Human Molecular Genetics, 2003, Vol. 12, No. 10 1121–1130
DOI: 10.1093/hmg/ddg130

Functional polymorphisms in the paternally expressed XLαs and its cofactor ALEX decrease their mutual interaction and enhance receptor-mediated cAMP formation

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Received December 19, 2002; Revised February 21, 2003; Accepted March 17, 2003

The paternally expressed extra-large stimulatory G protein gene (*XLαs*) is a splice variant of the stimulatory G-protein gene (*Gsα*) consisting of XL-exon1 and exons 2–13 of *Gsα*. A second open reading frame (ORF) in XL-exon1, that completely overlaps the XL-domain ORF, encodes ALEX, which is translated from the XLαs mRNA and binds the XL-domain of XLαs. We previously demonstrated that a paternally inherited functional polymorphism in XL-exon1, consisting of a 36 bp insertion and two nucleotide substitutions, is associated with Gs hyperfunction in platelets, leading to an increased trauma-related bleeding tendency and is accompanied by neurological problems and brachydactyly in two families. Here, we describe eight additional patients with brachydactyly, who inherited the same XLαs polymorphism paternally and who show Gs hyperfunction in their platelets and fibroblasts. All carriers also have an elongated ALEX protein, as a consequence of the paternally inherited insertion. The *in vitro* interaction between the two elongated XLαs and ALEX proteins is markedly reduced. Moreover, XLαs or ALEX can be co-immunoprecipitated with an antibody against either ALEX or XLαs in platelets from a control but hardly from patients with the XLαs/ALEX insertion. In contrast to the strong interaction between the two wild-type proteins, we suggest that this defective association results in unimpeded receptor-stimulated activation of XLαs. The paternally inherited double XLαs/ALEX functional polymorphism is also associated with elevated platelet membrane Gsα protein levels. Both phenomena contribute to increased Gs signaling in patients with platelet hypersensitivity towards Gs-agonists and may be accompanied by neurological problems or growth deficiency.

INTRODUCTION

Gs mediates signal transduction across cell membranes, thus coupling extracellular membrane-bound receptors to adenylyl cyclase; it consists of an α, β and γ subunit, each encoded by different genes (1). The ubiquitously expressed Gsα-subunit contains the guanine nucleotide binding site, has intrinsic GTPase activity, and confers receptor specificity. The *Gsα* gene (MIM 139320) is located on chromosome 20q13 and was originally described as a gene comprising 13 exons (2). Due to alternative splicing, the existence of four different *Gsα* transcripts has been established, each starting from the classical Gsα promoter and resulting in the 45 and 52 kDa forms of the Gsα protein (3).

Mutations in G protein-coupled receptors (GPCR) and G proteins can be either loss- or gain-of-function mutations (4,5). Mutations in an ubiquitously expressed gene such as Gsα will cause more generalized manifestations than those caused by mutations in a gene such as a receptor that is more restricted in expression (6). Amino acid substitutions at two specific codons (201 and 227) lead to constitutive activation of Gsα. Such gain-of-function mutations are found in a variety of sporadic

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endocrine tumors and in the McCune–Albright syndrome (MAS; MIM 174800), characterized by autonomous endocrine hyperfunction associated with fibrous dysplasia of bone and skin hyperpigmentation (7–9). Heterozygous loss-of-function *Gsα* mutations result in the dominantly inherited condition known as Albright hereditary osteodystrophy (AHO; MIM 103580) (10). Within AHO kindred, some patients have the somatic features of AHO alone without overt biochemical abnormalities (pseudopseudohypoparathyroidism = PPHP) while others have AHO in association with resistance to multiple hormones (parathyroid hormone, PTH; thyroid-stimulating hormone, TSH) (pseudohypoparathyroidism type Ia = PHPIa) (11,12). *Gsα* is believed to be imprinted at least in some tissues since inactivating *Gsα* mutations result in different clinical manifestations depending on the parental origin of the gene defect: transmission of Gsα mutations through the maternal germline results in PHPIa, whereas their inheritance from the father causes PPHP (13). More evidence for imprinting as a regulatory mechanism for *Gsα* expression comes from the recent knowledge that only paternally inherited inactivating *Gsα* mutations cause progressive osseous heteroplasia (POH; MIM 166350), an autosomal dominant disorder characterized by extensive dermal ossification and widespread heterotopic ossification of skeletal muscle and deep connective tissue (14).

In men and mice, *Gsα* was indeed found to be subject to genomic imprinting and is located in the complex GNAS1 gene cluster with multiple promoters generating multiple alternative transcripts by splicing of different upstream exons to exon 2 of the 'classical' *Gsα* gene (15–17). One promoter located ~47 kb upstream of *Gsα* is only active on the maternal allele, is methylated on the paternal allele and generates a transcript encoding NESP55, a chromogranin-like neurosecretory protein (18). In contrast, a paternally derived third GNAS1 transcript starting from exon1A was found (15). This group and others illustrated that absence of methylation of the exon1A allele is associated with pseudohypoparathyroidism type Ib (PHPIb) (19–21). Another promoter located ~35 kb upstream of *Gsα* and active on the paternal allele, generates a transcript encoding *XLαs*, a *Gsα* isoform with a long NH_2-terminal extension XLαs (22). XLαs is characterized by a bipartite structure consisting of the N-terminal half (XL-domain) encoded by the >1 kb XL-exon1 and the C-terminal half (αs-domain) encoded by exons 2–13 of *Gsα* (22). XLαs can copy the functions of Gsα as *in vitro* studies show that XLαs binds to βγ-subunits and interacts with adenylyl cyclase (23,24). A recent report demonstrates the coupling of XLαs to Gs-coupled receptors such as the β2-adrenergic receptor and the receptors for PTH, TSH, and corticotropin releasing factor (CRF) (25).

