Selective Resistance to Parathyroid Hormone Caused by a Novel Uncoupling Mutation in the Carboxyl Terminus of \( G_\alpha_s \)

**A CAUSE OF PSEU.DOHYPO.PARATHYROIDISM TYPE Ib* 

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\( G_\alpha \) is a heterotrimeric (\( \alpha, \beta \), and \( \gamma \) chains) \( G \) protein that couples heptahelical plasma membrane receptors to stimulation of adenylyl cyclase. Inactivation of one \( GNAS1 \) gene allele encoding the \( \alpha \) chain of \( G_\alpha \) (\( G_\alpha_s \)) causes pseudohypparathyroidism type Ia. Affected subjects have resistance to parathyroid hormone (PTH) and other hormones that activate adenylyl cyclase plus somatic features termed Albright hereditary osteodystrophy. By contrast, subjects with pseudohypparathyroidism type Ib have hormone resistance that is limited to PTH and lack Albright hereditary osteodystrophy. The molecular basis for pseudohypparathyroidism type Ib is unknown. We analyzed the \( GNAS1 \) gene for mutations using polymerase chain reaction to amplify genomic DNA from three brothers with pseudohypparathyroidism type Ib. We identified a novel heterozygous 3-base pair deletion causing loss of isoleucine 382 in the three affected boys and their clinically unaffected mother and maternal grandfather. This mutation was absent in other family members and 15 additional unrelated subjects with pseudohypparathyroidism type Ib. To characterize the signaling properties of the mutant \( G_\alpha_s \), we used site-directed mutagenesis to introduce the isoleucine 382 deletion into a wild type \( G_\alpha_s \) cDNA, transfected HEK293 cells with either wild type or mutant \( G_\alpha_s \) cDNA, plus cDNAs encoding heptahelical receptors for PTH, thyrotropic hormone, or luteinizing hormone, and we measured cAMP production in response to hormone stimulation. The mutant \( G_\alpha_s \) protein was unable to interact with the receptor for PTH but showed normal coupling to the other coexpressed heptahelical receptors. These results provide evidence of selective uncoupling of the mutant \( G_\alpha_s \) from PTH receptors and explain PTH-specific hormone resistance in these three brothers with pseudohypparathyroidism type Ib. The absence of PTH resistance in the mother and maternal grandfather who carry the same mutation is consistent with current models of paternal imprinting of the \( GNAS1 \) gene.

The term pseudohypparathyroidism (PHP)\(^1\) (1) describes a group of disorders characterized by biochemical hypoparathyroidism (i.e. hypocalcemia and hyperphosphatemia), increased secretion of PTH, and target tissue unresponsiveness to the biological actions of PTH (1). Patients with PHP type I show neither a phosphaturic nor a nephrogenous cyclic AMP response to administration of exogenous PTH. These findings have implicated a defect in the PTH receptor-G-protein-adenyl cyclase complex in cells of the proximal renal tubule as the basis for impaired PTH responsiveness. In one form of PHP, the type Ia variant (2), patients are resistant to PTH as well as multiple other hormones (3) that bind to receptors that are coupled by the stimulatory G protein (\( G_\alpha \)) to activation of adenyl cyclase. In addition to hormone resistance, patients with PHP type Ia also manifest a peculiar constellation of developmental and somatic defects, including short stature, round faces, brachydactyly, and subcutaneous ossifications, that are collectively termed Albright’s hereditary osteodystrophy (AHO) (1). The diverse clinical manifestations of AHO have been attributed to heterozygous mutations in the \( GNAS1 \) gene that lead to widespread deficiency of the \( \alpha \) subunit of \( G_\alpha_s \) (4, 5). Although most subjects with \( GNAS1 \) mutations have hormone resistance, and thus PHP type Ia, in many families some members have AHO and normal hormone responsiveness, a condition termed pseudo-PHP (PPHP) (4–6). In a second form of PHP, termed type Ib, patients have normal \( G_\alpha_s \) activity in accessible cells, lack features of AHO (7, 8), and have hormone resistance that is limited to PTH (3). Specific resistance of target tissues to PTH and normal \( G_\alpha_s \) activity had implicated decreased expression or function of the classical or type 1 PTH receptor (\( PTHR1 \)) that is expressed in bone and kidney as the cause for hormone resistance. However, molecular studies have failed to disclose mutations in the coding exons (9) and promoter regions (10) of the \( PTHR1 \) gene or its mRNA (11). Moreover, mice (12) and humans (13) in which one \( PTHR1 \) allele is inactivated do not manifest PTH resistance or hypocalcemia. Indeed, inheritance of two defective-type \( PTHR1 \) alleles results in Blomstrand chondrodysplasia, a lethal genetic disorder that is characterized by advanced endochondral bone maturation (13). 

