Developmental differences in the expression of the cholera toxin sensitive subunit (Gsα) of adenylate cyclase in the rat small intestine

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Abstract

Background—The stimulatory guanosine triphosphate (GTP) binding protein α subunit (Gsα) of adenylate cyclase is the target protein for cholera toxin.

Aims/methods—The expression of this signal transducer was analysed in the small intestine of developing rats by RNA transfer (northern blot) analysis by immunoblotting, and by ADP-ribosylation of membrane proteins.

Results—Intestinal Gsα mRNA (about 1·9 kb) was increased in the neonate compared with the adult rat. Two isoforms of Gsα proteins, a 45 000 and a 52 000 form, were expressed in the small intestinal epithelial cell and both were ADP-ribosylated by cholera toxin. A significant increase in the larger isoform (52 000) and in its ribosylation was noted in the 2 week old suckling compared with post-weaned older animals. The protein content or ribosylation of the smaller form (45 000) did not significantly change with age.

Conclusion—These data show that a developmental decline of intestinal Gsα expression seems to be, in part, regulated at the mRNA level. An increased Gsα expression in the immature intestine may help to explain a previously reported, dose dependent increased adenylate cyclase response and an increase in fluid secretion to cholera toxin in neonates compared with adults.

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The signal transducing guanosine triphosphate (GTP) binding protein (G protein) that regulates adenylate cyclase (EC 4.5.1.1) is an αβγ heterotrimer. Under physiological conditions, the binding of a hormone or neurotransmitter to the cell surface β-adrenergic receptor activates Gs, the stimulatory regulator of adenylate cyclase. The activation is initiated by a release of bound GDP and the subsequent binding of GTP to the Gsα subunit of Gs. Gsα bound to GTP dissociates from the βγ subunit to yield an active Gsα-GTP form, which is directly responsible for activation of the cyclase catalytic unit. Hydrolysis of bound GTP to GDP by the GTPase intrinsic to Gsα terminates the signal.

During infection, cholera toxin (CT) is produced in the upper small intestine by Vibrio cholerae. The interaction of this toxin with the enterocyte results in a toxicogic diarrhoal disease that principally affects young children. CT is an ADP-ribosylating toxin that uses Gsα as its substrate. It is an oligomeric protein of 84 000, composed of one A-subunit (active component) associated non-covalently with five B-subunits (binding components). The A subunit contains two polypeptides, denoted A1 and A2, linked by a single disulphide bond. The CT B-subunit binds to a GM1 glycolipid receptor on the enterocyte surface, and the A-subunit dissociates from B and enters into the cell, presumably via the formation of endosomes. Cleavage of A2 from the A-subunit, by a still undefined cellular reaction, activates the ADP-ribosyltransferase of A1. The enzyme activity of A1 is further increased by another cellular factor, namely ADP-ribosylation factor (ARP). The activated A1 then catalyses ADP-ribosylation of Gsα, which inhibits the GTPase activity of A1 and prevents the activation of the CT toxin. As a result, adenylate cyclase is persistently activated to produce cAMP from ATP. Cyclic AMP accumulation subsequently opens the Cl⁻ channel in intestinal crypt cells, and inhibits the NaCl co-transporter in intestinal villus cells, resulting in a changed ion flux that causes massive diarrhoea. Recent studies show that the CFTR (cystic fibrosis transmembrane conductance regulator) itself could be the cAMP-responsive chloride channel14 and it is proposed that patients with cystic fibrosis may be less susceptible to CT induced diarrhoea.

The human Gsα gene protein on human chromosome 20 contains 13 exons and spans about 20 kb. In the coding region, the nucleotide sequence homology between human and rat Gsα is 95%. The tissue specific expression of the Gsα isoform results from an alternative splicing of a single mRNA precursor. In the human brain, Gsα protein exists predominantly as both a small (45 000) and large (52 000) protein, which can be ADP-ribosylated. In the rabbit intestine, CT catalyses the ADP-ribosylation of proteins of 40 and 47 000 respectively. The proportion of these isoforms varies among tissues and cells and their functional differences have not yet been clarified.

Despite recent extensive studies of G protein, little is known about the gene expression and regulation of Gsα in the enterocyte, the natural target cell for CT during development. Previously, we have reported an increase in adenylate cyclase activity and in fluid...
secretion in the small intestine of suckling rats compared with older animals. We have hypothesised that a variation in amounts of \( \text{Gsa} \) protein during intestinal development might be one of the factors that contributes to this changed host responsiveness. To test this hypothesis, we have studied the ontogeny of \( \text{Gsa} \) expression in the rat small intestine. Our results show that there is indeed an age-dependent decline in intestinal \( \text{Gsa} \) mRNA expression, protein concentration, and ribosylation during development. Accordingly, we have concluded that an increase in gene expression for \( \text{Gsa} \) in the immature gut may contribute, in part, to the increased enterocyte responsiveness to CT in young mammals.