In three patients with markedly prolonged bleeding time and a variable degree of mental retardation, we have recently detected a 36 bp insertion and 2 bp substitutions flanking this insertion in the paternally inherited XL-exon1 (26). The prevalence of this genetic defect is 2.2% in the general population and is associated with increased Gs function in platelets only when inherited paternally, defining the defect as a functional polymorphism. The present study evaluates the effect of this polymorphism in other patients and in other cells than platelets in order to define a more precise phenotype for this Gs hyperfunction syndrome and to gain new insights in the biochemical consequences of the polymorphism.

A second open reading frame (ORF) in the XL-exon1 and completely overlapping with the ORF of the XL-domain, encodes ALEX (the alternative gene product encoded by the XL-exon) (27). XLαs and ALEX are translated from the same mRNA, are interacting co-factors possibly involved in signal transduction at the plasma membrane, and are specifically co-expressed in neuroendocrine cells (27). Since ALEX is translated from the same nucleotide sequence as XLαs, the polymorphism should also be present in ALEX from these patients. Here, we describe the consequences of the functional polymorphism in both XLαs and ALEX in terms of the patients' phenotype, the cAMP response in their platelets and fibroblasts and finally the disturbed interaction between these two paternally expressed and mutated co-factors.

RESULTS

Patient descriptions

We have identified eight children with at least one of the clinical characteristics of the three patients, whom we have described above (patients 1–3): unexplained mental retardation, brachymetacarpia, hypertrichosis, hypotonia, growth deficiency or bleeding problem. All had a variable degree of shortness of the metacarpals and/or metatarsals. These patients could be further subdivided into two groups: the C (cerebral)-type group with psychomotor retardation and a variable degree of feeding and gastro-intestinal motility problems, disturbed behavior, hypotonia, and facial dysmorphy ($n = 6$ patients, including patients 1–3 from the previous study) and the GD (growth deficient)-type group with a pre- and/or postnatal growth failure (height for chronological age below −2 SD; $n = 5$). In contrast to patients from the GD-type group, five out of six patients from the C-type group have a prolonged bleeding time and/or suffer from an enhanced bleeding tendency after trauma or surgery. Patients from the C-type group include two sisters (patients 1 and 2 from 26) and a monozygotic twin while all patients of the GD-type group are unrelated. In both groups the bone alkaline phosphatase activity can be increased.

These eight additional identified children carried the polymorphism in the internal repeat region of the XL-domain of XLαs, as detected by PCR on genomic DNA. Sequencing the XLαs exon 1 in all patients revealed the same 36 bp insertion and the 2 bp substitutions flanking this insertion. Parental DNA was available and all eight patients were confirmed to have inherited the insertion paternally. The schematic presentation of the XL-domain with its normal repeats and the location of the inserted and mutated amino acids are illustrated in Figure 1. The shorter protein ALEX is also translated from XLαs mRNA and thus also consists of a repeat region with the normal and mutated repeats shown in the Figure 1.

Association between the XLαs/ALEX insertion on the paternal allele and Gs hyperfunction in platelets and fibroblasts

Platelets from these eight children were functionally tested in a platelet aggregation inhibition test. They all manifested hyperfunction of the Gs pathway, similarly to the three

XLαs domain αs domain

XLαs M- Internal repeat region -THRLLLLG + exon 2 - 13

1 74 225 381

βγ

ALEX M- Internal repeat region

1 9 152 324

XLαs

NORMAL REPEATS **PATIENT REPEATS**

```
74  SPGYGSPAAGAA
    SADTAARAAPAA
    PADPDSSGAT
    PEDPDSGTA
    PADPDSSGAF
    AADPDSGAAPAA          AADPDSGAAPDA
    PADPDSGAAPDA          PADPDSGAAPDA
    PADPDSGAAPDA          PADPDSGAAPDA
    PADPDSGAAPEA          PADPDSGAARDA
    PAAPAAAETRAAHVA
    PAAPDAGAPTA
    PAASATRAAQVR
    RAASAAPASGARRKI 225
```

ALEX

NORMAL REPEATS **PATIENT REPEATS**

```
9   PLRGTDPLPPGQ
    PQRIPLPGQPLQ
    PQPILTPGQ
    PQKIPTPGQ
    HQPILTPGH
    SQPIPTPGQPLPP         SQPIPTPGQPLTP
     QPIPTPGRPLTP          QPIPTPGRPLTP
     QPIPTPGRPLTP          QPIPTPGRPLTP
     QPIQMPGRPLRL          QPIPTPGRPVTP
     PPPLRLLRP
    GQPMSPQLRG
    TQGLPLPQPLLPP 152
```

Figure 1. Schematic representation of XLαs and ALEX. The repeat regions in XLαs and ALEX are indicated by gray boxes and represent regions of poor inter-species homology. The terminal 8 amino acids of XLαs are identical to those encoded by exon 1 of Gsα and connect the XL-domain with the αs-domain. These amino acids are responsible for βγ-binding. ALEX is shorter but completely overlaps the ORF encoding the XL-domain. The normal internal repeat regions of XLαs and ALEX are compared with these of the patients, with an extra identical repeat of 12 amino acids and two codon substitutions (underscored).

originally described patients: all patients' platelets had a significantly increased sensitivity towards a Gs agonist, the prostacyclin analogue Iloprost (Fig. 2A).