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‡ The abbreviations used are: PHP, pseudohypparathyroidism; PPHP, pseudopseudohypparathyroidism; PTH, parathyroid hormone; PTHrP, parathyroid hormone-related protein; \( PTHR1 \), type 1 PTH receptor; G protein, guanine nucleotide-binding protein; \( G_\alpha_s \), stimulatory G protein; \( \alpha \) chain of \( G_\alpha \), \( \alpha \)Ile382,G\( ^a \), mutant with deletion of Ile382 AHO, Albright hereditary osteodystrophy; PHP, pseudohypparathyroidism; TSH, thyrotropic hormone; PCR, polymerase chain reaction; DGGE, denaturing gradient gel electrophoresis; RT-PCR, reverse transcription-PCR; GTP\(_S\), guanosine 5’-3-O-(thio)triphosphate; LH, luteinizing hormone; TSH, thyrotropic hormone.
Membranes were suspended in 25 mM HEPES and 1 mM dithiothreitol with the tonic lysis buffer (5 mM HEPES, pH 7.4, 0.5 mM EDTA) for 30 min at transfected HEK293 cells, cultured cells were incubated with a hypotonic lysis as described previously (17), and activity of detergent- and stored at 2 °C and subsequently harvested by scraping with a Teflon policeman. The proband (subject III-3 in Fig. 1) and his two brothers were referred for evalua-
tion of PHP when they were discovered to have elevated levels of serum PTH during an investigation of hypocalcemia. The three brothers had normal intellectual function and were at or above the 50th percentile for height; only one (III-3) of the three boys was obese (39 kg, >95th percentile) (Table I). Physical examination of the three boys, their unaffected sister, and their parents failed to disclose evidence of subcutaneous ossifications, brachydactyly, or other features of AHO, and radiographs of hands and feet were normal. All three brothers had elevated levels of intact PTH levels and failed to show a significant increase in the urinary excretion of nephrogenous cAMP after intravenous infusion of 200 units of synthetic human PTH-(1–34) (Table II). By contrast, their mother showed a normal urinary cAMP response to administration of human PTH-(1–34). Serum levels of magnesium, 25-(OH)-vitamin D, thyroxine, triiodothyronine, and TSH were within the normal range, and serum levels of testosterone were appropriate for age in all three boys (Table II). Subjects III-1 and III-2 showed pubertal LH and follicle-stimulating hormone responses to intravenous infusion of synthetic gonadotropin-releasing hormone (100 μg), and subject III-3 showed a prepubertal response. All studies were approved by the Joint Committee on Clinical Investigation of The Johns Hopkins University School of Medicine, and written informed consent was given by the study subjects or their parents.

Semi-quantitative immunoblot analysis was performed in triplicate and demonstrated that levels of Goα protein (Fig. 1) were not reduced in erythrocyte membranes prepared from the three affected brothers (134 ± 8%) as compared with other unaffected family members (99.3 ± 2.1%) or a control group of unrelated normal subjects (92 ± 8%). To examine the functional activity of the erythrocyte Goα protein, we assessed the ability of detergent-solubilized Goα from erythrocyte membranes to reconstitute a hormonally responsive adenyl cyclase system in membranes prepared from the cyc–clone of S49 murine lymphoma, which genetically lacks Goα protein (27). Addition of increasing amounts of erythrocyte membrane extract to constant amounts of cyc–membranes produced linear increases in L-isoproterenol (10 μM)-stimulated adenyl cyclase activity, with regression slopes for the three affected members (III-1, III-2 III-3; 60.8 ± 4.3) and two members who were unaffected carriers (I-1 and II-2; 69.3 ± 2.0) that were equivalent (Fig. 2).

