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Characterization of the Extra-large G Protein α-Subunit XLαs

II. SIGNAL TRANSDUCTION PROPERTIES*

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In the preceding paper (Pasolli, H. A., Klemke, M., Kehlenbach, R. H., Wang, Y., and Huttner, W. B. (2000) *J. Biol. Chem.* 275, 33622–33632), we report on the tissue distribution and subcellular localization of XLαs (extra large αs), a neuroendocrine-specific, plasma membrane-associated protein consisting of a novel 37-kDa XL domain followed by a 41-kDa αs domain encoded by exons 2-13 of the Gαs gene. Here, we have studied the signal transduction properties of XLαs. Like Gαs, XLαs undergoes a conformational change upon binding of GTPγS (guanosine 5′-*O*-(thio)triphosphate), as revealed by its partial resistance to tryptic digestion, which generated the same fragments as in the case of Gαs. Two approaches were used to analyze XLαs-βγ interactions: (i) ADP-ribosylation by cholera toxin to detect even weak or transient XLαs-βγ interactions and (ii) sucrose density gradient centrifugation to reveal stable heterotrimer formation. The addition of βγ subunits resulted in an increased ADP-ribosylation of XLαs as well as an increased sedimentation rate of XLαs in sucrose density gradients, indicating that XLαs interacts with the βγ dimer. Surprisingly, however, XLαs, in contrast to Gαs, was not activated by the β2-adrenergic receptor upon reconstitution of S49cyc$^-$ membranes. Similarly, using photoaffinity labeling of pituitary membranes with azidoanilide-GTP, XLαs was not activated upon stimulation of pituitary adenylyl cyclase-activating polypeptide (PACAP) receptors or other Gαs-coupled receptors known to be present in these membranes, whereas Gαs was. Despite the apparent inability of XLαs to undergo receptor-mediated activation, XLαs-GTPγS markedly stimulated adenylyl cyclase in S49cyc$^-$ membranes. Moreover, transfection of PC12 cells with a GTPase-deficient mutant of XLαs, XLαs-Q548L, resulted in a massive increase in adenylyl cyclase activity. Our results suggest that in neuroendocrine cells, the two related G proteins, Gαs and XLαs, exhibit distinct properties with regard to receptor-mediated activation but converge onto the same effector system, adenylyl cyclase.

In the preceding paper (32), we show that XLαs (for extra large αs), an unusual type of G protein α subunit, is predominantly associated with the plasma membrane of certain neuroendocrine cells. XLαs consists of a novel 37-kDa XL domain followed by a 41-kDa αs domain encoded by exons 2-13 of the Gαs gene (1, 2) and, hence, contains most of the functional domains of Gαs including receptor and effector binding sites. Given the subcellular localization of XLαs and its domain structure and in light of the observation that the C-terminal sequence of the XL domain shows a high homology to the exon 1-encoded portion of Gαs (1), which promotes binding to the βγ complex, it is important to determine whether or not XLαs functions, like Gαs, in signal transduction.

In the present study, we addressed the following questions. First, does XLαs exchange guanine nucleotides and, if so, does this guanine nucleotide exchange lead to a conformational change of XLαs, as has been reported for Gαs (3)? Second, does XLαs interact with the βγ complex? Third, does XLαs couple to heptahelical receptors and, if so, to which ones? And, finally, does XLαs activate adenylyl cyclase, the classical Gαs effector?

EXPERIMENTAL PROCEDURES

Antibodies

The rabbit antiserum RK5 (anti-XL) against the EPAA-repeats in the XL domain of XLαs was that described in the preceding paper (32). The rabbit antiserum against the C-terminal decapeptide of Gαs and XLαs (anti-αs C terminus) was the same as described previously (1).

Plasmids

The plasmid CDM8-XLαs, originally called CDM8-XL, contains a 2.6-kilobase insert starting at nucleotide 380 of the originally published sequence (1) and encodes the entire XLαs protein sequence (see correction of translational start (2)) under the control of the cytomegalovirus promotor.