We next studied the function of the Gs pathway in these patients using other cells than platelets. Fibroblasts were only available from five patients of the C-type group. We measured cAMP in fibroblasts after stimulation of the β_2-adrenergic receptor, coupled to Gsα and possibly also to XLαs (25). The cAMP levels following exposure to a low concentration of isoproterenol (1 μM f.c.) rose markedly in the patient cells compared with controls (Fig. 2B). These experiments were performed in the presence of a phosphodiesterase inhibitor (IBMX 400 μM f.c.). Stimulations with forskolin (5 μM f.c.) in the presence of IBMX showed no differences in cAMP

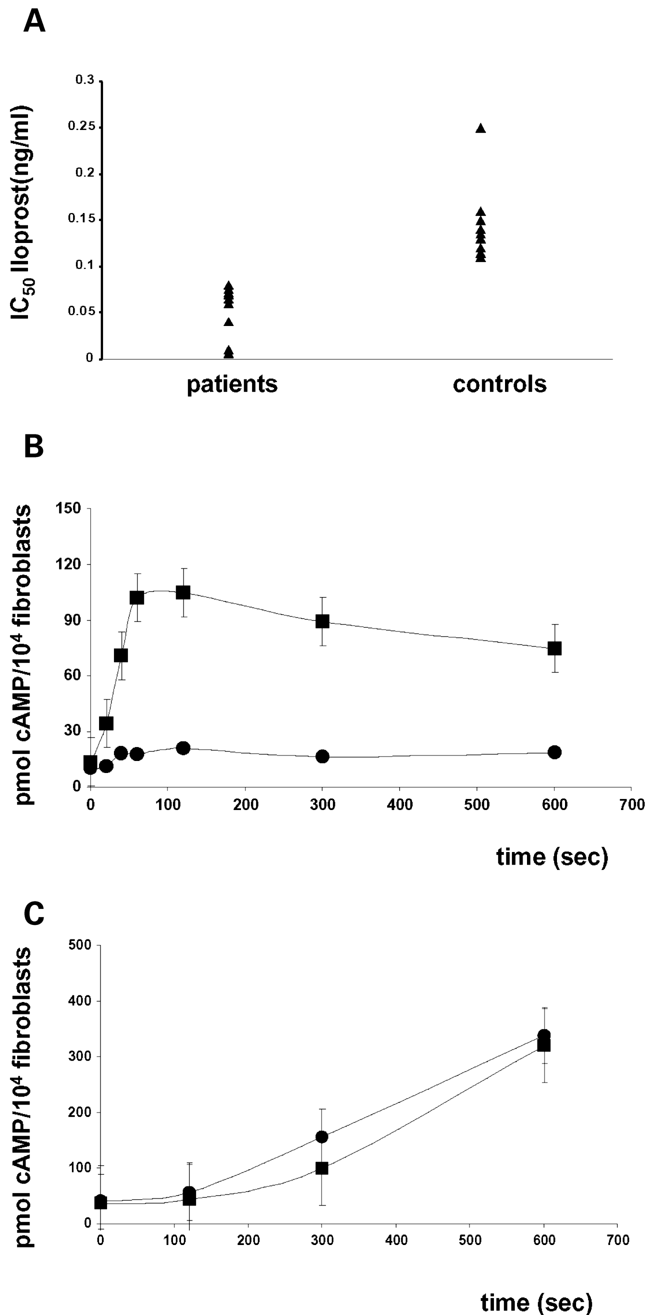

Figure 2. Gsα activity in platelets and fibroblasts. (**A**) IC_{50} values for inhibition by the Gs agonist Iloprost of U46619 (1.3 μM)-induced aggregation of platelets from eight patients and eight controls. The patients showed an enhanced inhibition of platelet aggregation upon stimulation with the Gsα agonist Iloprost ($P < 0.0001$). cAMP levels in fibroblasts before and after stimulation with (**B**) isoproterenol (1 μM f.c.) or (**C**) forskolin (5 μM f.c.) for different time-intervals, given as mean ± SD for five different controls (solid circles) and five patients (solid squares).

response between patient and control fibroblasts (Fig. 2C). These results exclude differences in intrinsic adenylyl cyclase or in phosphodiesterase activity between patients and controls.

Interaction between XLαs and ALEX

The hypothesis that ALEX plays a role in regulating signal transduction by interacting with XLαs (27) suggested that the altered Gs-signal transduction in carriers of the polymorphism results from a disturbed interaction between the mutated XLαs and ALEX proteins. We studied their interaction by an *in vitro* GST pull-down assay and found that in comparison to the binding between the two wild-type proteins the interaction between the mutated XLαs (XL + insert) and mutated ALEX (ALEX + insert) was strongly decreased (Fig. 3A). The other two binding experiments (XL to ALEX + insert and XL + insert to ALEX) seemed normal but are in fact artificial conditions (Fig. 3B). Because XLαs and ALEX are only monoallelically expressed from the paternal allele and translated from the same mRNA, patients with the insertion can only express both XLαs + insert and ALEX + insert (15,16,27 and results below). The *in vitro* binding between XLαs and ALEX is highly specific since XLαs binding did not interact with aspecific GST-coupled beads (Fig. 3B).

XLαs and ALEX mRNA and protein expression in platelets and fibroblasts

XLαs and ALEX are co-expressed in the GNAS1 cluster from only the paternal allele, as illustrated in Figure 4A. We could detect mRNA for both *XLαs* and *ALEX* in fibroblasts and rather weakly in platelets, probably due to the known poor stability of RNA in the latter. Fibroblasts of the patients only express mRNA for mutated *XLαs* and *ALEX* which is 36 bp longer than the wild-type mRNA (Fig. 4B).