Identification and Confirmation of GNAS1 Mutation—No abnormalities were found in exons 1–12 of the proband’s gene for Goα. Polycrylamide gel electrophoresis (Fig. 3B) and DGGE (Fig. 3C) of DNA fragments amplified from exon 13 revealed additional bands indicating the presence of an abnormal allele. Although the typical pattern for heterozygous alleles on a denaturing gradient gel is composed of four bands, of which two bands represent homoduplex fragments and two bands represent heteroduplex fragments (28–30), DGGE of this PCR product consistently resolved only three bands. Thus, it is likely that under the conditions we employed the two slowly migrating heteroduplex fragments migrated as a single band on the denaturing gradient gel. The exon 13 PCR products were ligated into the plasmid vector pCRII, and DNA from individual clones was sequenced. Of 10 clones sequenced, 4 contained the wild type sequence for Goα exon 13. In addition, 6 clones contained inserts in which there was a 3-base pair deletion (CAT) that eliminated an isoleucine residue at position 382 (ΔIle382) of Goα protein (Fig. 4). RT-PCR of Goα mRNA from peripheral blood mononuclear cells amplified equivalent amounts of wild type and mutant cDNA, indicating that mRNA derived from the mutant allele was stable (data not shown).

To genotype the rest of the family, PCR products of exon 13 from all available members were analyzed by polycrylamide gel electrophoresis and DGGE. DNA from the clinically unaffected mother (II-1, Fig. 3) and maternal grandfather showed patterns consistent with heterozygosity for the ΔIle382 mutation, whereas DNA from all other family members migrated as single bands indicating homozygosity for the wild type GNAS1 allele (Fig. 3 and not shown). The ΔIle382 mutation was not present in genomic DNA from 15 additional unrelated subjects with PHP type 1b or from 30 other normal, unrelated subjects (data not shown).

Functional Analysis of Recombinant Goα Protein—The ΔIle382 mutation occurs in the middle of the α5 helix at the carboxyl terminus of the protein, a region that contributes importantly to receptor selectivity. The characteristics of the ΔIle382 mutation were assessed by transiently expressing wild type and mutant forms of Goα plus cDNAs encoding G protein-coupled receptors in HEK293 cells at 37 °C. Immunoblot analyses revealed that the Goα containing the ΔIle382 mutation was expressed at a level that was similar (118 ± 11%) to that of the wild type recombinant Goα protein under all experimental conditions (Fig. 5). Moreover, cells that expressed mutant or wild type Goα proteins produced similar agonist-dependent increases in cAMP when incubated with various concentrations of L-isoproterenol, which activates endogenous β-adrenergic receptors (Fig. 6B). However, as the Bmax for isoproterenol stimulation was slightly reduced for the mutant Goα protein, we cannot exclude a subtle defect in coupling to β-adrenergic receptors. To assess the functional effects of the ΔIle382 mutation on receptor coupling, wild type and mutant forms of Goα were transiently coexpressed in HEK293 cells with specific receptors that can mediate activation of Gβ but that are not found in HEK293 cells (31), and after incubation with various concentrations of hormones the production of cAMP was measured. Cells transfected with the human PTHR1 showed a concentration-dependent increase in intracellular cAMP accumulation after incubation with 10−11 to 10−7 M bPTH–(1–34), and this response was significantly enhanced in cells that had been cotransfected with the wild type Goα cDNA (Fig. 6A). By contrast, HEK293 cells that had been cotransfected with the cDNAs for the mutant Goα and hPTHR1 showed cAMP responses to PTH (Fig. 6A) and PTHrP (data not shown) that were no greater than those in cells that had been transfected with only the hPTHR1.

To assess the ability of the mutant Goα protein to couple to other heptahedral receptors, HEK293 cells were cotransfected with wild type or mutant Goα cDNAs plus cDNAs encoding the TSH receptor or LH receptor. After incubation with various concentrations of either bTSH or human choriomic gonadotropin, the accumulation of cAMP was measured. At all concentrations of hormone tested, cells expressing the wild type or mutant Goα cDNAs showed similar increases in cAMP that were significantly greater than those in cells expressing the receptor alone (Fig. 6. C and D).