For construction of CDM8-XLαs-wt, the plasmid pGEM-Gαs (kindly provided by Dr. Peter Gierschik, University of Ulm), that encodes the entire rat Gαs protein sequence, was cut with *Eco*47III and *Nsi*I. The resulting 667-nucleotide fragment, corresponding to amino acid residues 165–386 of Gαs, was cloned into the *Eco*47III and *Nsi*I sites of CDM8-XLαs, resulting in a predicted XLαs protein sequence containing a leucine at position 519 (corrected translational start (2)) instead of a proline as in CDM8-XLαs (amino acid residue 650 of originally published sequence (1)). The nucleotide exchange and the ligation sites were confirmed by sequencing.

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The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™/EBI Data Bank with accession number(s)AF116268.

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For construction of CDM8-XLαs-Q548L, the plasmid pVL-1393-Gαs-Q227L (kindly provided by Dr. Peter Gierschik, University of Ulm), which encodes the entire human Gαs protein sequence, was cut with *Eco*47III and *Nsi*I. The resulting 667-nucleotide fragment, corresponding to amino acid residues 165–386 of Gαs, was cloned into the *Eco*47III and *Nsi*I sites of CDM8-XLαs, resulting in a predicted XLαs protein sequence carrying a single point mutation (Gln → Leu) at amino acid residue 548 (corrected translational start (2); amino acid residue 679 of originally published sequence (1). The other differences in nucleotide sequence between human pVL-1393-Gαs-Q227L and the rat CDM8-XLαs do not cause any amino acid sequence variation between the two species.) The nucleotide exchange and the ligation sites were confirmed by sequencing. The plasmid CDM8-Gαs (kindly provided by Yanzhuang Wang of our laboratory) encodes the entire rat Gαs protein sequence under the control of the cytomegalovirus promotor.

In Vitro Transcription

After linearization by *Nde*I, 10 μg of each plasmid (CDM8-Gαs and CDM8-XLαs-wt) was *in vitro* transcribed for 4 h at 37 °C in a final volume of 100 μl containing 20 μl of 5× transcription buffer (MBI Fermentas), 3 μl each of ATP, GTP, CTP, and UTP (100 mM each), 2 μl of RNase Inhibitor (40 units/μl), 3 μl of T7 RNA polymerase (40 units/μl), and nuclease-free distilled H_2O. Two h after the addition of the T7 RNA polymerase, another 3 μl of the polymerase were added. Two μl of a 1:10 dilution of the total *in vitro* transcription mixture were used directly for *in vitro* translation.

In Vitro Translation

Cell-free translation of *in vitro* transcribed RNAs was carried out at 30 °C for 1 h using the Promega nuclease-treated reticulocyte lysate following the manufacturer's instructions. Briefly, a typical translation mixture contained 35 μl of the reticulocyte lysate, 7 μl of nuclease-free distilled H_2O, 1 μl of RNase Inhibitor (40 units/μl), 1 μl of the amino acid mixture without methionine, 4 μl of the L-[^{35}S]Met/Cys ProMix™ (Amersham Pharmacia Biotech, 1000 Ci mmol^{-1}), and 2 μl of the 1:10 diluted total *in vitro* transcription mixture containing the RNA template. The non-radioactive *in vitro* translation for ADP-ribosylation and the reconstitution of S49cyc$^-$ membranes was performed with 1 μl of the amino acid mixture without methionine and 1 μl of the amino acid mixture without cysteine.

Immunoprecipitation

All steps were performed at 4 °C. *In vitro* translated Gαs and XLαs were mixed with two volumes of immunoprecipitation buffer (3% Triton X-100, 1.5% sodium deoxycholate, 0.3% SDS, 450 mM NaCl, 3 mM EDTA, 3.75 mM phenylmethylsulfonyl fluoride (PMSF),[1] and 30 mM Tris-Cl, pH 8.0) and incubated for 30 min. Insoluble material was removed by centrifugation for 20 min at 14,000 × *g*, and the supernatant was incubated for 30 min with 50 μl of a 50% slurry of protein A-Sepharose and centrifuged for 5 min at 800 × *g*. The supernatant was used for immunoprecipitation using the antiserum against the common C-terminal decapeptide of Gαs and XLαs or the anti-XL antiserum. The samples were incubated with the antibody overnight followed by the addition of protein A-Sepharose (50 μl of a 50% slurry) and further incubation for 2 h. The Sepharose beads were pelleted and washed twice with buffer A (0.2% Triton X-100, 150 mM NaCl, 2 mM EDTA, 10 mM Tris-Cl, pH 8.0) and buffer B (0.2% Triton X-100, 450 mM NaCl, 2 mM EDTA, 10 mM Tris-Cl, pH 8.0) and once with buffer C (0.1% Triton X-100, 10 mM Tris-Cl, pH 8.0). Immunoprecipitated material was analyzed by SDS-PAGE and phosphoimaging.

