAI, CAII and CAVI and 0.128 U ml^{-1} in the case of CAIV. Dilution of antibodies and detection were carried out as described in Materials and methods.

respectively. Antibodies against human CAI, CAII, CAIV and CAVI show no reaction with human colonic mucus whereas the same amount of CA activity in the corresponding positive control lanes produces reactions at the expected molecular weights. The western blots confirm for human mucus the above conclusions, i.e. that mucus CA is not identical to either CAI, CAII nor CAIV. In addition, Fig. 2 provides evidence that it is not CAVI that is responsible for the CA activity in the human colonic mucus.

It may be added that we have also studied human gastric mucus by western blotting with anti-human CAVI antibody (results not shown). In agreement with the results of Parkkila et al. (1997), we find CAVI in some samples of gastric mucus, but not in others. In each case, the intensity of the immunostaining signal of the bands of gastric mucus was less than that of control samples of human saliva containing identical amounts of CA activity. This suggests that the gastric mucus contains not only (small variable amounts of) CAVI but also some other CA isozyme, which may possibly be the same isozyme that we describe here in the colonic mucus.

To study whether guinea pig mucus CA – like human and mouse mucus CA – has properties distinct from the intraepithelial CAI and CAII, we isolated CA from guinea pig colonic mucus for further characterization of the enzyme. The molecular weight was determined to be 30 kDa by SDS polyacrylamide electrophoresis. Results of inhibition studies with the isolated mucus enzyme from guinea pigs agree well with the results obtained from inhibition experiments with mucus samples. The isolated CA could be fully inhibited by SDS and the inhibition with 7 mmol l^{-1} KI is about 36%, i.e. guinea pig mucus CA is moderately anion-sensitive as is mouse mucus CA (Table 3). Western blot analyses with anti-guinea pig CAI and CAII are shown in Figs 3 and 4. It is apparent that there is some reaction of mucus samples with both antibodies. However, it is clear that at identical amounts of CA activity applied to the gel, the bands seen for CA in whole mucus are with anti-CAI as well as with anti-CAII markedly weaker than those seen with isolated CAI or CAII, respectively. It can be hypothesized that mucus samples are either contaminated to a minor extent with CAI and CAII, or that both antibodies cross react with mucus CA. In view of the other evidence presented below, the latter possibility appears more likely. That isolated mucus CA as well as the

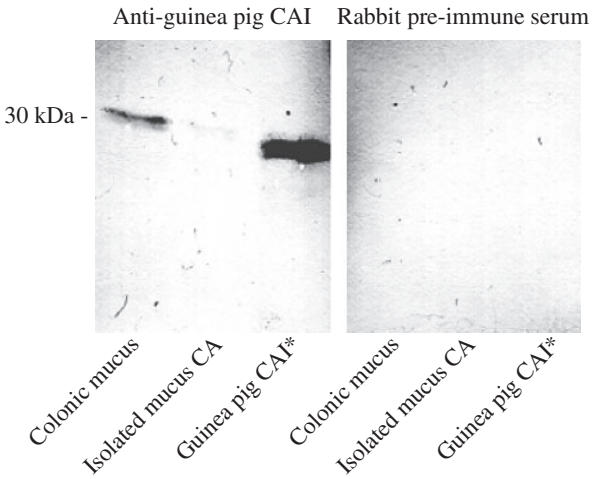

Fig. 3. Western blot analysis of mucus CA from guinea pig. For each analysis identical amounts of CA activity applied with the samples were: guinea pig colonic mucus with 0.87 U ml^{-1} CA activity, isolated mucus CA with 5.3×10^{-3} U ml^{-1} CA activity. *Guinea pig CAI was applied at an amount of 0.87 U ml^{-1} CA activity as a positive control for guinea pig mucus. Applying CA I at 5.8×10^{-3} U ml^{-1} as a control for isolated a mucus CA did not result in a visible band (not shown).

corresponding positive controls do not react with the two antibodies is due to the very the low amounts of enzyme applied to the gel in these cases (see legend to Figs 3 and 4); this could not be improved upon because of lack of sufficient amounts of purified mucus CA.