Western blot analysis had previously shown a variable degree of increased Gsα protein levels in the platelet membranes of patients 1 and 2 (26); however this difference in expression level was less obvious when studying cytosol or total platelet or fibroblast protein fractions. We compared the Gsα expression in platelet membrane, cytosol and total protein fractions of patients from the C-type versus the GD-type group and found again increased membrane-bound Gsα and moderately increased cytosol fractions but no differences between the two groups of patients (Fig. 5A). We had already analyzed XLαs protein expression in human platelets and found a band of about 98 kDa (21). In contrast to the altered Gsα expression levels, patients with the XL/ALEX insertion did not differ in XLαs platelet expression. We also studied the presence of ALEX in platelets and detected normal expression levels in the patients, but the corresponding bands were slightly higher than wild-type ALEX due to the insertion (Fig. 5A). The real direct binding of human ALEX to XLαs in platelets was also studied by a double *ex vivo* immunoprecipitation assay. XLαs and ALEX can be co-immunoprecipitated with either an antibody against ALEX or XLαs in platelets from a control but this co-interaction is much weaker in patients with the XL/ALEX insertion (Fig. 5A).

To exclude the fact that disturbed binding between XLαs + insert and ALEX + insert could be due to a different anti-ALEX antibody specificity for wild-type versus mutated ALEX, some additional controls were performed. The immunoblot analysis of CHO cells transfected with either an

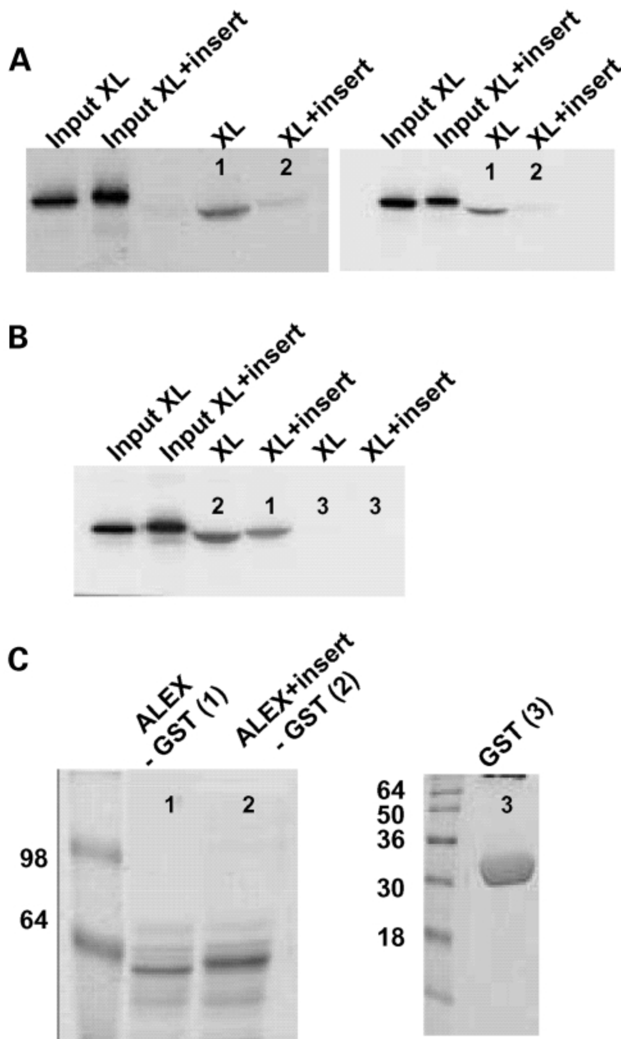

Figure 3. ALEX/XLαs *in vitro* interaction studies. The *in vitro* transcription/translated ^{35}S-labeled XL-domain (XL) and mutated XL-domain (XL + insert) were incubated with various GST fusion proteins (ALEX or 1 and ALEX + insert or 2) immobilized on glutathione-sepharose beads. After washing the beads, bound proteins were eluted and resolved by SDS–PAGE. (**A**) The autoradiogram shows the amount of input (10%) ^{35}S-labeled XL or XL + insert and the amount of ^{35}S-labeled XL or XL + insert that was retained by their respective binding to ALEX (1) or ALEX + insert (2). This binding experiment was done in duplicate. (**B**) The autoradiogram shows the amount of input (10%) ^{35}S-labeled XL or XL + insert and the amount of ^{35}S-labeled XL or XL + insert that was retained by their respective binding to ALEX + insert (2) or ALEX (1). ^{35}S-labeled XL or XL + insert did not bind to the aspecific GST-coupled beads (negative control) (3). (**C**) The lower panel shows the Coomassie blue staining of the gel confirming equal ALEX-GST and ALEX + insert-GST fusion protein sample loading. GST alone (3) was used as negative control.

empty vector or with normal or mutated ALEX showed that the antibody is highly specific (Fig. 5B). We observed the same discrepancy between the electrophoretic mobility of this protein (about 48 kDa) and its predicted molecular mass (38 kDa), as described by others (26). Immunoprecipitation with the anti-ALEX antibody showed no differences in the binding between ^{35}S-labeled wild-type or mutated ALEX (Fig. 5C).

Presence of the Gβ3 C825T polymorphism in patients from the C-type versus GD-type group

Western blot analysis of Gsα, ALEX, and XLαs or the *ex vivo* XLαs/ALEX co-immunoprecipitations never showed any differences between patients of the C-type versus the GD-type group (Fig. 5A). Therefore, we hypothesized that the phenotypic differences are more likely to be the result a deficiency of other players in the same signal transduction pathway. The functional C825T polymorphism in the G-protein β3-subunit alters signal transduction via pertussis toxin-sensitive G proteins in lymphoblasts and fibroblasts from patients with hypertension (28). The presence of this polymorphism was studied by PCR and restriction digestion in all patients with the XL/ALEX insertion. We found that all patients except the two sisters (patients 1 and 2 from 26) from the C-type group carried the Gβ3 polymorphism (C/T or T/T allele) while patients from the GD-type group only have the C/C allele (data not shown). The presence of the T allele is associated with a *Gβ3* splice variant (Gβ3-short) lacking part of exon 9, which is translated into a shorter Gβ3 protein as could be detected in platelets from carriers with the polymorphism (28). We found that wild type Gβ3 (Gβ3-WT) and Gβ3-short are capable of binding to both Gsα and XLαs, but only XLαs showed a different binding affinity for these two Gβ3 proteins with a stronger affinity for Gβ3-WT (Fig. 6). No differences in the binding affinity of Gβ3 for XLαs with or without the insertion were observed (data not shown).