Tryptic Digestion

Tryptic digestion of Gαs and XLαs was performed as described previously (4). Briefly, *in vitro* translated ^{35}S-labeled proteins were incubated for 10 min at 37 °C in TMED (25 mM Tris-Cl, pH 8.0, 10 mM $MgCl_2$, 1 mM EDTA, 1 mM DTT) in the absence or presence of 100 μM GTPγS and then digested for 1 h at 30 °C in the presence of various concentrations (0–0.5 μg/μl) of trypsin (as L-1-tosylamido-2-phenylethyl chloromethyl ketone-treated; Sigma). Digestion was stopped by the addition of SDS sample buffer immediately followed by boiling of the samples for 5 min at 95 °C.

Cholera Toxin-catalyzed ADP-ribosylation

ADP-ribosylation of *in vitro* translated XLαs was performed by a modification of the procedure of Audigier (3). *In vitro* translated α-subunit (20 μl) was mixed with 30 μl of 20 mM HEPES-KOH, pH 7.2, 2 mM $MgCl_2$, and 1 mM EDTA. After 15 min of incubation on ice, 0.5 μl of buffer (20 mM Tris-Cl, pH 8.0, 2 mM $MgCl_2$, 1 mM EDTA, 2 mM DTT, and 11 mM CHAPS) either lacking or containing 43 ng $\beta_1\gamma_2$ was added to the mixture, followed by the immediate addition of 17.5 μl of 0.5 M Na_3PO_4, pH 7.2, 60 mM thymidine, 5 mM ATP, 0.5 mM GTP, 5 mM $MgCl_2$, and 10 μCi of ^{32}P-NAD$^+$ (800 Ci mmol^{-1}, PerkinElmer Life Sciences). Cholera toxin-catalyzed ADP-ribosylation was initiated by the addition of 10 μl of a 0.5 mg/ml solution of cholera toxin A-subunit activated with 25 mM dithiothreitol for 30 min at 37 °C before use. After 60 min of incubation at 30 °C, the reaction mixture was transferred on ice, and 4 volumes of immunoprecipitation buffer (100 mM Tris-HCl, pH 7.5, 100 mM KCl, 5 mM $MgCl_2$, 1% Triton X-100, 1% sodium deoxycholate, 0.5% SDS, and 1 mM PMSF) were added. Insoluble material was removed by centrifugation for 15 min at 14,000 × *g* at 4 °C. The supernatant was incubated overnight with 20 μl of the rabbit antiserum against the C-terminal decapeptide of Gαs and XLαs. Immune complexes were collected using protein A-Sepharose in PBS and analyzed by SDS-PAGE and autoradiography.

Sedimentation Analysis

Sedimentation analysis using sucrose density gradient centrifugation was performed as described previously, with minor changes (5). Briefly, 5 μl of Gαs or XLαs translation medium was incubated for 24 h at 0 °C in the absence or presence of 150 ng of purified unlabeled $\beta_1\gamma_2$ subunits (kindly provided by Dr. Christiane Kleuss, Free University of Berlin) in a buffer containing 50 mM Tris-Cl, pH 8.0, 10 mM EDTA, 1 mM DTT, and 1 mM GDPβS in a final volume of 100 μl. Samples are loaded on top of linear 5–30% sucrose density gradients prepared from solutions also containing 20 mM Tris-Cl, pH 8.0, 1 mM EDTA, and 10 mM β-mercaptoethanol. Gradients were centrifuged for 18 h in a Beckman SW60 rotor at 55,000 rpm at 4 °C with the deceleration setting "slow," and 20 fractions were collected per gradient.

