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$G_{i2}\alpha$ Protein Deficiency: A Model for Inflammatory Bowel Disease

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Mice deficient for the G protein subunit $G_{i2}\alpha$ were obtained by gene targeting. They displayed a growth retardation that was apparent at 6 weeks of age. They subsequently developed diffuse colitis with clinical and histopathological features closely resembling those of ulcerative colitis in humans. Seven of 20 $G_{i2}\alpha$-deficient mice with colitis also developed adenocarcinomas of the colon. $G_{i2}\alpha$-deficient thymocytes displayed two- to fourfold increases in mature $CD4^+8^-$ and $CD4^-8^+$ phenotypes, an approximately threefold increase in high-intensity CD3 staining and enhanced proliferative responses to T-cell receptor stimuli. Stimulation of $G_{i2}\alpha$-deficient peripheral T cells induced a hyperresponsive profile of interleukin-2, tumor necrosis factor, and interferon-γ production, which may reflect a heightened response of primed cells or a defective negative regulation. We suggest that $G_{i2}\alpha$-deficient mice may represent a useful animal model for dissecting the pathomechanisms of inflammatory bowel disease and also for the development of novel therapeutic strategies.

KEY WORDS: G protein; transmembrane signaling; gene targeting; inflammatory bowel disease; T cells.

INTRODUCTION

G proteins couple membrane-bound receptors to effectors such as enzymes and ion channels. They consist of α-, β-, and γ-subunits. To date, 16 α-subunit, 4 β-subunit, and 7 γ-subunit genes have been cloned. For most of the α-subunits, we now know at least some of their biochemical roles: α-subunits stimulate adenylyl cyclase and some Ca^{2+} and K^+ channels; α_i-subunits inhibit adenylyl cyclase and stimulate other types of K^+ channels; the members of the α_q family stimulate certain types of phospholipase C (reviewed in Ref. 1). The assignment of roles for heterotrimeric G proteins in the regulation of biological pathways such as growth, differentiation, and homeostasis is a complex task which requires a thorough understanding of their biochemical actions on the physiology of the cell in which they occur. Moreover, each cell type and tissue contribute to the organization and maintenance of a living organism. Unfortunately, little is known about the biological functions of the three α-subunits of G_{i1}, G_{i2}, and G_{i3}, which are more than 85% identical at the amino acid level. These can be tested for with pertussis toxin (PTX), which by ADP-ribosylating their α-subunits, impedes their role as intermediaries between an incoming signal and an effector. Recently, a carboxy-terminal putatively pertussis toxin-insensitive splice variant of $G_{i2}\alpha$, $sG_{i2}\alpha$, has been identified by molecular cloning, which is localized to the Golgi apparatus and may be involved in membrane transport (2). Table I (3–31) lists a set of functions and roles that directly or indirectly involve a pertussis toxin-sensitive G protein, and G_{i2} in particular. Of these, perhaps the most interesting is the recent implication of $G_{i2}\alpha$ as a regulator of the MAP kinase system. Activating mutations of $G_{i2}\alpha$ have been identified in human adrenal and ovarian tumors (3). Fibroblasts transfected with a mutationally activated $G_{i2}\alpha$ form tumors in nude mice (4), thus identifying $G_{i2}\alpha$ as a protooncogene.

To gain further insights into the biological role of $G_{i2}\alpha$, we inactivated the $G_{i2}\alpha$ gene in mice by gene targeting.

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Table I. Proven and Potential Roles of G_{i2}

	Ref. No.(s.)
A. Proven effects/roles of $G_{i2}\alpha$	
1. Inhibition of adenylyl cyclase	5–7
2. Stimulation of the inwardly rectifying K^+ channel(s) of heart and endocrine cells	8, 9
3. Stimulation of the ATP-sensitive K^+ channel(s) in pancreatic (RIN) cells and heart	10, 11
4. Protooncogene in Rat-1 cells	4, 12
5. Stimulation of MAP kinase pathway	13
6. Regulation of fibroblast (NIH-3T3) proliferation	14
7. Regulation of neonatal growth–development	15
B. Roles potentially ascribable to G_{i2} due to pertussis toxin sensitivity of the pathway	
1. Stimulation of PLA2 by the α_1-adrenergic receptor in FRTL-5 thyroid cells	16
2. Increased PLC activity in rat liver by EGF	17
3. Stimulation of EGF P-Tyr phosphatase in pancreatic (MIA PaCa-2) cells	18
4. Mediation of action of CSF-1 in promacrophages	19
5. Mediation of TNF action in promacrophages	20
6. Zona pellucida-induced acrosome reaction of capacitated sperm cells	21
7. Negative regulation of vesicle budding from Golgi membranes	22
8. Mitogen-induced intracellular alkalinization in lung (CCL39) fibroblasts	23
9. Stimulation of DNA synthesis by LPA	24
10. Endosomal acidification	25
11. Maintenance of differentiated state of cultured primary hepatocytes	26
12. Chemotactic peptide-induced signaling in leukocytes	27
13. Thymocyte maturation	28
14. T-cell homing	29
15. Regulation of cell proliferation	30, 31