To decide whether it may be cytosolic CAIII that occurs in mucus, we determined the sensitivity of mucus CA for sulfonamides. CAIII is known to be present in type I skeletal muscle fibers and exhibits a uniquely high resistance towards sulfonamides. We find that mucus CA from guinea pig possesses a high sensitivity towards these CA inhibitors: K_i-values of guinea pig colonic and caecal mucus for acetazolamide are about 6×10^{-9} mol l^{-1}, whereas K_i of CAIII for acetazolamide is between 10^{-4} and 10^{-3} mol l^{-1} (Gros and Dodgson, 1988). We conclude that the CA activity in the mucus of the gastrointestinal tract is not due to CAIII. The partial sequences of human mucus CA (see below) are also not compatible with CAIII.

To investigate whether the mucus CA is synthesized by the

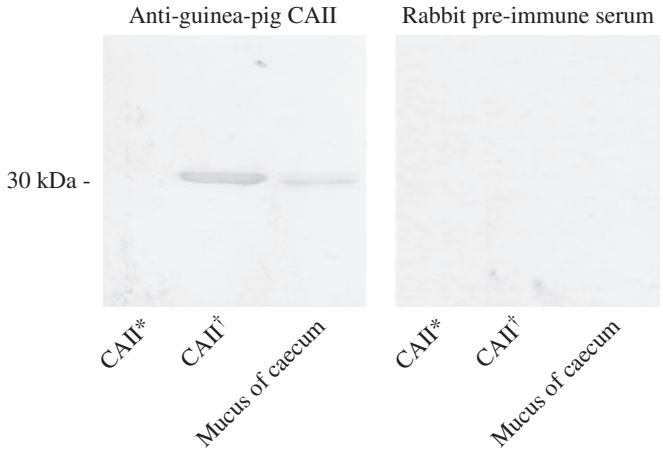

Fig. 4. Western blot analysis of mucus CA from guinea pig. Mucus from guinea pig caecum was applied at an activity amount of 0.87 U ml^{-1}. Positive controls are (*) isolated guinea pig CA II at 5.1×10^{-3} U ml^{-1} CA activity and (†) isolated CA II at 0.79 U ml^{-1}. Applying isolated mucus CA from the guinea pig at an activity amount of 5.3×10^{-3} U ml^{-1} did not result in a visible band.

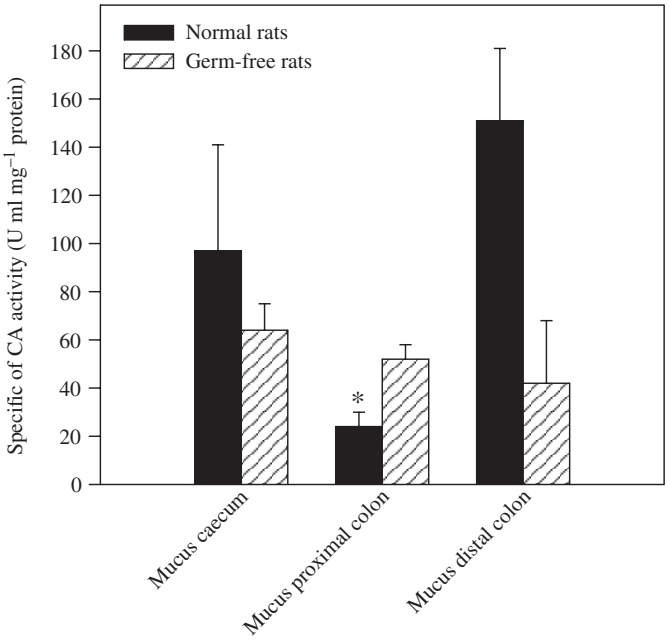

Fig. 5. Comparison of specific mucus CA activity in the caecum and colon of normal and germ-free rats. Means ± S.D. (*N*=5).

tissues of the gastrointestinal tract or by gastrointestinal bacteria, we compared the CA activities in the mucus of normal and germ-free rats. As seen in Fig. 5, we find no significant decrease in the mucus CA activity of germ-free compared to normal rats in caecum and colon. We conclude that the CA observed in the mucus of the gastrointestinal tract is not due to bacteria. It may be noted that studies of Lönnerholm et al. (1988) have shown that there is no difference in intracellular CA activity in the epithelial cells of colon and caecum between normal and germ-free rats.