### Methods

#### Animals

Sprague-Dawley rats (Charles River Breeding Laboratories, Wilmington, MA) were housed in an animal room with a 12 hour light/dark cycle, fed rat chow (Purina, St Louis, MO), and permitted water ad libitum. In all studies, animals remained with their mothers until 2 weeks when one half of each litter was removed for separation of epithelial cells. The remaining animals were weaned at 3 weeks and examined at 8 weeks. For mRNA studies, four litters of 10 pups each were examined, litters being split at 2 weeks with five pups removed from each litter. Differences in \( \text{Gsa} \) protein were examined in a separate litter and a further litter was used to study differences in ribosylation. All experiments were approved by the Subcommittee on Research Animal Care at the Massachusetts General Hospital.

#### Enterocyte isolation

Enterocytes were isolated from full length rat small intestine according to a modification of a method described by Weiser. A protease inhibitor PMSF (1 mM final concentration) was added to all buffers. Intestinal sacs were filled with the buffer and incubated at 4°C. Enterocytes from postweaned rats were collected every 20 minutes for four consecutive periods and pooled. The collecting time was reduced to 15 minutes for neonatal enterocytes. For studies of \( \text{Gsa} \) mRNA, isolated enterocytes were homogenised in guanidine salts. For protein preparations, enterocytes were transferred to homogenising buffer (see later).

#### Nucleic acid probes

Hybridisation experiments were performed using (1) \( \text{Gsa} \) cDNA for \( \text{Gsa} \) was made from the polymerase chain reaction (PCR) products of reverse transcription of rat epithelial cell mRNA using \( \text{Gsa} \)-specific oligonucleotides as primers. Primers were synthesised from published sequences Genbank M12673. After reverse transcription using 50 U/µl of reverse transcriptase, cDNA was amplified in four separate reaction tubes with each combination of the following upstream primers (\( \text{Gsa} \) 331s: CAGCTGCAAG AGGACAGC; \( \text{Gsa} \) 483s: TGCAAGGAGC AACAGGCG) and downstream primers (\( \text{Gsa} \) 849as: CGTCTGACCC TCTGGAATCT; \( \text{Gsa} \) 931as: GATGAAGCGGC GCA AAGTGAG). The size of four PCR products was as predicted by the distance between the primers. Confirmation of their identity was obtained by transferring DNA to nylon membranes (Southern blot) and hybridising with a separately synthesised 39-base oligonucleotide \( \text{Gsa} \) probe (DuPont/NEB, Boston, MA). (2) \( \gamma \)-actin: cDNA as a BamHI/HindIII insert in a pSP64 vector (a gift of Dr Herman Eisen, Massachusetts Institute of Technology, Cambridge, MA). DNA was labelled with [\( \text{32P} \)]dCTP (3000 Ci/mmol, DuPont/New England Nuclear, Boston, MA) with Klenow fragment after random hexanucleotide priming with Prime-IT II labelling system (Stratagene, Anaheim, CA).

#### RNA transfer (northern blot analysis)

The epithelial cell pellet was dissolved into at least 20 vol GIT buffer (4 M guanidine isothiocyanate, 50 mM TRIS (pH 7.6), 2% Sarkosyl and 100 mM 2-mercaptoethanol). The RNAs were deproteinised by selective precipitation from GIT buffer and by extraction with phenol, chloroform, and isooamyl alcohol. RNA was quantified by absorbance at 260 nm and stored at -20°C. For RNA transfer blots (northern blots), RNAs (5 µg per well) were separated in morpholinopropane sulfonic acid-formaldehyde agarose gels and transferred to GeneScreen Plus membranes (DuPont/NEB) by capillarity. Blots were hybridised and washed according to manufacturer's recommendations (DuPont/NEB). Washed blots were autoradiographed between intensifying screens at -70°C. The amount of hybridisation of labelled \( \text{Gsa} \) chain cDNA to RNA transfer blots was determined by measuring the optical density of the \( \text{Gsa} \) mRNA by scanning the autoradiographs utilising Molecular Dynamics (Molecular Dynamics, laser densitometer Sunnyvale, CA) as described later. Once \( \text{Gsa} \) had been measured, blots were reprobed with \( \gamma \)-actin whose expression does not change with development in epithelial cells. The amount of \( \text{Gsa} \) mRNA of the intestinal epithelium of each individual rat was expressed as the ratio of \( \text{Gsa} \) mRNA optical density to \( \gamma \)-actin mRNA optical density. RNA samples from any one litter were always electrophoresed, transferred, probed, and measured together as a single unit. RNA from different litters were examined on different membranes. It is possible therefore to examine the changes in \( \text{Gsa} \) within a litter, but absolute values cannot be compared between litters.