Cell Culture and Transfection

PC12 cells were plated on 15-cm dishes and were grown to subconfluency in Dulbecco's modified Eagle's medium supplemented with 10% horse serum and 5% fetal calf serum at 10% CO_2 and 37 °C. For transient transfection, cells harvested from a subconfluent 15-cm dish after trypsinization were subjected to electroporation (Bio-Rad Gene Pulser; 960 μF, 300 V) in 0.8 ml of phosphate-buffered saline containing 45 μg of circular plasmid DNA. Transfected cells were plated on a 15-cm dish and used 2 days after transfection, with 10 mM sodium butyrate added during the last 16 h to increase the expression of the transgene (6). S49cyc$^-$ cells were grown in flasks to a density of $1 \times 10^5 - 2 \times 10^6$ cells/ml in Dulbecco's modified Eagle's medium (4.5 mg/ml glucose) supplemented with 10% fetal calf serum at 5% CO_2 and 37 °C.

Membrane Preparations

PC12 Membranes—A post-nuclear supernatant (PNS) from PC12 cells was prepared as described (7). For the determination of adenylyl cyclase activity, total membranes were prepared from the PNS by centrifugation (1 h, 100,000 × *g*, 4 °C), resuspended in 20 mM HEPES-KOH, pH 7.2, at 1–2 mg of protein/ml, and snap-frozen in liquid nitrogen.

S49cyc$^-$ Membranes—S49cyc$^-$ cells (50 ml, ~10^6 cells/ml) were pelleted for 5 min at 800 rpm in a Heraeus Megafuge at 4 °C and washed once in ice-cold phosphate-buffered saline containing 0.5 mM PMSF. The cells were resuspended in 10 ml of homogenization buffer (0.25 M sucrose, 1 mM EDTA, 1 mM magnesium acetate, 1 mM DTT, 0.5 mM PMSF, and 10 mM HEPES-KOH, pH 7.4) and pelleted for 5 min at 1600 rpm in a Heraeus Megafuge. The cells were resuspended in 800 μl of homogenization buffer and homogenized by passage through a 22-gauge needle attached to a 1-ml syringe followed by 10 passes through a cell cracker (EMBL, 12-μm clearance). The homogenate was centrifuged for 10 min at 900 × *g* at 4 °C, and the resulting PNS was centrifuged for 1 h in a Beckman TLA45 rotor at 43,000 rpm at 4 °C. The membrane pellet was resuspended in 10 mM HEPES-KOH, pH 7.4, 1 mM DTT to a final protein concentration of 2 mg/ml, snap-frozen in liquid nitrogen, and stored at −80 °C.

[1] The abbreviations used are: PMSF, phenylmethylsulfonyl fluoride; DTT, dithiothreitol; AA-GTP, [^{32}P]GTP-azidoanilide; PNS, post-nuclear supernatant; PAGE, polyacrylamide gel electrophoresis; GDPβS, guanosine 5′-*O*-(2-thiodiphosphate);GTPγS, guanosine 5′-3-*O*-(thio)triphosphate; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonic acid; PACAP, pituitary adenylyl cyclase-activating polypeptide; CGS 21680, 2-(4-[2-carboxyethyl]-phenethylamino)adenosine-5′-*N*-ethylceronamide.

Rat Pituitary Membranes—Adult rats (Wistar strain) were anesthetized with chloroform and killed by cervical dislocation. The pituitaries were removed from the skull and transferred into ice-cold HBS (0.3 M sucrose, 1 mM $MgCl_2$, 1 mM EDTA, 1 mM PMSF, and 10 mM HEPES-KOH, pH 7.4). The pituitaries were homogenized in 5 volumes of HBS using a motor-driven glass-Teflon homogenizer at 1000 rpm. The homogenate was centrifuged for 10 min at 1000 × *g* at 4 °C. The resulting PNS was centrifuged for 1 h at 100,000 × *g* at 4 °C. The membrane pellet was resuspended to a protein concentration of 2 mg/ml in 10 mM Tris-Cl, pH 7.4, snap-frozen in liquid nitrogen, and stored at −80 °C.

Reconstitution of S49cyc⁻ Membranes with in Vitro Translated Gαs and XLαs

Reconstitution of the S49cyc⁻ membranes was performed as described previously (8). Briefly, 1 volume of the *in vitro* translation mixture was mixed with 1 volume of S49cyc⁻ membranes (2 mg of protein/ml) and incubated for 30 min at 30 °C, followed by centrifugation for 30 min in a Beckman TLA45 rotor at 43,000 rpm at 4 °C. The resulting membrane pellet was resuspended to a protein concentration of 1 mg/ml in 10 mM Hepes, pH 7.4, 1 mM DTT. The reconstituted membranes were kept on ice and immediately used for the adenylyl cyclase assay.