To characterize further the human mucus CA, we obtained partial sequences of the isolated enzyme. A comparison of these sequence fragments with human CAI, CAII, CAXIII and CAIII is shown in Fig. 6. It is apparent that there are remarkable differences from CAII, excluding this enzyme as a candidate, and also from cytosolic CAXIII and CAIII. The differences from CAI, on the other hand, are much smaller.

Discussion

Carbonic anhydrase activity in native mucus

To assess a possible physiological role of colonic mucus, it is desirable to estimate its activity in the intact mucus layer *in situ*. We found it difficult to obtain representative samples of *in situ* mucus because: (1) this requires first washing away the luminal contents, which unavoidably leads to a dilution at least of the surface portion of the mucus; and (2) scratching mucus from the apical epithelial surface is associated with lesions of the epithelial cells, which themselves contain substantial CA activity, which then will contaminate the mucus samples. Another possibility is to use the protein concentration of native mucus in conjunction with the specific mucus CA activities as given in Table 1. Rechkemmer (1981) has reported a protein concentration of native mucus from the proximal colon of guinea pigs of 10 mg ml^{-1}. Using the specific CA activity for proximal colonic mucus of guinea pigs given in Table 1, 185 U ml mg^{-1}, we obtain an *in situ* mucus CA activity of ~2000 units, meaning that the velocity of CO_2 hydration in the mucus layer is sped up by a factor of 2000 when compared with the uncatalysed hydration reaction. This same acceleration factor is about 10,000 within human red cells, which are known to possess an exceptionally high CA activity. Thus, the CA activity in colonic mucus would appear to be very substantial and would result in reducing the half-time of the CO_2 hydration from its uncatalysed value of ~7 s to ~4 ms.

Identity of mucus carbonic anhydrase

The results of the described experiments seem to exclude the presence of five CA isoenzymes in the mucus of the gastrointestinal tract of some of the investigated species. (1) CAI is excluded for human and for guinea pig colonic mucus by the absence of reactivity with anti-CAI antibodies (except for some possible cross-reactivity in the case of guinea pig mucus), and by the lack of complete inhibition by KI in the cases of the mucus of normal and CAII-deficient mice and of human and guinea pig colonic mucus. Conversely, the partial sequences of the human mucus CA might be taken to suggest that mucus CA has some similarity although not identity with the CAI isoform. (2) CAII as a mucus isozyme is ruled out by the absence of reactivity with anti-CAII antibody (in the case of human colonic mucus and – except for a possible minor cross-reactivity – also guinea pig colonic mucus), by the partial sequences of human mucus CA, and by the finding of an unaltered CA activity in the gastrointestinal mucus of CAII-deficient mice. (3) CAIII cannot account for the CA activity in mucus because it possesses a low sensitivity towards

sulfonamides while mucus CA exhibits high sensitivities for these CA inhibitors (guinea pig mucus). Also, the sequence of Fig. 6 is not compatible with that of human CAIII. (4) CAIV is a known apical membrane-bound CA isoenzyme. Its presence in mucus would be conceivable if it was cleaved from its GPI-anchor at the apical epithelial membrane and retained in the mucus layer. However, CAIV is resistant against 0.2% SDS while mucus CA is fully inhibited by this concentration