**DISCUSSION**

PHP type Ib is characterized by isolated resistance to PTH and an absence of features of AHO, and thus appears to be biochemically and clinically distinct from PHP type Ia. The identification of a novel germ line mutation, ΔIle382, in the gene encoding Goα in three boys with PHP type Ib provides a new
Selective Uncoupling of Goα from PTH Receptor in PHP Type Ib

|             | II-2 | III-1 | III-2 | III-3 | III-4 |
|-------------|------|-------|-------|-------|-------|
| Serum calcium (8.8–10.4 mg/dl) | 9.1  | 7.4   | 9.1   | 8.0   | 9.6   |
| Serum phosphate (3.8–6.0 mg/dl, child) | 2.7  | 7.4   | 5.5   | 4.9   | ND*   |
| Intact PTH-(1–84) (10–65 pg/ml) | 55   | 416   | 276   | 200   | 40    |
| PTH infusion, peak UcAMP (nmol/dl GF) | 118  | 4     | 4     | 3     | ND    |
| Testosterone | ND   | 154 ng/dl | 84 ng/dl | 11 ng/dl | ND    |
| Free testosterone | ND   | 22.7 pg/ml | 11.5 pg/ml | 1.3 pg/ml | ND    |
| GnRH infusion* | Peak LH | 9.0 pg/ml | 9.9 pg/ml | 2.4 pg/ml | ND    |
| Peak FSH* | 3.3 pg/ml | 1.4 pg/ml | 2.0 pg/ml | ND    |
| TSH (0.3–5.0 microunits/ml) | 1.9  | 3.4   | 3.3   | 3.2   | ND    |

* ND indicates not determined.
* GnRH indicates gonadotropin-releasing hormone.
* FSH indicates follicle-stimulating hormone.

Fig. 2. Erythrocyte Goα activity. Goα activity in detergent extracts of erythrocyte membranes was determined by measuring the ability of various amounts of the extract to reconstitute hormone-response adenylyl cyclase activity in membranes prepared from the cyc− clone of S49 murine lymphoma cells, which genetically lack Goα (27). Addition of increasing amounts of erythrocyte membrane extract to constant amounts of cyc− membranes produced linear increases in l-isoproterenol (10 μM)-stimulated adenylyl cyclase activity, with regression slopes for the three affected members with PHP (III-1, III-2, and III-3; 60.8 ± 4.3) and two unaffected carriers with “PPHP” (1-2 and II-2; 69.3 ± 2.0) of the kindred that were equivalent. Results are representative of three separate experiments, each performed with triplicate determinations. Results are expressed as pmol of cyclic AMP per mg protein per 20 min of incubation.

Fig. 3. Analysis of exon 13 of GNAS1 gene. A shows the pedigree of a portion of the family; symbols are as described in Fig. 1. Exon 13 of GNAS1 was amplified by the polymerase chain reaction using genomic DNA as described under “Experimental Procedures.” PCR products were analyzed by electrophoresis through nondenaturing polyacrylamide gels (B) or through polyacrylamide gels containing a linearly increasing concentration of the denaturants urea and formamide (C). Although the typical pattern for heterozygous alleles on a denaturing gradient gel is comprised of four bands, of which two bands represent homoduplex fragments and two bands represent heteroduplex fragments (28–30), DGGE of this PCR product consistently resolved only three bands. The two lower bands represent the mutant (lowest) and wild type (middle band) Goα sequences, whereas the upper-most band consists of the two slowly migrating heteroduplex fragments.
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boxy terminus of Goα, an essential region for receptor coupling and selectivity (reviewed in Ref. 36). The crystallographic structure of the 394-amino acid Goα molecule indicates that Ile382 lies within the α5 helix, which together with the α4–β6 loop form a plane on the back of Goα that may interact with receptors (37). Additional evidence that the α5 helix and α4–β6 loop contribute importantly to the receptor binding surface comes from patterns of evolutionary conservation (38) as well as from biochemical and genetic analyses. For example, peptide-specific antibodies directed against the last 10 amino acids of G-protein α chains can block receptor-mediated regulation of adenylyl cyclase activity (39, 40), and experimentally produced mutations in this region uncouple Goα from receptors (41). In addition, naturally occurring mutations in the carboxyl terminus of Goα that inhibit coupling to all receptors have been identified in the unc gene of the S49 murine lymphoma cell line (Arg389 → Pro, (42, 43)) and in a patient with PHP type Ia (Arg385 → His, (33)). Deletion of Ile382 could alter the kink in the midsection of the α5 helix, thereby disrupting contact between the α5 and α4–β6 regions of the Goα chain. Specific uncoupling of the ΔIle382 Goα chain from only the PTHR1 would imply that the interaction of Goα with the PTHR1 is particularly sensitive to this change in the three-dimensional structure of the α chain. Support for the extraordinary specificity of this defect in receptor interaction derives from observations of differential efficacy of Goα-coupled receptors to activate chimeric forms of G proteins in which only the last five amino acid residues are derived from Goα (44). These studies suggest that other amino acids in the α5 helix must contribute to the fidelity of receptor-G protein interaction.