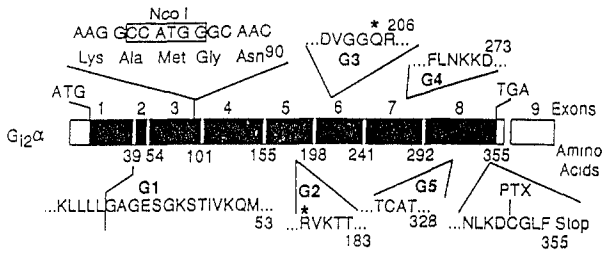

Fig. 1. Disruption of the $G_{i2}\alpha$ gene. Intron–exon boundaries of the cDNA coding for $G_{i2}\alpha$ and location of key amino acid sequences. Boxes represent exons, with white representing untranslated and black representing translated sequences. Exon numbering is shown above and the number of the last amino acid of each exon is shown below the cDNA. The *Nco*I site in exon 3 that was used to disrupt the gene is shown. G1 through G5: regions responsible for binding and hydrolysis of GTP. *R in G2: site of $G_{i2}\alpha$-to-*gip*2 mutation found in some adrenocortical adenomas and carcinomas and in ovarian granulosa and theca-cell tumors. *Q in G3: regulates GTPase activity of $G_{i2}\alpha$, which is reduced upon mutation to L. Pertussis toxin (PTX) ADP-ribosylates Cys at −4 from the C terminus.

STUDY DESIGN

Insertion vector IV-1 (32) was opened at a *Not*I site that has been engineered to replace a deleted 105-bp *Kpn*I–*Bgl*II fragment 0.8–0.9 kb upstream of the disrupting neomycin resistance (*neo*) cassette and electroporated into AB1 ES cells. Targeted colonies were identified first by a polymerase chain reaction (PCR) detecting the gap repair that is predicted to accompany homologous recombination events and by Southern blot analyses. Clone 31A probably arose by target conversion and looks like a clone targeted with a replacement vector since the vector backbone was not integrated into the target locus. The *neo* resistance marker inserted into the *Nco*I site of exon 3 is predicted to create a $G_{i2}\alpha$ null allele (see Fig. 1). The mutant allele can be identified by hybridization of a 3′-flanking probe to a specific 4.0-kb *Sac*I–*Sac*I fragment, whereas the same 3′-flanking probe hybridizes to a 8.4-kb fragment in the wild-type allele. Targeted stem cells were injected into blastocysts, which were implanted into pseudopregnant foster mothers. Chimeras derived from clone 31A were demonstrated to contain the mutant $G_{i2}\alpha$ allele in the germline (32). The mutation was then bred to homozygosity. Tissues of homozygous mutant mice were analyzed for the absence of $G_{i2}\alpha$ protein by ^{32}p-ADP-ribosylation. No PTX-labeled band migrating on urea-gradient/sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) gels with the mobility expected for G_{i2} could be found in heart, thymus, skeletal muscle, embryonic fibroblasts, and adipocyte membranes. Only α-subunits migrating as α_{i1} or α_{i3} are found in fat, skeletal muscle, and thymus of $\alpha_{i2}-/-$ mice. These findings indicate that our targeted allele is a true null allele and that neither fat nor thymus nor skeletal muscle expresses significant quantities of α_o, of which the type 1 splice variant has an electrophoretic mobility similar to that of α_{i2} and should now be visible, and the type 2 splice variant migrates more rapidly than all other PTX-sensitive α-subunits (i.e., α_{i1}, α_{i2}, α_{i3}, and α_{o1}) and should also be visible.

The relative intensity of labeling of the α_{i2} band in heterozygous mouse embryonic fibroblasts, as well as heterozygous thymus homogenates, appears to be about one-half that found in the $\alpha_{i2}+/+$ counterparts. Thus, the level of α_{i2} in membranes is sensitive to gene dosage.

