The role of Gαq/Gα11 signaling in intestinal epithelial cells

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ABSTRACT

Intestinal homeostasis and the coordinated actions of digestion, absorption and excretion are tightly regulated by a number of gastrointestinal hormones. Most of them exert their actions through G-protein-coupled receptors. Recently, we showed that the absence of Gαq/Gα11 signaling impaired the maturation of Paneth cells, induced their differentiation toward goblet cells, and affected the regeneration of the colonic mucosa in an experimental model of colitis. Although an immunohistochemical study showed that Gαq/Gα11 were highly expressed in enterocytes, it seemed that enterocytes were not affected in Int-Gαq/Gα11 double knock-out intestine. Thus, we used an intestinal epithelial cell line to examine the role of signaling through Gαq/Gα11 in enterocytes and manipulated the expression level of Gαq and/or Gα11. The proliferation was inhibited in IEC-6 cells that overexpressed Gαq/Gα11 and enhanced in IEC-6 cells in which Gαq/Gα11 was downregulated. The expression of T-cell factor 1 was increased according to the overexpression of Gαq/Gα11. The expression of Notch1 intracellular cytoplasmic domain was decreased by the overexpression of Gαq/Gα11 and increased by the downregulation of Gαq/Gα11. The relative mRNA expression of Muc2, a goblet cell marker, was elevated in a Gαq/Gα11 knock-down experiment. Our findings suggest that Gαq/Gα11-mediated signaling inhibits proliferation and may support a physiological function, such as absorption or secretion, in terminally differentiated enterocytes.

1. Introduction

The architectural features of the intestinal epithelium are maintained by a rapid cellular turnover through the continuous replication of multipotential stem cells residing in the niches in the lower part of the crypt [1]. Stem cells give rise to progenitor cells, which are amplified by constant division along the bottom two-thirds of the crypts [2]. These daughter cells migrate up as they differentiate. In the transit amplifying (TA) zone near the top of the crypt, these cells terminally differentiate into the four main cell types: absorptive enterocytes and three secreting cell types: mucus-secreting goblet cells, antimicrobial peptide-secreting Paneth cells and hormone-secreting enteroendocrine cells [3].

Proliferation, differentiation and morphogenesis in the intestinal epithelium are tightly regulated by a number of molecular pathways. Cells adopt an absorptive or secretory cell fate according to the balance between Wnt and Notch signaling, regulating transcription networks that further define the differentiation of intestinal epithelial cells (IECs) [4,5]. The most established effects of Wnt/β-catenin in IECs are those involved in cellular proliferation, in particular the maintenance of the proliferative state of progenitors [4]. The Notch cascade mediates cell-to-cell signaling and has been shown to be essential for the maintenance of the proliferative crypt compartment, as well as for the formation of absorptive enterocytes [4].

The digestive tract consists of a variety of tissues. The coordination of the complex functions of the digestion, absorption, and excretion of a meal is largely achieved by molecules of neuroendocrine origin [6]. Most of the gastrointestinal hormones regulate their target cells through G-protein coupled receptors (GPCRs). Secretin, glucose-dependent insulino tropic polypeptide (GIP), and glucagon-like-peptide-1 (GLP-1)
exert their signals through the Gα family of heterotrimeric G proteins [7]. In contrast, cholecystokinin (CCK), gastrin, and acetylcholine (ACh) exert their signals through the Gαq family of G proteins. Mammals express four Gαq class α-subunits, of which two—Gαq and Gα11—are widely expressed [8].

In order to evaluate the physiological relevance in the regulation of intestinal homeostasis, we generated Int-Gαq/Gα11 double knock-out (DKO) (C57BL/6 J; Gαqfl/fl; Gα11fl/fl) mice and investigated the role of Gαq/Gα11-mediated signaling in the intestine [9]. The absence of Gαq/Gα11-mediated signaling especially impaired Paneth cell maturation and positioning, and regeneration in an experimental model of colitis.

In an immunohistochemical study, the expression of Gαq/Gα11 was abundantly detected near the basolateral membrane of enterocytes, but not in the Paneth cells. Although the Paneth cells in the Int-Gαq/Gα11 DKO intestine showed severe alteration, the enterocytes did not show any alteration. Thus, we used a rat intestinal epithelial cell line (IEC-6) to explore the role of Gαq/Gα11-mediated signaling in enterocytes. We manipulated the expression of Gαq/Gα11 in IEC-6 cells and evaluated the changes in proliferation, intracellular signaling, and differentiation.