That the same mutation could result in PHP type Ib in the three brothers but fail to produce any clinical effects in their mother and maternal grandfather is a remarkable discrepancy in genotype-phenotype relations (Figs. 1 and 2). The discrepancy in the maternal grandfather (I-1) might be explained by the presence of undetected mosaicism, but this explanation could not account for the lack of phenotypic features in his daughter (II-2), the mother of the three affected boys. The inconstant phenotypes exhibited in members of this family who share the same GNAS1 mutation are reminiscent of the peculiar pattern of inheritance of hormone resistance exhibited by patients with other GNAS1 gene mutations, in whom maternal transmission of Goα deficiency leads to PHP type Ib, whereas paternal transmission of the defect leads to PPHP (6), a variant characterized by somatic features of AHO but normal hormone responsiveness (45–48). These observations first suggested imprinting of the GNAS1 locus as a mechanism for gene regulation but could not anticipate the complex pattern of genomic imprinting now identified. Two upstream promoters, each associated with a large coding exon, lie 35–40 kilo base pairs upstream of GNAS1 exon 1 and generate unique proteins. In addition, a third alternative first exon, termed 1A, is only about 3 kilo base pairs upstream of exon 1, and lacks translated sequences (49). The more 5’ of these exons encodes NESP55, which is expressed exclusively from the paternal allele. By contrast, the XLo5 (50, 51) and 1A exons (52) are paternally expressed. Although initial studies in human tissues were consistent with biallelic expression of Goα (50, 51, 54), subsequent analyses in mice in which one Gnas allele is disrupted (Gnas+/−) have suggested a model of cell- or tissue-specific paternal imprinting of Goα. In this model, both Gnas alleles are expressed in most tissues, but only the maternal allele is expressed in some tissues (e.g. renal cortex) (55–57). Accordingly, Gnas +/− mice that inherit an inactivated Gnas allele mater-

FIG. 4. Sequence analysis of antisense strand of exon 13 of the GNAS1 gene. Exon 13 was amplified by PCR of genomic DNA from the propositus, and after ethanol precipitation the DNA was ligated into the plasmid vector pCRⅢ. DNA from individual clones was sequenced as described under “Experimental Procedures.” Sequence analysis showed a heterozygous mutation (wild type Normal Allele in left panel; mutant Abnormal Allele in right panel) in which one allele contained a 3-base pair deletion (CAT) that results in the in-frame loss of the isoleucine residue at codon 382.

FIG. 5. Immunoblot analysis of recombinant wild type and mutant Goα proteins. HEK293 cells were transfected with expression vectors (pcDNA1/Amp) encoding the wild type (WT) or mutant (M) Goα proteins, plus expression vectors (pcDNA1/Amp) with no cDNA insert (Vector) or cDNA encoding the PTHR1 (PTH-Rc), the TSH receptor (TSH-Rc), or the LH receptor (LH-Rc). Cells were harvested by scraping with a Teflon policeman in a hypotonic lysis buffer (5 mM HEPES, pH 7.4, 0.5 mM EDTA) and were disrupted by 20 strokes of a Dounce homogenizer (loose-fitting) on wet ice. Membrane fractions were collected by centrifugation, and expression of Goα protein was determined by immunoblot analysis in which 50-μg samples of membrane protein were resolved by denaturing 10% SDS-PAGE, transferred to polyvinylidine difluoride membranes, and incubated with a rabbit polyclonal antibody (RM/1) directed against a carboxyl-terminal decapetide of Goα. Antibody binding was detected using a 125I-labeled goat anti-rabbit antibody and a PhosphorImager. Markers indicate the overexpressed 52-kDa Goα as well as the endogenous 45-kDa Goα. At least two additional experiments for each cotransfection condition were performed and gave similar results.