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Hum CA I      ASPDWGYDDKNGPEQWSKLYPIANGNNQSPVDIKTSETKHDTSLKPISVSYNPATAKEIINVGHSFHVNFEDNDN 75
Hum Mucus-CA                                        HDTSLKPISISYNPAGAKEIINVGHVFHVNFEDD
Hum CA II     -SHHWGYGKHNGPEHWHKDFPIAKGERQSPVDIDTHTAKYDPSLKPLSVSYDQATSLRILNNGHAFNVEFDDSQD
Hum CA XIII                                         ydsslrplsikydpssakiisnsghsfnvdfddt
Hum CA III                                          hdpslqpwsvsydggsaktilnngktcrvvfddt

Hum CA I      RSVLKGGPFSDSYRLFQFHFHWGSTNEHGSEHTVDGVKYSAELHVAHWNSAKYSSLAEAASKADGLAVIGVLMKV 150
Hum Mucus-CA                                              YSSAAEAISKPDGLAIIGVLIK
Hum CA II     KAVLKGGPLDGTYRLIQFHFHWGSLDGQGSEHTVDKKKYAAELHLVHWNT-KYGDFGKAVQQPDGLAVLGIFLKV
Hum CA XIII                                               ypsfveaahepdglavlgvflq
Hum CA III                                                yntfkealkqrdgiavigiflk

Hum CA I      GEANPKLQKVLDALQAIKTKGKRAPFTNFDPSTLLPSSLDFWTYPGSLTHPPLYESVTWIICKESISVSSEQLAQ 225
Hum Mucus-CA
Hum CA II     GSAKPGLQKVVDVLDSIKTKGKSADFTNFDPRGLLPESLDYWTYPGSLTTPPLLECVTWIVLKEPISVSSEQVLK

Hum CA I      FRSLLSNVEGDNAVPMQHNNRPTQPLKGRTVRASF-                                      261
Hum Mucus-CA               SVPKQHDNRPTQ
Hum CA II     FRKLNFNGEGEPEELMVDNWRPAQPLKNRQIKASFK
Hum CA XIII                aaflvsnhrppq
Hum CA III                 pvplvsnwrppq
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Fig. 6. Comparison of partial sequences of isolated human mucus-CA with human CA I, CA II and relevant regions of human CA XIII and CA III. Partial sequences of human mucus-CA were obtained by Edman degradation of tryptic cleavage products separated by µHPLC. Alignment of the mucus-CA peptides was done in such a way as to maximize agreement with the CA I sequence. Numbering of the alignment is based on human CA I. Underlined sites show identity to the human mucus-CA peptide sequences. GenBank sequences used: human CA I (#P00915); human CA II (#P00918); human CA XIII (#NP_940986); human CA III (#P07451).