DISCUSSION

We recently demonstrated that a paternally inherited functional polymorphism in the extra-large stimulatory G-protein gene is associated with increased platelet membrane-bound Gsα protein levels, a Gs-inducible hyperfunction in platelets and seems to be a risk factor for bleeding (26). Here, we show that the insertion with associated 2 bp substitutions not only exists in the coding sequence of the XL-domain of XLαs but also in its overlapping ORF coding for ALEX. We have now characterized another eight children who all have inherited the double XLαs/ALEX polymorphism paternally and share some phenotypic features with the three previously described children, such as brachydactyly in combination with either mental or growth retardation. Apart from an overexpression of membrane-bound Gsα in the patients' platelets, a clear inducible Gs hyperfunction was found in their platelets and fibroblasts, where receptor stimulation with a low dose of Gs agonists had already resulted in high levels of cAMP. In contrast to the McCune–Albright syndrome due to a somatic gain of function mutation in *Gsα*, normal basal cAMP levels were found in the patients with the polymorphism, who exhibit a Gs hyperfunction only when this pathway is stimulated.

The main component in Gs-signaling leading to intracellular generation of cAMP is the Gsα protein. Gsα may share this function in some cells or during some stages of the development with XLαs, which has the same αs-domain and was shown to interact with the same Gs-coupled receptors, including the β-adrenergic receptor (24). Klemke *et al.* (27) hypothesize that XLαs may only undergo receptor-mediated GDP-GTP exchange when ALEX is not bound to XLαs. This

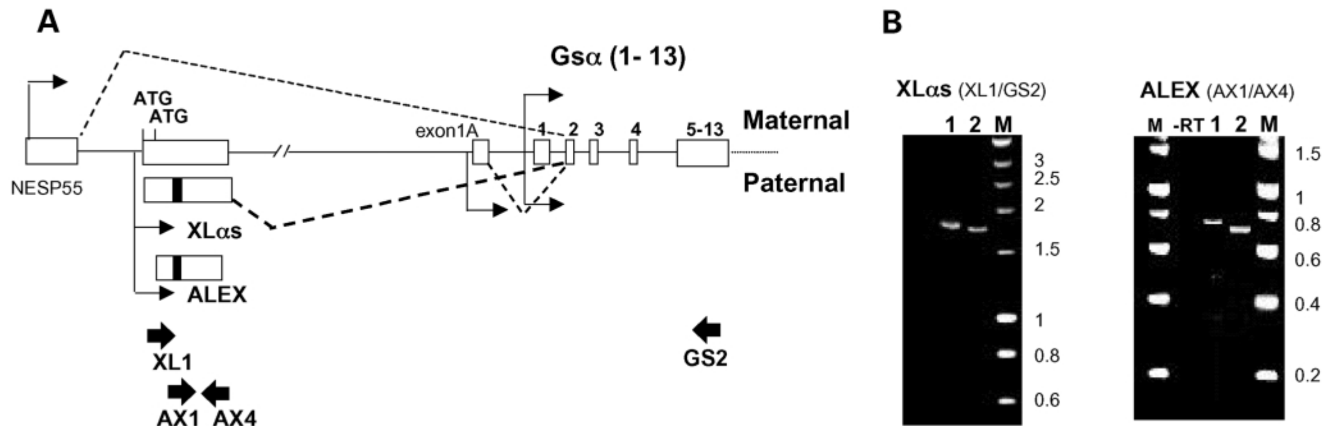

Figure 4. Schematic representation of the GNAS1 cluster. (**A**) All different first exons are represented by white boxes and indicated by their names. The alternative splicing and parent-specific transcription are indicated by dashed lines and arrows, respectively. The mutation in the paternally expressed XLαs and ALEX is indicted by a black box and results in a repeat extension in both proteins. (**B**) XLαs (1.8 kb) and ALEX (0.78 kb) mRNA detection in fibroblasts from a patient with the insertion (number 1) and from a control (number 2). The primers used for RT–PCR are indicated in (A). 'M' represents the internal standard in kb. Since ALEX contains no introns, a sample without RT was added as negative control to exclude gDNA contamination.

knowledge in combination with the fact that the mutated ALEX and XLαs lose much of their mutual affinity, allows us to suggest that the inducible Gs hyperfunction in the carriers of the polymorphism is also due to increased XLαs signaling. A model for this hypothesis in combination with the previous finding that patients with the XLαs/ALEX insertion have increased membrane-bound Gsα protein levels (26) is proposed in Figure 7. The enhanced cAMP response towards Gs-coupled receptor stimulation in the patients seems to be the result of two additional effects: increased Gsα signaling due to higher Gsα expression levels and more XLαs signaling due to reduced XLαs/ALEX binding.

XLαs is primarily expressed in neuroendocrine tissues, possibly explaining how mutated XLαs contributes to neurological symptoms in the C-type group of carriers. Findings in mice with heterozygous disruption of Gnas exon 2 suggest a functional role for XLαs, as mice with a paternally disrupted Gnas (thus no XLαs) die within 24 h of birth, while mice with a maternally disrupted Gnas (thus normal Xlαs) survive into the first week (29). XLαs is therefore believed to be important in fetal and neonatal development, and its mutation may contribute to growth deficiency, characteristic for the GD-type group of carriers.