PTHR1 receptor. Thus, isolated resistance to PTH, and the subsequent development of PHP type Ib in the three affected subjects in this family, can be explained by a GNAS1 mutation that produces an abnormal Goα molecule that, although widely expressed, is apparently unable to interact with only the PTHR1. By contrast, subjects with PHP type Ia have defective GNAS1 alleles that produce inadequate amounts of Goα or Goα molecules that are unable to interact with all receptors (33–35). The basis for AHO in subjects with PHP type Ia or their relatives with PPHP and normal hormone responsiveness remains unexplained, but the lack of AHO in these three affected boys provides further confirmation that defective signaling through the PTHR1 is unlikely to be the cause. Thus, abnormal growth of tubular bones, the presumptive basis for brachydactyly in AHO patients with GNAS1 mutations that completely inactivate Goα may result from defective signaling through other adenylyl cyclase-coupled receptors that are expressed in the growth plate.

Selective receptor uncoupling in the patients described here was due to deletion of a single amino acid located in the car-
nally will lack any functional Goα protein in tissues in which Gnas is paternally imprinted, such as the PTH-sensitive renal proximal tubule, and will consequently develop PTH resistance. By contrast, Gnas +/− mice that inherit the inactive Gnas allele paternally will express only the wild type maternal allele in these tissues. As a consequence, these mice will have “normal” levels of Goα protein in renal cells and, like subjects with PPHP, will have normal PTH responsiveness. Finally, Gnas +/− mice and humans will have a 50% reduction in Goα expression in nonimprinted tissues, which express both Goα alleles. Biallelic expression would explain the similar 50% reduction in Goα activity in erythrocytes and cultured fibroblasts from subject with PHP type Ia or PPHP (19, 46), who inherit a defective GNAS1 allele from their mother or father, respectively. Moreover, hormone responsiveness in tissues that express both GNAS1 alleles would be normal (e.g. renal medulla and vasopressin) or mildly impaired (e.g. thyroid and TSH) based on whether 50% of the normal complement of Goα is sufficient for normal signal transduction. Biallelic expression of GNAS1 in bone cells could also explain the normal response of cultured bone cells from a patient with PHP type Ia to PTH treatment in vitro (58), as well as the development of hyperparathyroid bone disease by many patients with PHP type I who have elevated serum levels of PTH (53).

The prevalence of mutations in the GNAS1 gene as a cause of PHP type Ib remains to be determined, but our current studies indicate that defects in coding exons are unlikely to be common. However, recent molecular genetic analyses show linkage of PHP type Ib to the chromosomal region that composes the GNAS1 gene locus, as well as apparent paternal imprinting of PTH resistance (15), thus suggesting that mutations in or near the promoter region of GNAS1 are likely to be present in these patients. Such mutations may selectively reduce Goα expression in the renal proximal tubule and thereby impair PTH-dependent signaling in the kidney. Identification of these mutations will provide additional confirmation that defective regulation of Goα signaling is a common mechanism for PTH resistance in these two distinctive forms of PHP type I.

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FIG. 6. Functional studies of the recombinant wild type and mutant Goα proteins. HEK293 cells were transfected with expression vectors (pcDNAs.1) encoding the wild type (WT) or mutant (MUT) Goα proteins, plus expression vectors (pcDNA1/Amp) encoding the PTHR1 (A), the TSH receptor (C), or the LH receptor (D). The cells were incubated for 10 min in the presence of increasing concentrations of the indicated agonist, and cyclic AMP was extracted and measured by radioimmunoassay. Each point represents the mean (±S.E.) of at least three experiments in which triplicate determinations were performed. The level of cyclic AMP is expressed in terms of maximal agonist-induced accumulation (100%) by cells expressing wild type Goα. Cells were transfected with 4 μg of plasmid containing PTHR1 or LH receptors plus 4 μg of Goα plasmid and 6 μg of plasmid containing TSH receptor plus 2 μg of Goα plasmid.