2. Materials and methods

2.1. Cell culture

IEC-6 cells (RBRC-RCB0993), a rat intestinal epithelial cell line, were purchased from RIKEN Cell Bank (Tsukuba, Japan). IEC-6 cells were cultured in Dubcco’s modified Eagle’s medium (DMEM) containing 10% fetal bovine serum, 25 mM glucose, 100 U/ml penicillin, and 100 μg/ml streptomycin at 37 °C in a humidified environment of 95% air and 5% CO2. The medium was replaced every 1–3 days, depending on the harvest time and the degree of confluence. All of the experiments were carried out using IEC-6 cells at the 10–25th passage.

2.2. Construction of retroviruses expressing GNAQ and GNA11

The GNAQ and GNA11 retroviral expression vectors were prepared in a bicistronic vector pMXs-ires-EGFP and pMXs-ires-Neo, respectively (Cell Biolabs, Inc., San Diego, CA, USA). The human full-length GNAQ gene (GenBank Accession No. NM_002072) was cloned by a PCR using total RNA from CaCo2 cells as a template and the following primers: sense 5′-CTCAGAGCCACAGTGATCGGATCCATGATGC-3′ and antisense 5′-GGGCGCCGCTTACGAGCATGTCTCCTCAG-3′. The human full-length GNA11 gene (GenBank Accession No. NM_002072) was amplified by a PCR using total RNA from CaCo2 cells as a template and the following primers: sense 5′-CTCAGAGCCACAGTGATCGGATCCATGATGC-3′ and antisense 5′-GGGCGCCGCTCAACAGGTTGTACTCCTTCAG-3′. The PCR products were digested with BamHI and were inserted into the pMXs-IRES-GFP and pMXs-IRES-Neo vector, respectively. The expression vectors (pMXs-GNAQ-IRES-GFP, pMXs-GNA11-IRES-GFP) were transfected into the medium using a FACS Vantage system (Beckton Dickinson, NJ, USA) into PLAT-E cells to obtain the viruses. The IEC-6 cells that were used as a negative control were transfected with X-Fluc (Gatobio, Basel, Switzerland) and the start codon is indicated by bold typeface.

2.3. Construction of GNAQ- and/or GNA11-overexpressing IEC-6 cells

The expression vectors (pMXs-GNAQ-ires-EGFP, pMXs-GNA11-ires-EGFP) and the mock vectors (pMXs-ires-EGFP, pMXs-ires-Neo) that were used as a negative control were transfected with X-tremeGENE 9 DNA transfection reagent (Roche Applied Science, Basel, Switzerland) into PLAT-E cells to obtain the viruses. The IEC-6 cells were infected with the viruses, and the cells expressing GFP were sorted 2.72 h after infection. The IEC-6 cells expressing neomycin-resistant gene were selected with the addition of 400 μg/ml G418 (Invitrogen, Carlsbad, CA, USA) into the culture medium. The cells were named IEC6-cont1 (pMXs-ires-EGFP vector), IEC6-cont2 (pMXs-ires-Neo vector), IEC6-Gq, IEC6-G11, and IEC6-Gq/11, respectively.

2.4. Small interfering RNA (siRNA) transfection

The siRNAs specific for rat Gnaq (Stealth siRNAs RSS330736, RSS330737, RSS372821) and Gna11 (Stealth siRNAs RSS340230, RSS340231, RSS340232) and the matched negative control were purchased from Invitrogen. The Gnaq, Gna11 and negative control siRNAs were transfected twice on two consecutive days using Lipofectamine 2000 (Invitrogen) according to the manufacturer’s instructions. The cells were named IEC6-siGnt (negative control siRNA), IEC6-Gaq (Gnaq siRNA), IEC6-Ga11 (Gna11 siRNA) and IEC6-Gq/11 (Gnaq + Gna11 siRNA).