It still remains to be established how the paternally inherited polymorphism in XLαs leads to such a variable phenotype (C-type versus GD-type) and why the prevalence of the polymorphism in the general population can be as high as 2.2%, with an equal distribution of the parental origin. Our previous study showed that control subjects with the paternally inherited insert also had Gs hyperfunction in their platelets, whereas control subjects with the insert on the maternal allele did not. Why are some people with the paternally inherited polymorphism apparently asymptomatic? One reason may be that the polymorphism leads to an inducible rather than to a constitutional Gs hyperfunction, as is observed in the McCune–Albright syndrome. A second reason could be that the adenylyl cyclase pathway itself is subject to many regulations, through Gi, phosphorylation of Gsα, βγ polymorphisms, and so on. It is likely that alteration of additional genes is required to elicit a more or less extensive phenotype or even to explain the two observed phenotypes. Evidence for this exists as in contrast to all patients from the GD-type group, patients from the C-type group except for the two sisters also carry a known functional polymorphism in the G-protein β3-subunit (28). We found that the binding between XLαs and the polymorphic Gβ3-short is reduced compared with the binding with the Gβ3-WT protein. We show the first evidence that the combination between two functional polymorphisms in two proteins involved in the same pathway, could result in distinct phenotypes. However, more studies are needed to determine the real contribution of this Gβ3 polymorphism to the phenotypic differences of the two patient groups. Other factors probably contribute to the GD-type group specific characteristics. There is indeed increasing evidence that simple Mendelian disorders can be converted into complex traits by the influence of modifier genes (30,31).

In conclusion, we found that a common insert in the XLαs gene results in altered XLαs and ALEX proteins, with reduced mutual affinity; the latter together with increased Gsα levels may be responsible for increased adenylyl cyclase activation in platelets and fibroblasts, when the insert is inherited paternally.

MATERIALS AND METHODS

Antibodies

The primary anti-XLαs monoclonal antibody (referred to as anti-XLαs) (21) and the anti-ALEX polyclonal antibody (referred to as anti-ALEX) were produced in our laboratory upon injection of respectively mice or rabbits with recombinant fusion proteins consisting of the amino acids encoded by either the complete XL-exon1 or the complete ALEX sequence each coupled to glutathione *S*-transferase (GST). These recombinant fusion proteins were expressed in *Escherichia coli* and purified by affinity chromatography on immobilized glutathione

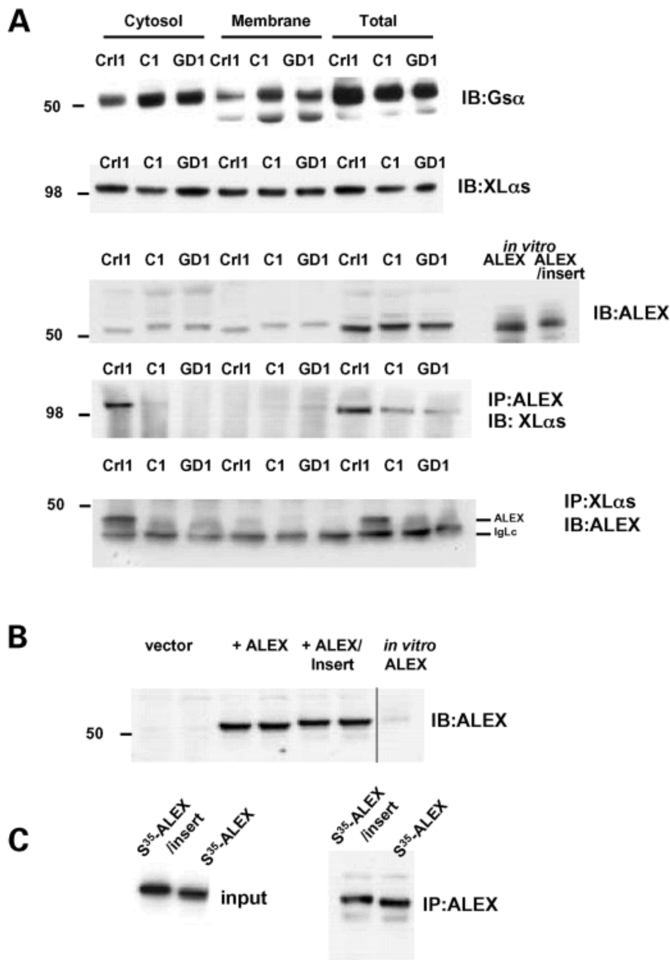

Figure 5. Gsα, XLαs and ALEX expression and binding. (**A**) Western immunoblot (IB) analysis of Gsα (52 and 45 kDa), XLαs (±98 kDa), ALEX (±50 kDa) and XLαs or ALEX co-immunoprecipitated (IP) with either an antibody against ALEX or XLαs in membrane, cytosol and total platelet protein fractions (25 μg) from a normal control (Crl1), a patient of the C-type (C1) and GD-type (GD1) group. Results are shown for one of the two series of normal control, C-type and GD-type patients. (**B**) Immunoblot analysis of ALEX in CHO cells transfected with an empty pcDNA3.1 vector, with pcDNA3.1-ALEX or with pcDNA3.1-ALEX + insert. *In vitro* transcripted/translated non-labeled ALEX was also included. (**C**) The left panel shows the amount of input ^{35}S-labelled ALEX or ALEX + insert while the right panel illustrates similar amounts of ALEX or ALEX + insert after immunoprecipitation with the polyclonal anti-ALEX antibody.

(Amersham Biosciences). The primary antibodies were purified on protein A Sepharose™ beads (Amersham Biosciences), controlled for their specificity by detection of their corresponding recombinant protein. Gsα was detected with a polyclonal antibody (Calbiochem).

Patients

Patients and/or their legal representatives consented to the determination of platelet and fibroblast Gs activity and molecular analysis of the GNAS1 cluster. The study was approved by the Institutional Review Board.