Adenylyl Cyclase Assay

The activity of adenylyl cyclase was determined by the method of Salomon (9) with minor modifications as follows. The activity of the S49cyc⁻ membranes (20 μg of protein) was assayed in a final volume of 100 μl containing 100 μM ATP, 10 mM $MgCl_2$, 500 μM cAMP, 10 mM creatine phosphate, 0.5 mg/ml creatine kinase, 1 mM DTT, 25 mM Tris-Cl, pH 8.0, 2 μCi of [α-^{32}P]ATP, and GTP, GTPγS, and isoproterenol as indicated in the figure legend. Reactions were carried out for 30 min at 30 °C.

The adenylyl cyclase activity in PC12 membranes was assayed in the presence of 165 μM ATP, 5 mM $MgCl_2$, 10 mM creatine phosphate, 0.5 mg/ml creatine kinase, 0.5 mM DTT, 3 μM GTP, 1 mg/ml bovine serum albumin, 3 mM HEPES-KOH, pH 7.2, and 1 μCi [α-^{32}P]ATP. Reactions were carried out for 30 min at 30 °C.

Photoaffinity Labeling of α-Subunits with [^{32}P]GTP-azidoanilide (AA-GTP)

Photoaffinity Labeling Using Pituitary Membranes—AA-GTP labeling was performed essentially as described previously (10). Briefly, membranes (100 μg of protein) were pelleted (10 min, 14,000 × *g*) and resuspended in 30 μl of incubation buffer (0.2 mM EDTA, 10 mM $MgCl_2$, 60 mM NaCl, 100 mM HEPES-KOH, pH 7.5, 2 mM benzamidine, and 2–200 μM GDP). Samples then received 10 μl without or with the indicated receptor agonists (see text under "Results" and the legend to Fig. 8), were incubated for 3 min at 30 °C, received 20 μl of [α-^{32}P]AA-GTP (1.4×10^6 cpm/μl), and were further incubated for 1–10 min at 30 °C. Samples were transferred on ice and centrifuged for 5 min at 14,000 × *g* at 4 °C. The membranes were rapidly resuspended in 60 μl of labeling buffer (0.1 mM EDTA, 5 mM $MgCl_2$, 30 mM NaCl, 50 mM HEPES-KOH, pH 7.5, 1 mM benzamidine, 2 mM glutathione) and immediately irradiated at 265 nm for 10–15 s at 4 °C. The samples were centrifuged as above, and the membranes were solubilized in 160 μl of immunoprecipitation buffer (1% Nonidet P40, 1% sodium deoxycholate, 0.5% SDS, 150 mM NaCl, 1 mM DTT, 1 mM EDTA, 0.2 mM PMSF, 10 μg/ml aprotinin, and 10 mM Tris-Cl, pH 7.4) for 10–15 min on ice. Insoluble material was removed by centrifugation as above, and the supernatant was used for immunoprecipitation using an antiserum (5 μl) against the common C-terminal decapeptide of Gαs and XLαs. The samples were incubated with the antibody overnight at 4 °C followed by addition of protein A-Sepharose (60 μl of a 10% slurry) and further incubation for 2 h at 4 °C. The Sepharose beads were pelleted and washed twice with buffer A (1% Nonidet P40, 0.5% SDS, 600 mM NaCl, 50 mM Tris-Cl, pH 7.4) and once with buffer B (300 mM NaCl, 10 mM EDTA, 100 mM Tris-Cl, pH 7.4). Immunoprecipitated material was analyzed by SDS-PAGE and phosphoimaging.

Photoaffinity Labeling Using PC12 Cell Membranes—Membranes were washed once with 50 mM Hepes-NaOH, pH 7.4, and the membrane proteins (50 μg) were subjected to photoaffinity labeling with [α-^{32}P]AA-GTP as described above in the presence of GTP or ATP as indicated in the figure and with the following modifications. (i) The incubation buffer was 1 mM $MgCl_2$ and 50 mM NaCl, and GDP was omitted; (ii) no receptor agonist was added; (iii) incubation was for 10 min.

SDS-PAGE

SDS-PAGE (7.5 or 10% gels) and immunoblotting was performed according to standard procedures. Dried gels were either autoradiographed followed by densitometric scanning or exposed to phosphoimager plates, and the intensity of the bands was determined using a Fuji phosphoimager and MacBAS software.