2.5. Measurement of cell growth and DNA synthesis

To measure cell growth, the cells were seeded at a density of 1 × 104 cells/ml in plastic 24-well plates and cultured. After 4, 7 and 10 days, the cells were detached by incubation with 0.05% trypsin/EDTA, and the number of cells was counted using a Cell Counter Plate (Wattson, Kobe, Japan). To evaluate DNA synthesis, IEC-6 cells were seeded at a density of 1 × 105 cells/ml in 96-well culture plates. Following serum starvation for 24 h, the cells were cultured for an additional 48 h. BrdU was added for the last two hours of incubation. The DNA synthesis was evaluated using a BrdU incorporation assay kit (Roche Diagnostics, Mannheim, Germany) according to the manufacturer’s instruction. CCK-8 was purchased from Peptide Institute (Osaka, Japan), and carbachol was obtained from Sigma-Aldrich (St. Louis, MO, USA).

2.6. Western blotting

IEC-6 cells were homogenized in a lysis buffer (100 mM NaCl, 20 mM Tris/HCl (pH7.5), 1% TritonX-100). After centrifugation, the crude extracts were boiled in Laemmli 2× sample buffer. Twenty to eighty micrograms of protein was loaded onto each lane of 10% sodium dodecyl sulphate-polyacrylamide gels and run at 200 V. The proteins were then transferred onto nitrocellulose membranes at 60 V for 4 h. The membranes were incubated sequentially with Blocking Ace (Snow Brand Milk Products, Sapporo, Japan), primary antibodies (Abs) and secondary Abs, then were detected using an enhanced chemiluminescence Western blotting detection reagent (Amersham Biosciences, Piscataway, NJ) to visualize the secondary Ab. The experiment was repeated independently at least three times. The densitometry analysis was performed using the ImageJ software program. The primary Abs used in this study were anti-GFP Ab from Thermo Fisher Scientific (Carlsbad, CA, USA); anti-Gq/11 and anti-Notch1 Abs from Abcam (Cambridge, UK); anti-phospho-PKC (pan), anti-PKCα, anti-PKCδ, and anti-Tcf1 Abs from Cell Signaling (Danvers, MA, USA); and anti-actin Ab from Santa Cruz (Dallas, TX, USA). The secondary Abs were horseradish-peroxidase-conjugated donkey anti-rabbit IgG and horseradish-peroxidase-conjugated donkey anti-goat IgG, purchased from Jackson Immuno Research (West Grove, PA, USA).

2.7. Quantitative real-time PCR (qPCR)

The following primers were used for the qPCR: Muc2, sense 5’-GAATGAGATTGAGTGGCA-3’ and antisense 5’-GGATCCGGTGTGGTACCTCAG-3’; β-actin, sense 5’-TGAGAGGGAAATCGTGCGTG-3’ and antisense 5’-TGTCGACGAAGGAGGAGG-3’. The reactions were performed using an ABI PRISM 7900HT system (Applied Biosystems), with denaturation at 95 °C for 15 s, and annealing and extension at 60 °C for 60 s.

2.8. Statistical analysis

All data are presented as the mean ± standard deviation. The statistical significance of the values obtained was evaluated by Student’s t-
allowed to grow over a 7-day period. To confirm positive selection with geneticin (G418), equal numbers of IEC6-cont1, IEC6-Gq, and IEC6-Gq/11 cells were transfected and selected with geneticin (G418). The retroviral transduction results in the stable integration of viral vectors to drive the expression of signed overexpression and knock-down systems using a non-transcribed plasmid. After sorting and/or cell sorting of G4q/G11 cells, the band of G4q/G11 expression was clearly detected with anti-G4q/G11 antibodies. As shown in Fig. 1, the band of G4q/G11 expression was detected using anti-phospho PKC (pan) Ab and the blots of PKC α isoform and the consequent calcium mobilization and PKC activation [10].