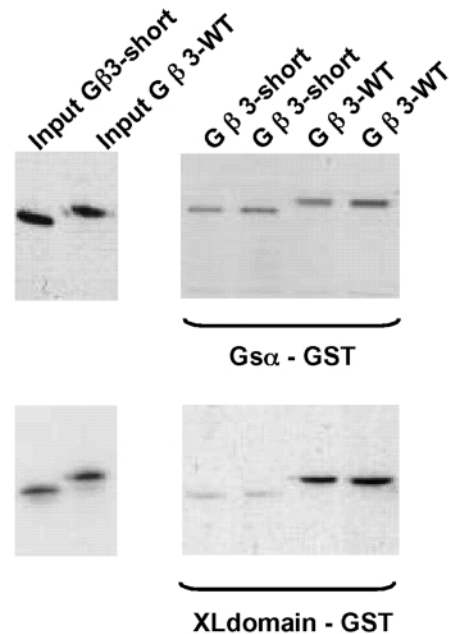

Figure 6. Gsα or XLαs/Gβ3 WT or short *in vitro* interaction studies. The *in vitro* transcription/translated Gβ3-WT or Gβ3-short were incubated with various GST fusion proteins (Gsα or the XL-domain that contains the βγ-interaction site of XLαs) immobilized on glutathione agarose beads. The autoradiograph shows the amount of retained ^{35}S-labeled Gβ3-WT versus mutant Gβ3-short for respectively Gsα and XLαs. The binding experiment was performed in duplicate.

Platelet aggregation-inhibition test

Platelet aggregation in plasma was measured on two dual-channel Chrono-Log Aggregometers (Chronolog Corp, Havertown, PA, USA). Aggregation-inhibition studies were performed as originally described (24) with some minor modifications: aggregation was induced by U46619 (1.3 μM; Sigma) in the presence of apyrase (1 U/ml; Sigma), following exposure of the platelet-rich plasma to several concentrations of the Gs agonist Iloprost. The 50% inhibitory concentration (IC_{50}) of Iloprost with accuracy value was calculated after curve fitting of the dose response using the software InStat 2.03, both for the patient and a simultaneously studied healthy control.

Cell culture

Skin fibroblasts were obtained via punch biopsy from the volar side of the upper arm. Fibroblasts were cultured in DMEM/H12 (Gibco BRL) supplemented with 10% fetal bovine serum and antibiotics (Gibco BRL), at 37°C in a 5% CO_2 humidified incubator. Only fibroblasts of low passage number (between 6 and 12) were used for DNA and RNA extraction and for cAMP measurements. Cells were grown to 100% confluence and then incubated in serum free medium for 24 h before cAMP measurements. Fibroblasts were plated in duplicate, one used for the assay, the other for cell counting and the obtained cAMP levels were thus adjusted to the cell number.

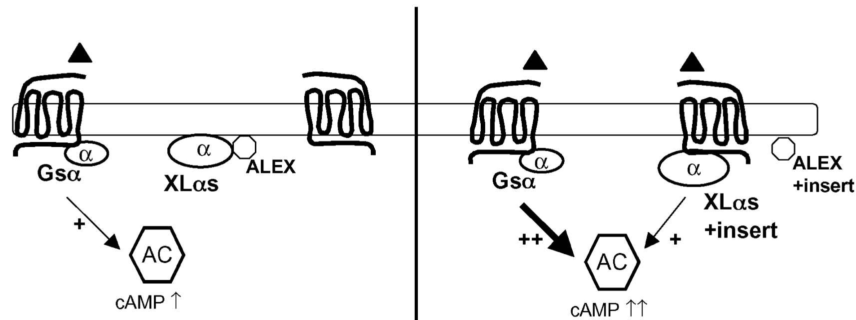

Figure 7. Hypothetical Gsα versus XLαs-ALEX signal transduction model. The left model illustrates the normal situation in which ALEX is strongly bound to XLαs making the latter incapable of binding to membrane receptors. Adenylyl cyclase activation fully depends on stimulation of Gsα. The model on the right represents the situation in the patients with the polymorphism: mutated ALEX and XLαs have a reduced mutual affinity and XLαs can undergo receptor-mediated activation and more Gsα is found in the platelet membrane fraction. Both observations will result in enhanced cAMP responses upon Gs-coupled receptor activation in patients with the polymorphism.

cAMP measurement in fibroblasts

Patient or control fibroblasts were stimulated with either the Gs agonist isoproterenol (Calbiochem) at 1 μM or the adenylyl cyclase activator forskolin (Calbiochem) at 5 μM in the presence of a phosphodiesterase inhibitor (3-isobutyl 1-methylxanthine, IBMX; 400 μM) and reactions were terminated by the cell lysis buffer, supplied with the kit. The cAMP levels were measured using a commercially available cAMP enzyme-immunoassay (Amersham, Pharmacia Biotech).

Detection of XLαs and ALEX mRNA expression

Total RNA was extracted from cultured fibroblasts using TRIzol reagent (Gibco BRL) according to the manufacturer's protocol. Before RT treatment, RNA samples were first treated with Dnase I (1 U/mg RNA, Gibco BRL) to eliminate genomic DNA contamination. Approximately 1 μg fibroblast RNA, in the presence of an RNaseI inhibitor (Gibco BRL), was used for oligo (dT)-primed first strand cDNA synthesis using M-MLV reverse transcriptase (Gibco BRL). The following primer sets were used to generate specific fragments: primers for the *XLαs* mRNA amplification were XL1-(5′-ATGGAGATCTCCGGA-CCCCCGTTC-3′) and GS2-(5′-TGCTGGCCACCACGAAG-ATGATG-3′) and for ALEX mRNA amplification were AX1-(5′-GAAGCAGCAGAGATGGAAGGAGCCG-3′) and AX4-(5′-CTGCTGCCCGCAGGCAGAGTCCGCCGC-3′). PCR was performed by adding 4 μl cDNA aliquots to the reaction mixture containing 25 pmol of each primer, PCR buffer (Gibco BRL), 0.75 mM $MgCl_2$, 10% DMSO, 200 mM dNTP, 1 U *Taq* polymerase (Gibco BRL) in a PTC-100™ programmable Thermal Controller (MJ research Inc., Biozym) using the following conditions: 95°C for 5 min followed by 35 cycles of 95°C for 30 s, 60°C for 1 min and 72°C for 1.5 min, and final extension at 72°C for 10 min.