#### Enterocyte protein preparations

All procedures were performed at 4°C. Isolated enterocytes were homogenised in a homogenising buffer (0-1 M TRIS-HCl, pH
bands were quantified using a laser densitometer (Molecular Dynamics, Sunnyvale, CA). The densitometry programme was established to subtract for background, which was recorded before other measurements. The optical density of bands relative to protein content was verified in other experiments using radioactive probes, where direct measurements of radioactivity correlated with densitometry.

**ADP-ribosylation of proteins with CT**

Membrane fractions prepared from isolated neonatal and adult enterocytes and adult brain homogenates were used. CT catalysed ADP-ribosylation was performed as described by Gill and Woolkalis. The reaction mixture contained 100 μg of membrane, 20 μg/ml of activated CT, 20 μM [32P]NAD (20 000 cpm/pmol), 20 mM isoniazid, 1 mM 3-acetylpyridine adenine nucleotide, 10 mM thymidine, 10 mM dithiothreitol, 0-1% Triton X-100, and 0-5 mM Gpp(NH)p. The reaction was incubated at 25°C for one hour. The membrane was recovered and subjected to SDS-PAGE on 10% gel. The gel was dried and subjected to autoradiography. Ribosylation of each isoform was determined by measuring densitometry of bands at the appropriate molecular weight on the autoradiograph.

**Statistics**
mRNA expression was examined by analysis of variance (ANOVA). The effects of age and litter on Gsa/actin mRNA were examined as independent variables. Differences were regarded as significant at p<0.05. Gsa protein experiments were all performed within one litter and the results of the change between 2 weeks and 8 weeks were compared using Student’s t test. Similarly ribosylation was also examined within a single litter. In these latter cases each point represents the mean and standard deviation of three experiments.

**Results**

**Developmental decline in Gsa mRNA expression**

To examine the effect of age on Gsa mRNA, four litters (40 pups) were examined. Twenty pups were killed at 2 weeks of age and the intestinal epithelial cell fraction of each animal was examined separately. The remaining 20 were weaned at 3 weeks and examined at 8 weeks. Hybridisation was observed at a band that corresponded to 1-9 kb, the size of Gso mRNA (Fig 1A). Northern blot analysis of Gsa mRNA/γ-actin mRNA from epithelial cells showed a significant decrease with age between 2 and 8 weeks (p<0.05) (Fig 1). Although each litter showed a reduction in Gso RNA in relation to that of γ-actin, there was a variation in the degree of this effect between litters, being 74%, 38%, 20%, and 54% in litters 1 to 4, respectively. The amount of γ-actin mRNA per cell is known to remain constant in intestinal epithelial cells during development.

**Gel electrophoresis and immunoblotting**

Membrane proteins were subjected to SDS-PAGE on a 10% gel, transferred to a nitrocellulose membrane, and probed with a rabbit anti-G protein (RM1/Gso) polyclonal antibody. The blot was subsequently incubated with a horseradish peroxidase conjugated donkey antirabbit immunoglobulin polyclonal antibody. The target proteins were then detected with an enhanced chemiluminescence western blotting system as described by the supplier (Amersham, Arlington Heights, IL). Protein

Figure 1: RNA transfer blot analysis of Gsa mRNA expression in the rat small intestine during perinatal development. (A) Northern blot of epithelial cell RNA probe with Gsa and γ-actin cDNA showing a reduction in Gsa mRNA ratio in small intestinal epithelial cells of rats between 2 weeks and 8 weeks (from a single litter). Five μg of RNA from each animal was electrophoresed and transferred to nylon membranes. (B) The blots were hybridised with Gsa and γ-actin cDNAs sequentially and the degree of hybridisation measured by densitometry. Each individual transfer blot contained all the specimens of a single litter at both time points. The ratio of the Gsa and γ-actin for each rat was calculated. The data are expressed as mean (SEM) of the Gsa mRNA to γ-actin mRNA ratios. Gsa/actin mRNA was significantly greater (p<0.05) in suckling animals than in 8 week rats.
Expression patterns of Gsα isoforms in the developing rat intestine
To identify intestinal Gsα and to determine if there was a decline in Gsα protein expression as noted with Gsα mRNA during development, intestinal membrane samples were analysed by immunoblotting using an antibody that detected Gsα isoforms with a single litter. Two predominant Gsα isoforms were detected in whole intestine (Fig 2A) as well as in isolated enterocytes, one a 45 000 (small form) and the other a 52 000 (large form). These two isoforms were also detected in rat brain, used here as a positive control (Fig 2A). An age related decline in the expression of the large (52 000) but not the small (45 000) Gsα isoform was noted in membrane samples prepared from both whole intestine and from isolated enterocytes. Densitometric analysis showed approximately a 4-5-fold decrease in the abundance of the 52 000 Gsα isoform expressed in whole intestine from 2 week old to preweaned compared with the 8 week old postweaned rat (Fig 2B). A similar decline was seen in Gsα isolated from isolated enterocytes (Fig 2C) (p<0.05).