3. Results

3.1. Generation of G4q and/or Gα11-overexpressed IEC-6 cells

To determine the functional role of G4q/Gα11 signaling, we designed overexpression and knock-down systems using a non-transformed small intestinal epithelial cell line, IEC-6. We generated retroviral vectors to drive the expression of GNAQ and/or GNA11 cDNA. The recombinant retroviral transduction results in the stable integration of the GNAQ and/or GNA11 cDNA transgene into the IEC-6 genome and the stable expression of GNAQ and/or GNA11. After sorting and/or positive selection with geneticin (G418), equal numbers of IEC6-cont1, IEC6-cont2, IEC6-Gq, IEC6-G11, and IEC6-Gq/G11/11 cells were plated and allowed to grow over a 7-day period. To confirm the overexpression of G4q and Gα11, we performed Western blotting using anti-G4q/Gα11 and anti-GFP antibodies. As shown in Fig. 1, the band of G4q/Gα11 was not detected in naïve IEC6, IEC6-cont1 or IEC6-cont2 cells. It was slightly expressed in IEC6-Gq and IEC6-G11 cells and was clearly overexpressed in IEC6-Gq/G11 cells. As expected, the expression of GFP was observed in IEC-cont1, IEC6-Gq, and IEC6-Gq/G11 cells, but not in naïve IEC6, IEC6-cont2 or IEC6-G11 cells. The activation of G4q and Gα11 results in the stimulation of the phospholipase C (PLC)-β isoform and the consequent inositol 1,4,5-triphosphate-mediated (IP3-mediated) intracellular calcium mobilization and PKC activation [10]. To confirm the functional expression of G4q and Gα11 in IEC-6 cells, the PKC phosphorylation was examined using anti-phospho PKC (pan) Ab and the blots of PKCa and PKCβ were used as internal controls. As shown, the induction of the G4q/Gα11 expression resulted in an increase in PKC phosphorylation.

3.2. The effect of G4q/Gα11-overexpression in IEC-6 cells

We first analyzed the proliferation of IEC-6 cells by counting the number of cells and measuring the incorporation of BrdU (Fig. 2A, B). We seeded IEC-6 cells at 1 × 10^4 cell/ml in plastic plates and counted the number of cells on days 4, 7, and 10. As shown, the proliferation was inhibited in IEC6-Gq, and IEC6-G11 cells, and severely inhibited in IEC6-Gq/G11 cells. In the BrdU incorporation assay, a similar result was obtained and the proliferation was significantly inhibited in IEC6-Gq/G11 cells.

Intestinal homeostasis is preserved by a number of molecular pathways, including Wnt/β-catenin and Notch signaling [4]. Wnt/β-catenin signaling was altered in the Int-Gq/Gq11 DKO intestine [9]. To evaluate Wnt/β-catenin and Notch signaling, we examined the expression of T-cell factor 1 (Tcf1; also known as transcription factor 7) and Notch1 intracellular cytoplasmic domain (NICD) by Western blotting. The expression of Tcf1 increased according to the overexpression of G4q/Gα11, while that of NICD decreased (Fig. 2C).

3.3. The effect of the knock-down of the G4q/Gα11 expression in IEC-6 cells

We knocked-down the expression of G4q and/or Gα11 in IEC-6 cells using siRNA. We investigated the effect of G4q/Gα11 knock-down with three types of siRNA. We used cellular homogenate of IEC6-Gq/G11 as a positive control and that of the Int-Gq/Gq11 DKO intestine as a negative control. We then selected RSS372821 for Gnaq and RSS340230 for Gna11 and confirmed the knock-down of the G4q/Gα11 expression by Western blotting (Fig. 3A, B).

We evaluated the effect on proliferation. IEC-6 cells were seeded at a density of 1 × 10^4 cells/ml in 96-well culture plates. We incubated the cells with siRNA for two consecutive days and performed the BrdU incorporation assay. As shown in Fig. 3C, the proliferation in IEC6-Ga4, IEC6-Ga11 and IEC6-Gαq/Gα11/11 cells was increased in comparison to IEC6-sicontrol cells.

We next examined the changes in Wnt/β-catenin and Notch signaling. The expression of Tcf1 did not change but the expression of NICD was markedly increased according to the downregulation of G4q/Gα11 (Fig. 3D). This was in sharp contrast to the results of the overexpression experiment (Fig. 2C).

3.4. The effect on the differentiation of IEC-6 cells

In the Int-Gq/Gq11 DKO mouse study, the maturation of Paneth cells was impaired and differentiation toward goblet cells was induced [9]. We then evaluated the relative mRNA expression of a goblet cell marker (Muc2) using a qPCR. As shown in Fig. 4, the relative expression of Muc2 was increased in IEC6-Ga11 and IEC6-Gαq/Gα11/11 cells, but it was not changed in the overexpression experiment.