Inhibition of adenylyl cyclase in heart homogenates in response to carbachol and in adipocyte membranes in response to phenylisopropyl adenosine, prostaglandin E_2 (PGE_2), and nicotinic acid was blunted by about 50% in $\alpha_{i2}-/-$ mice. In T antigen-immortalized $\alpha_{i2}-/-$ embryonic fibroblasts, adenylyl cyclase inhibition by lysophosphatidic acid was fully blocked by PTX. These findings indicate that not only G_{i2}, but also other G_i's (G_{i1} and/or G_{i3}) mediate inhibition of adenylyl cyclase and that the

four receptors tested for are coupled to G_{i2} as well as to another G_i. This contrasts with results in GH3 cells regarding the specificity of receptor–G_o interactions (33).

Thymus and spleen cells from 6- to 12-week $\alpha_{i2}-/-$ and age-matched $\alpha_{i2}+/+$ mice were analyzed by flow cytometry for T-cell subsets and activation/adhesion markers and cultured *in vitro* to assess proliferation and cytokine production. Thymus cells from $\alpha_{i2}-/-$ mice exhibited a two- to fourfold increase in the proportion of thymocytes with a mature $CD4^+8^-$ or $CD4^-8^+$ single-positive phenotype and an approximately threefold increase in the high-intensity CD3 staining characteristic of the most mature subset. Sixty to 80% of $CD3^{hi}$ thymocytes expressed a peripheral-like MEL-14^{hi}, $CD44^{lo}$, $J11d^-$ phenotype. Elevated expression of the mature thymocyte subset was also evident at 2 weeks of age. Spleen and lymph node $\alpha_{i2}-/-$ T cells appeared relatively normal in phenotype. $\alpha_{i2}-/-$ thymocytes also exhibited enhanced proliferative responses to T-cell receptor stimuli, including immobilized anti-CD3 [with or without phorbol myristate acetate (PMA)] and staphylococcal enterotoxin A (SEA), and in BALB/c(H-2^d) T-depleted mixed lymphocyte reactions. In each case $\alpha_{i2}-/-$ thymocytes demonstrated a response three- to fivefold higher than that of matched controls, roughly consistent with the increased frequency of mature single-positive thymocytes.

The cytokines interleukin-2 (IL-2), IL-4, interferon (IFN), and tumor necrosis factor (TNF) are produced by $CD3^{hi}$ thymocytes and affect thymocyte development and function (34). Immobilized anti-CD3 (with or without PMA), or PMA/ionomycin, stimulated $\alpha_{i2}-/-$ thymocytes to produce severalfold increased IL-2, IFN-γ, and TNF, but not IL-4, levels, even after normalization to 100% $CD3^{hi}$ cells for both $\alpha_{i2}-/-$ and $\alpha_{i2}+/+$ populations. Thus, $G_{i2}\alpha$-deficient thymocytes are responsive to T-cell stimuli and exhibit heightened proliferation that may reflect both increased proportion of peripheral T cell-like thymocytes and elevated cytokine levels.

Spleen and lymph node $G_{i2}\alpha$-deficient T cells appeared relatively normal in phenotype, except for a somewhat more heterogeneous display of CD3. $G_{i2}\alpha$-deficient thymocytes thus emigrate properly to spleen and lymph nodes and also exhibit an unaffected proliferative response. Stimulated $G_{i2}\alpha$-deficient spleen T cells produced IL-2, IFN-γ, and TNF levels that were elevated up to 80-fold, depending on the cytokine and stimulus, while IL-4 was more modestly enhanced. Thus, stimulation of $G_{i2}\alpha$-deficient peripheral T cells also induces cytokine production with a hyperresponsive profile, which may reflect a heightened response of primed cells or defective negative regulation.

Analysis of the B-cell phenotype in bone marrow and spleen of $G_{i2}\alpha$-deficient mice by IgM, IgD, B220, and CD23 expression did not reveal any substantial defects in the development of pre-B cells ($B220^{dull}/IgM^-$), immature B cells ($B220^{dull}/IgM^+IgD^-$), and mature B cells ($B220^{bright}/IgM^+/IgD^+$) in comparison to wild-type mice. Similarly, in the spleen no significant differences were noted in the number of mature B cells. Additional analysis of bone marrow spleen B cells for expression of CD23 revealed no differences between the $G_{i2}\alpha$-deficient and the wild-type mice.