CT catalysed ADP-ribosylation of membrane proteins in rat enterocytes
To determine if the two immunodetectable Gsα isoforms could serve as substrates for CT in ADP-ribosylation, CT catalysed [32P]ADP-riboseylation was performed using membrane proteins prepared from isolated enterocytes. CT catalysed the ADP-ribosylation of both the 45 000 and 52 000 Gsα isoforms. The expression of the large isoform (52 000) declined from 2 to 8 weeks of age (p<0.025); however, there was no change in the smaller protein (45 000).

In accordance with the data examining Gsα protein expression (discussed earlier), there was a significant fall in ribosylation activity in enterocytes from 2 weeks old compared with 8 weeks old rats (Fig 3). These experiments...
therefore show that Gsa mRNA, protein levels, and ribosylation activity are under developmental regulation.

Discussion
CT induced secretory diarrhoea occurs more commonly in young infants than in older children and adults. It is the best characterised disease mediated by a Gs/oAMP signalling pathway. Previous studies from our laboratory have shown an increased sensitivity in the adenylate cyclase response to CT in the small intestine of suckling rats. This was accompanied by an increased responsiveness in toxin induced fluid secretion. In this study, we have examined these findings and provide evidence to suggest that the molecular basis for this changed host responsiveness might be related to a developmental variation in Gsa gene expression. Several lines of evidence show that Gsa gene expression may be modulated by developmental and hormonal regulation as well as by disease states. For example, an age dependent decrease in the expression of the 1.9 kb Gsa mRNA transcript was noted in rat brain and rat testes. Treatment of F9 mouse teratocarcinoma cells with retinoic acid for five days resulted in a 20-fold increase in steady state levels of a 2.0 kb Gsa mRNA, accompanied by an increase in the expression of the 45 000 and 52 000 Gso polypeptides. In addition, a decreased Gsa mRNA level was associated with a fall in Gs protein and in adenylate cyclase activity in compensated left ventricular hypertrophy. In a neuroblastoma α glioma hybrid, NG108-15 cells, the mechanisms of an ethanol induced change in adenylate cyclase entails a decrease in gene expression of Gsa, resulting in a decrease in the quantity of Gsa mRNA and Gsa protein in those cell membranes exposed longterm to ethanol.

In the small intestine, our results have shown an increased level of Gsa mRNA expression in the suckling compared with the mature rat although the exact degree of decrease varied with the litter examined (Fig 1). A dependence on litter is a feature of a number of developmentally regulated genes. The 52 000, but not the 45 000, Gso protein isoform concentration was also significantly greater in suckling compared with post-weaned rats. This was reflected in a reduction of ADP-ribosylation of the 52 000 isoform with development although both isoforms were ADP-ribosylated by CT in vitro.

Under physiological conditions, intestinal Gsa is used by vasoactive intestinal peptide as a signal transducer, coupling the β-adrenergic receptor to adenylate cyclase. Chastre et al reported that intestinal cells are more sensitive to vasopressin than to vasopressin's vasodilator action. The adenylate cyclase response in 17 and 19 day old fetuses than in adult rats. They further showed that the difference might result from a developmental variation at the level of vasoactive intestinal peptide receptors.

It is noteworthy that in enterocytes, the catalytic unit of adenylate cyclase is located on the basolateral membrane, while depending on species, the distribution of the enzyme's regulatory unit is not necessarily restricted to the basolateral membrane. In rat enterocytes, the CT targeted Gsa is found on the basolateral membrane. In contrast, in the small intestines of rabbits, CT catalyses ADP-ribosylation of Gso proteins in the microvillus membrane and these proteins then migrate to the basolateral membrane where they activate the catalytic component of adenylate cyclase. The basis for this species specific sorting difference of Gso proteins in polarised enterocytes is currently unknown.

In summary, this study suggests that a postnatal decline in Gsa gene expression in rat enterocytes may in part contribute to an age dependent difference in adenylate cyclase responsiveness to stimulation by bacterial toxins. We wish to thank Ms Suzette McCarron and Ms Sally Burke for their expert typing of this manuscript. We are also indebted to David Schoenfeld, PhD for statistical advice. This work was supported by grants from the National Institutes of Health (RO1 HD31852 and RO1 HD12437).


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