4. Discussion

The gastrointestinal system is a rich source of neuroendocrine hormones that interact with at least 10 families of GPCRs containing more than 30 known receptor subtypes [6]. Although the physiological relevance of the regulation of intestinal homeostasis is unclear, the sheer number of potential Gαq/Gα11-coupled receptors suggests the importance of this G protein family members in the regulation of the intestinal functions [6]. In our previous in vivo study, the enterocytes in the Int-Gq/Gq11 DKO intestine showed no alterations; however, an immunohistochemical study clearly showed that Gαq/Gα11 were abundantly expressed near the basolateral membrane of enterocytes [9]. In the present study, we used a rat intestinal epithelial cell line (IEC-6) and investigated the role of Gαq/Gα11 in enterocytes, in which Gαq/Gα11 was overexpressed or downregulated with a retroviral system or siRNA.

When Gαq/Gα11 was overexpressed in IEC-6 cells, the number of cells and the incorporation of BrdU were clearly inhibited (Fig. 2A, B). In contrast, when the expression of Gαq/Gα11 was knocked-down, the incorporation of BrdU was increased (Fig. 3C). These results suggested that signaling through Gαq/Gα11 inhibited the proliferation of IEC-6 cells. Potential ligands interacting with GPCRs accompanied by Gαq/Gα11 in intestine are CCK, gastrin, and ACH. Therefore evaluated the BrdU incorporation in naive IEC-6 cells with the addition of CCK or carbachol, a stable muscarinic acetylcholine receptor (mAChR) agonist, to the culture medium. Cholecystokinin-2 receptor (CCK2R, originally known as the CCK-B receptor) is a member of the G-protein-coupled seven transmembrane domains receptor superfamily that binds both amidated gastrin and CCK [11]. As shown in Supplementary Fig. 1, CCK did not affect the proliferation, but carbachol dose-dependently inhibited the cell growth with a maximum effect at 1 mM in naive IEC-6 cells. The peptide hormone gastrin is a well-recognized growth factor for the colonic epithelium [12,13]. The deletion of the functional gastrin gene resulted in decreased colonic proliferation in mice [14,15].
The overexpression of CCK2R has been shown to mediate the rapid progression of colorectal cancer [16], and gastrinomas have been linked in case reports to increased colon polyposis [17]. CCK2R is expressed in murine basal colonic crypts [18], where the stem cells and progenitor cells are located. Gastrin and CCK may therefore play a proliferative role in immature cells but not in mature enterocytes.

In rat and human intestinal epithelial cells, the main subtype of mAChR is the m3 receptor with perhaps a minor contribution by the m1 receptor [19]. The m3 mAChR mainly mediates secretory responses, such as ions and enzymes, but it has been reported to be capable of stimulating colon cancer cell growth [20]. ACh is reported to stimulate the proliferation of neural stem cells and stem cell-derived progenitor cells expressing m2, m3, m4 mRNA, and m2 protein during neural cell lineage progression in vitro [21]. The m3 mRNA was reported to be localized to the lower two-thirds of the villi and not to the crypt in rat jejunum by in situ hybridization [22], overlapping with the localization of enterocytes. Reynolds et al. reported that the m3 receptor was localized to the basal membrane of human colonic epithelium in immunohistochemistry [23]. Carbachol dose-dependently inhibited the cell growth of IEC-6 cells. Therefore, ACh may inhibit the proliferation of enterocytes and play a role in the secretory or absorptive functions.