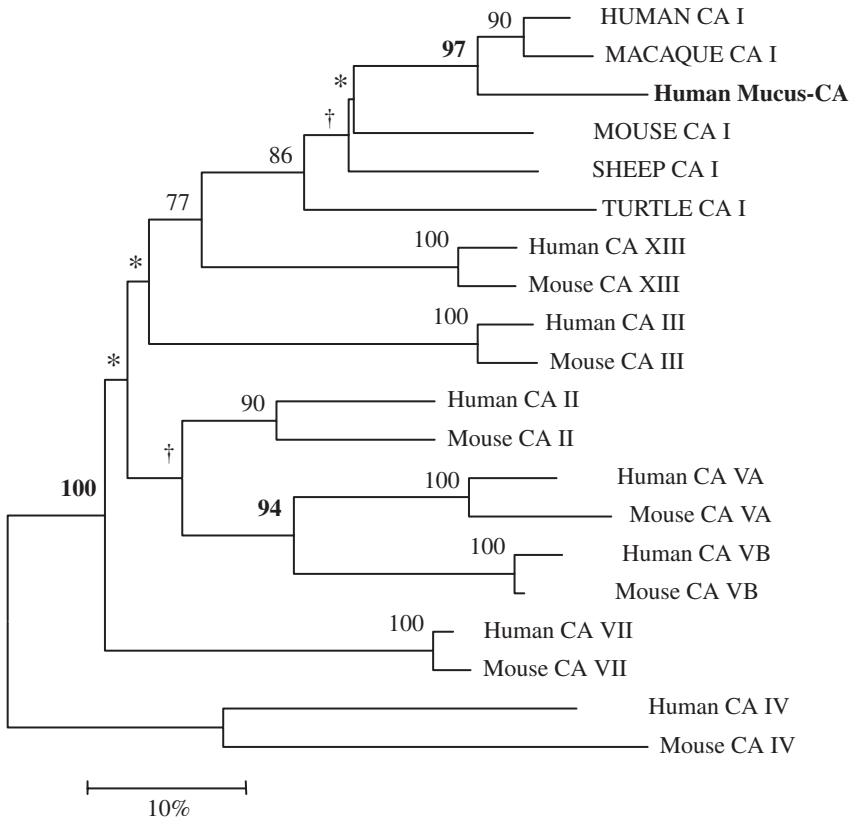

Fig. 7. Evolutionary relationships of human mucus carbonic anydrase. Distances between protein sequences were not corrected for underestimated multiple mutations. Tree was built by the neighbor-joining method using a 68 amino acid alignment of the three human mucus CA peptides with selected CA isozyme sequences. The numbers on the branches represent the bootstrap replicates; only values above 75% are shown (for details see Hewett-Emmett and Tashian, 1996). GenBank protein sequence accessions: human CA I, II, III, XIII are given in the Fig. 6 legend; human CA IV (#P22748); CA VA (#NP_001730), CA VB (#NP_009151), CA VII (#P43166); Macaca nemestrina CA I (#P35217); mouse CA I (#NP_033929), CA II (#NP_033931), CA III (#NP_031632), CA IV (#P031633), CA VA (#NP_031634), CA VB (#NP_851832), CA VII (#NP_444300), CA XIII (#AAK16672); sheep CA I (#P48282); turtle CA I (cf. Table 1 in Hewett-Emmett and Tashian, 1996). *Bootstrap value <50%. †50%< bootstrap value <75%. Bold: bootstrap values >85% and support clustering of different CA genes.

of SDS. This is shown here for gastrointestinal mucus CA of guinea pigs, mice and humans. In addition, intestinal mucus CA from guinea pigs is found in the water phase during phase separation experiments with Triton X-114, while CAIV is normally found in the Triton phase. Lastly, in the case of human colonic mucus, there is no immunoreactivity with anti-CAIV and the partial sequences of Fig. 6 are not compatible with human CA IV. (5) CAVI is known to be secreted into saliva and might pass intact through the gastrointestinal tract into caecum and colon. We report here lack of immunoreactivity of human colonic mucus in western blots with anti-human CAVI, eliminating CAVI as a possible major colonic mucus CA. In addition, the partial sequences of the human colonic mucus CA are not compatible with the sequence of human CA VI.

We conclude therefore that colonic mucus CA in humans – and likely in several other species as well – does not appear to be identical with any of the tested CA isoforms. The interpretation of the partial sequences obtained for human mucus CA is not entirely clear. They may suggest an isoform similar to CAI; the lack of CAI antibody to react with human mucus CA, however, argues against this view. In the case of mouse, human and guinea pig mucus the moderate anion sensitivities in addition are clearly not compatible with the high anion sensitivity known for CAI. It should be mentioned that the mucus CA sequences shown in Table 6 are also incompatible with the isoenzymes CAVA, CAVB, CAVII, CAIX, CAXII, CAXIII, CAXIV and CAXV as well as the 'acatalytic' CA-related protein isoforms CA-RP VIII, CA-RP X and CA-RP XI. It appears likely therefore that mucus CA represents a hitherto unknown CA isoenzyme.

A search in the human genome databases for the mucus CA sequences of Table 6 was not successful. It may be noted that Chegwidden et al. (1995) and Chegwidden et al. (2001) reported a variant CAI-like DNA sequence encoding an isozyme which they named CAIB. Their inferred CAIB protein sequence is quite distinct from the mucus-CA peptide sequences shown in Fig. 6. Like our sequence, however, it cannot presently be found in the human genome or other DNA sequence databases.