Plasma IgM levels were similar in $\alpha_{i2}-/-$ and $\alpha_{i2}+/+$ mice. In contrast, plasma IgG and IgA levels were approximately twice as high in $\alpha_{i2}-/-$ mice. Immunoglobulins in large and small intestinal secretions showed that IgA predominates in both $\alpha_{i2}+/+$ and $\alpha_{i2}-/-$ mice and was elevated in $\alpha_{i2}-/-$ mice. IgG was markedly elevated in the large intestine of $\alpha_{i2}-/-$ mice (409 ± 100 vs 29 ± 34 μg/ml) and more modestly in the small intestine, while IgM was elevated in the large intestine and undetectable in the small intestine of both groups. Spleens of 8-week-old $\alpha_{i2}-/-$ mice contained significantly larger numbers of granulocytes in comparison to $\alpha_{i2}+/+$ mice, as was also found in peripheral blood ($20.7 \pm 6.5 \times 10^3$ vs $0.8 \pm 0.1 \times 10^3$/ml).

$\alpha_{i2}-/-$ mice did not gain weight as well as wild-type mice. The growth retardation was apparent at 6 weeks of age. Subsequently, some of the mice began losing weight. The mean age at spontaneous death was approximately 20 weeks. Necropsies revealed colons with irregular dilatation and focally thickened walls. Foci of serosal discoloration and increased vascularity were also seen, as was perforation and peritonitis. Twenty-one of 26 animals examined displayed an inflammation of the large bowel; the inflammatory reaction was restricted to the rectum and colon, was diffuse and not patchy, and resembled ulcerative colitis. Heavy chronic infiltration with lymphocytes and active plasma cells was associated with ulceration, loss of mucus from goblet cells of crypts, atypia, and loss of crypts. In advanced stages, regeneration of glands was observed with typical characteristics of adenocarcinomas (back-to-back growth without intervening stroma, loss of nuclear polarity, and severe crowding). These glands penetrated the submucosa and extended to the smooth muscle layer (muscularis propria).

DISCUSSION

Inflammatory bowel disease (IBD) is a group of chronic inflammatory disorders involving the gastrointestinal tract (reviewed in Ref. 35). Crohn's disease

(CD), or regional enteritis, is often localized to the terminal ileum but can occur anywhere in the digestive tract. Ulcerative colitis (UC) is characterized by a continuous inflammation of rectum and colon.

Recently, it was reported that mice lacking IL-2 (36), IL-10 (37), and functional $\alpha\beta$ T cells (38) develop chronic intestinal inflammations. IL-2-deficient mice developed splenomegaly, lymphadenopathy, and severe anemia, so that at 9 weeks of age, half of the animals had already died. The surviving animals developed an inflammatory bowel disease limited to the rectum and colon and widespread amyloidosis (liver, kidneys, spleen). Germ-free mice did not develop clinical symptoms or histological signs of inflammatory bowel disease, indicating that the normal gut flora seems to be responsible for the inflammatory process (36). IL-10-deficient mice develop a chronic enterocolitis that may involve the entire gastrointestinal tract but affects predominantly the duodenum, adjoining jejunum, and colon. These animals also developed an anemia (37). In $\alpha\beta$ T cell-deficient mice the inflammation was limited to the rectum and colon, but there were no mucosal ulcerations present (38). $G_{i2}\alpha$-deficient mice also developed an inflammatory bowel disease, with histological features that included excess plasma cells and lymphocytes in the mucosa with neutrophil collections in the crypts. The process started in the rectum and distal colon and was followed by continuous diffuse colonic involvement, mucus depletion, and progressive superficial ulceration. The most intense inflammation was found distally. This process is characteristic of ulcerative colitis, as is the development of invasive adenocarcinomas of the colon without preference for the rectum or distal colon. Of the original 20 $G_{i2}\alpha$-deficient mice which developed an inflammation of the large intestine, 3 mice displayed unique features: mild focal jejunitis, severe duodenitis, and small penetrating ulcers without surrounding inflammation in the proximal colonic mucosa. Granulomas, skip areas, or upper intestinal tract lesions, which would suggest a Crohn's disease-like phenomenon, were not found. Therefore, by clinical and histological criteria, $G_{i2}\alpha$-deficient mice have striking similarities to human inflammatory bowel disease. It is currently not clear how the $G_{i2}\alpha$ deficiency leads to the development of inflammatory bowel disease. It will be important to identify cell types whose lack of $G_{i2}\alpha$ deficiency leads to disease initiation and/or disease progression.

Bone marrow transplantation might demonstrate whether the lack of $G_{i2}\alpha$ in gut epithelial cells or in bone marrow-derived cells is of functional significance. Furthermore, it will be important to identify the receptors and effectors with which $G_{i2}\alpha$ normally interacts to regulate immune responses. Having several mouse strains available with distinct and specific genetic lesions that develop forms of inflammatory bowel disease opens new avenues for developing an understanding of etiology, pathogenesis, and perhaps, novel treatment strategies.

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