In vitro XLαs/ALEX and XLαs or Gsα/Gbeta3-WT or Gbeta3-short binding by the GST pull-down assay

ALEX and ALEX + insert (residues 1–324) or Gsα (residues 1–394) and the complete XL-domain (residues 1–389) fragments were generated by PCR, confirmed by DNA sequencing and cloned in the expression vector pGEX-4T-2 (Pharmacia Biotech). The XL-domain and the XL-domain + insert (residues 1–389) or Gβ3-WT and Gβ3-short (residues 1–340 and with deletion of residues 167–207) were cloned in pcDNA3.1/His (Invitrogen) and ^{35}S-labeled XL-domain or ^{35}S-labeled Gβ3 proteins were produced by *in vitro* transcription/translation using the TNT system (Promega). Primer sequences are available on request. A 4 μl aliquot of the translation mixture containing the ^{35}S-labeled XL-domain (with or without insert) or ^{35}S-labeled Gβ3 was incubated for 3 h at 4°C in 300 μl binding buffer [150 mM NaCl, 20 mM Tris pH 7.5, 0.1% Igepal CA-630 (Sigma Chemical), 0.25% bovine serum albumin, 1 mM β-mercaptoethanol, 1.5 mM phenylmethylsulfonyl fluoride] containing 50 μl of glutathion-Sepharose beads with respectively an equal amount of either GST-ALEX (with or without insert) or Gsα versus XL-domain fusion protein bound to it. After three washing steps with 500 μl of binding buffer, the amount of retained labeled XLαs or Gβ3 bound to the GST-ALEX fusion protein on the beads was analyzed by SDS-PAGE and autoradiography.

Preparation of platelet cell extracts and immunoblot analysis

To obtain the total platelet protein fraction: platelets were isolated from citrated blood were directly lysed in solubilisation buffer [ice-cold PBS containing 1% igepal CA-630 (Sigma), 1 mmol/l EDTA, 1 mmol/l phenylmethylsulfonyl fluoride, 2 mmol/l DTE, 1 protease inhibitor cocktail tablet/50 ml] and incubated on ice for 60 min. Lysates were cleared of insoluble debris by centrifugation at 14 000*g* for 20 min at 4°C. To obtain separate membrane and cytosol platelet protein fractions, platelets were isolated from citrated blood, resuspended in lysis buffer (10 mM Tris–HCl buffer, pH 7.5, 1 mM EDTA, 140 mM NaCl, 250 mM sucrose, 1 mM dithiotreitol, protease inhibitor cocktail tablet) and membranes were separated from cytosol via centrifugation at 14 000*g* for 15 min. Membranes were lysed in solubilisation buffer, incubated on ice for 1 h and centrifuged at 14 000*g* for 15 min.

Platelet protein fractions (25 μg) were mixed with Laemmli sample buffer (5% SDS reducing buffer), resolved by SDS–PAGE on 12.5% (for ALEX), 7% (for XLαs) or 10%

(for Gsα) acrylamide gels, and transferred to Hybond ECL-nitro-cellulose membrane (Amersham, Pharmacia Biotech). The blots were blocked for 1 h at room temperature in Tris-buffered saline with Tween (TBS-T; 0.1% Tween-20) supplemented with 5% non-fat dry milk. Incubation with primary (overnight at 4°C) and with secondary antibody (2–3 h at room temperature) was performed in TBS-T with 5% non-fat milk. Blots were revealed with either a polyclonal or monoclonal antibody, the commercial anti-Gsα antibody was used as indicated and the antibodies made in our laboratory were used at 50 μg/ml. The secondary antibody was conjugated with HRP and staining was performed with the Western blotting ECL detection reagent (Amersham, Pharmacia Biotech).

Ex vivo XLαs/ALEX binding assay by immunoprecipitation

Immunoprecipitation was performed by incubating 25 μg of either a rabbit polyclonal anti-ALEX antibody or a monoclonal anti-XLαs antibody, with protein A Sepharose™ beads, and 100 μg of cell lysate proteins (cell extracts) in 300 μl binding buffer [150 mM NaCl, 25 mM Tris; pH 7.5, 0.1% Igepal CA-630 (Sigma), 0.25% BSA, 1 mM β-mercaptoethanol, protease inhibitor cocktail tablet per 50 ml] for 3 h at 4°C. After centrifugation and washing the protein A-coupled ALEX or XLαs antibody beads three times in 500 μl binding buffer, the respectively bound XLαs or ALEX was determined by western blotting.

Detection of the Gβ3 C825T polymorphism

DNA genotyping was performed as described by PCR and *BseD*I restriction digestion. The unrestricted PCR product (TT genotype) has a size of 268 bp while complete restriction (CC genotype) results in bands of 116 and 152 bp.

ACKNOWLEDGEMENTS

This work was supported by research grant G.0371.01 and KAN2000-1523100 from the FWO-Vlaanderen and OT/00/25, KULeuven. C.V.G. and F.d.Z. are holders of a clinical-fundamental research mandate of the FWO and M.V.H. has a research grant of the Belgian Study Group for Pediatric Endocrinology. J.V. is holder of the Aventis Chair of Hemostasis Research.

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