Proliferation, differentiation and morphogenesis in the intestinal epithelium are tightly regulated by a number of molecular pathways, including Wnt/β-catenin and Notch signaling [4]. Wnt/β-catenin signaling and the Tcf family transcription factors play a central role in proliferation during intestinal development [24]. Western blotting using homogenates of the mucosa of the Int-Gq/G11 DKO intestine revealed that the expression of Tcf1 was decreased, while that of NICD was unchanged [9]. We then examined the expression of Tcf1 and NICD in IEC-6 cells. In Wnt/β-catenin signaling, the expression of Tcf1 was increased according to the overexpression of Gαq/Gα11 (Fig. 2C), which was a sharp contrast to the result of Int-Gq/G11 DKO intestine [9], and was unchanged in the knock-down experiment (Fig. 3D). Wnt/β-catenin signaling plays a pivotal role in the proliferation and differentiation of intestinal stem and progenitor cells [4], but may act differently in terminally differentiated cells. The Wnt pathways include canonical Wnt signaling, which is referred to as the Wnt/β-catenin pathway, and noncanonical Wnt signaling, which is β-catenin-independent and
subdivided into two general categories: the Wnt/Ca\(^2+\) and Wnt/JNK pathways. Fzd is a seven-pass transmembrane receptor and is associated with G\(\alpha_q\)/G\(\alpha_{11}\) in the Wnt/Ca\(^2+\) pathway. This signaling pathway is known to antagonize the canonical Wnt/\(\beta\)-catenin signaling\[25\]. If this pathway plays a role in G\(\alpha_q\)/G\(\alpha_{11}\)-overexpressing cells, the expression of Tcf1 is expected to be decreased. Thus, signaling through G\(\alpha_q\)/G\(\alpha_{11}\) affects the Wnt/\(\beta\)-catenin pathway in a different fashion from Wnt/Ca\(^2+\) pathway in this cell system.

As for Notch signaling, the expression of NICD was decreased with the overexpression of G\(\alpha_q\)/G\(\alpha_{11}\), where the proliferation was inhibited and increased in the knock-down experiment where the proliferation was enhanced (Figs. 2C and 3D). During the regeneration of the intestinal epithelia, the activation of Notch promoted proliferation by suppressing goblet cell differentiation \[26\]. Thus, the effect on the proliferation of IEC-6 cells through G\(\alpha_q\)/G\(\alpha_{11}\) affects the Wnt/\(\beta\)-catenin pathway in a different fashion from Wnt/Ca\(^2+\) pathway in this cell system.

As for Notch signaling, the expression of NICD was decreased with the overexpression of G\(\alpha_q\)/G\(\alpha_{11}\), where the proliferation was inhibited and increased in the knock-down experiment where the proliferation was enhanced (Figs. 2C and 3D). During the regeneration of the intestinal epithelia, the activation of Notch promoted proliferation by suppressing goblet cell differentiation \[26\]. Thus, the effect on the proliferation of IEC-6 cells through G\(\alpha_q\)/G\(\alpha_{11}\) signaling relies more on Notch signaling than on Wnt/\(\beta\)-catenin signaling. However, when we evaluated the expression of Muc2, a goblet cell marker, the expression of Muc2 was elevated to 2–3 fold in the knock-down experiment (Fig. 4), which suggested that goblet cell differentiation was not suppressed. The elevated Muc2 expression in the knock-down experiment was somewhat consistent with the results in the Int-G\(\alpha_q\)/G\(\alpha_{11}\) DKO intestine, in which the differentiation of Paneth cells toward goblet cells was induced \[9\].

In IEC-6 cells, the proliferation of G\(\alpha_q\)/G\(\alpha_{11}\)-overexpressing cells was inhibited, while that of G\(\alpha_q\)/G\(\alpha_{11}\)-knock-down cells was increased. Wnt/\(\beta\)-catenin signaling and Notch signaling were changed according to the level of G\(\alpha_q\)/G\(\alpha_{11}\) expression, which was not consistent with the results obtained with Int-G\(\alpha_q\)/G\(\alpha_{11}\) DKO mice. Signaling through G\(\alpha_q\)/G\(\alpha_{11}\) probably works in a context-dependent fashion, based on whether the cells are stem cells, progenitor cells, proliferating cells, or terminally differentiated cells. In differentiated enterocytes, signaling through G\(\alpha_q\)/G\(\alpha_{11}\) inhibits the proliferation and may have an effect on physiological functions, such as absorption or secretion.
Fig. 4. The relative mRNA expression of goblet cell marker, Muc2. A quantitative real-time PCR was performed. The expression of β-actin was used as an internal control. The data are expressed as the mean ± standard deviation from n = 3 per cell type. Two independent experiments showed similar results. Representative figures are shown. **P < 0.01.

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Appendix A. Transparency document

Transparency document associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.bbrep.2018.01.003.

Appendix B. Supplementary material

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