We have constructed an evolutionary tree (Fig. 7) using just the part of the alignment covering the three peptides (68 amino acids) that have been characterized in the human mucus CA (Fig. 6). We included CAI from the Old World monkey *Macaca nemestrina* (Hopkins et al., 1995), and from mouse, sheep and turtle, plus human and mouse CAXIII, CAII, CAIII, CAVA, CAVB, CAVII and (as root) CAIV. This tree indicates that the mucus CA I-like gene most likely resulted from a gene duplication between 30 and 75 million years ago, i.e. it is probably restricted to primates although it might be absent from the earliest diverging primate lineages (lemurs and lorisoids). A less straightforward possibility, however, is that the gene duplication predated the mammalian radiation generating a pair of CAI genes, one of which became expressed in all mammalian intestinal mucosae. The close similarity of the gene pair in primates could have arisen by intergenic sequence exchange, i.e. gene conversion of the mucus CAI gene by the well characterized CAI gene, giving a more recent apparent date for the gene duplication based on the limited peptide sequence data presently available.

Possible physiological significance of mucus carbonic anhydrase

What might be the physiological function of CA in gastrointestinal mucus? The apical membrane of the colon of the guinea pig, as well as other species, is a membrane through which several acid-base-relevant transport processes occur: Na^+-H^+ exchange (proximal colon), K^+-H^+ pump (distal), HCO_3^- secretion by a short-chain-fatty-acid/HCO_3^- exchanger and possibly by a $Cl^-HCO_3^-$ exchanger, proton consumption on the apical surface by the uptake of undissociated short chain fatty acids through the apical membrane (for overview see von Engelhardt et al., 1994). These transport processes can pose a challenge to the apical surface pH; but the latter can in addition be challenged by high P_{CO_2} values in the colonic lumen or by high loads of fixed acid associated with low pH values in the luminal contents. It has been shown that under a large variety of luminal conditions the pH in the mucus layer, the so-called pH microclimate that exists in a layer ~0.5 mm thick on the apical epithelial surface, is held rather constant, indicating that there is a regulatory system for this surface pH (Rechkemmer 1981; McNeil et al., 1987; Rechkemmer et al., 1986; Said et al., 1986). Most, although not all, of the molecules involved in challenging as well as regulating the apical surface pH are the molecules and ions that participate in the CO_2 hydration reaction: H^+, HCO_3^-, and CO_2. In view of rapidly changing luminal conditions and in view of high transcellular fluxes of acid–base relevant ions, it would appear very beneficial for maintaining a constant pH on the apical surface that these three species can rapidly achieve chemical equilibrium. When it takes about 1 min for the uncatalysed hydration–dehydration reaction to reach chemical equilibrium, as would be the case in the absence of carbonic anhydrase, pH regulatory transport processes cannot be expected to re-establish the pH microclimate after a challenge faster than this. It is likely, therefore, that an extracellular CA in the intestinal mucus contributes to maintaining the pH microclimate. Experimental proof demonstrating this has yet to be provided.

This hypothesis does not answer the question whether such an extracellular CA would have necessarily to be located in the mucus layer rather than directly on the membrane surface as a membrane-bound CA on the apical membrane. In fact, it would appear logical to have a CA right on the external surface of the membrane across which acid–base transports occur, because this would ensure that the reaction partners establish chemical equilibrium immediately after they have crossed the membrane. Conversely, we have recently shown (Endeward et al., 2003) that a CA distributed throughout the entire mucus layer may be useful for another possible function of the epithelial barrier. We have demonstrated theoretically that due to the continuous mucus production (and mucus flow from the apical membrane across the mucus layer towards the lumen)

the CO_2 partial pressure at the apical membrane may be considerably lower than that which prevails in the lumen. This is accomplished by the flow of mucus in conjunction with the very high buffer power of the mucus and the presence of a substantial CA activity all across the entire mucus layer. Mucus CA may, therefore, have an important role in protecting the epithelium against the very high CO_2 partial pressures that can occur in the lumina of various sections of the gastrointestinal tract.

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