Identification of Mouse XLαs—The IMAGE cDNA clone 1499201 was identified by BLAST searching with the rat and human XLαs sequences. It was subsequently obtained from Research Genetics and completely sequenced. The clone encodes the full-length mouse XLαs protein. The sequence was submitted to GenBank™ (accession number AF116268).

RESULTS

Construction of an XLαs cDNA for in Vitro Transcription/Translation—The originally published sequence of XLαs contains a point mutation in the αs-part (1) that leads to a single amino acid exchange (L → P) at position 519 (corrected translational start (2); amino acid residue 650 of originally published sequence (1)). This point mutation was not found in XLαs mRNAs isolated from rat pituitary or PC12 cells as revealed by reverse transcription-polymerase chain reaction analysis (data not shown), suggesting that it reflects an aberration from the physiological XLαs sequence introduced in the course of construction of the cDNA library from which the original XLαs cDNA was isolated (1). We therefore constructed an XLαs cDNA, referred to as XLαs-wt, in which this point mutation in the αs-part of XLαs was reversed to the physiological sequence. This cDNA was used in the *in vitro* transcription/translation experiments performed in this study.

In Vitro Translation of XLαs and Gαs—*In vitro* translation in the rabbit reticulocyte lysate of a Gαs mRNA, obtained by *in vitro* transcription of a Gαs cDNA, gave rise to a 46-kDa labeled band (Fig. 1, *lane 1*). Translation of the *in vitro* transcribed XLαs-wt mRNA generated a major band of M_r 94,000 and additional bands of lower apparent molecular weight (Fig. 1, *lane 4*). Immunoprecipitation of the translation products using an antiserum against the C-terminal decapeptide of Gαs showed that XLαs (Fig. 1, *lane 6*) contains the same C-terminal epitope as Gαs (Fig. 1, *lane 3*). Upon immunoprecipitation using an antiserum against the EPAA repeats in the N-terminal region of the XL domain (1), referred to as anti-XL antibody, only XLαs mRNA-derived translation products of $M_r \leq$ 94,000 (Fig. 1, *lane 5*), but no Gαs mRNA-derived translation products (Fig. 1, *lane 2*), were obtained. This showed that (i) the anti-XL antibody was indeed specific for the XL domain of XLαs, and (ii) the major XLαs mRNA-derived translation products of $M_r < 94{,}000$ (Fig. 1, *lanes 4* and *5*) were truncated in the αs domain. *In vitro* translated Gαs and XLαs, as shown in Fig. 1, *lanes 1* and *4*, were used in the subsequent experiments.

Trypsin Resistance of the αs Domain of XLαs in the GTP-bound State—The activation of Gα subunits, *i.e.* the replacement of GDP by GTP, is associated with a conformational change that is reflected in their differential sensitivity to degradation by trypsin (3). As shown in Fig. 2, the presence of GTPγS affected the tryptic digestion of *in vitro* translated XLαs in the same way as it was described previously for Gαs (3). In the absence of GTPγS, XLαs, like Gαs, was fully degraded by increasing concentrations of trypsin. In contrast, for both Gαs and XLαs in the presence of GTPγS, 37–35-kDa fragments were protected from tryptic digestion. This strongly suggests that (i) XLαs binds GTPγS and (ii) upon GTPγS binding, the αs domain undergoes the same conformational change as Gαs.

Formation of an XLαs-βγ Heterotrimer—In XLαs, the exon 1-encoded portion of Gαs is replaced by the XL domain (1). The exon 1-encoded portion of Gαs is involved in the interaction of Gαs with the βγ complex (5, 11). Interestingly, the C-terminal region of the XL domain shows a high degree of homology to the

exon 1-encoded portion of Gαs and other types of Gα subunits (1) (Fig. 3, *A* and *B*). In particular, the residues that are known to directly contact the βγ dimer (11, 12) are conserved not only between rat, mouse, and human XLαs (Fig. 3*B*) but also between XLαs and the various types of Gα subunits (1). It is therefore possible that XLαs, like Gαs, binds to the βγ complex. To investigate this issue, we used (i) ADP-ribosylation by cholera toxin to detect even weak or transient XLαs-βγ interactions and (ii) sucrose density gradient centrifugation to search for stable heterotrimer formation.

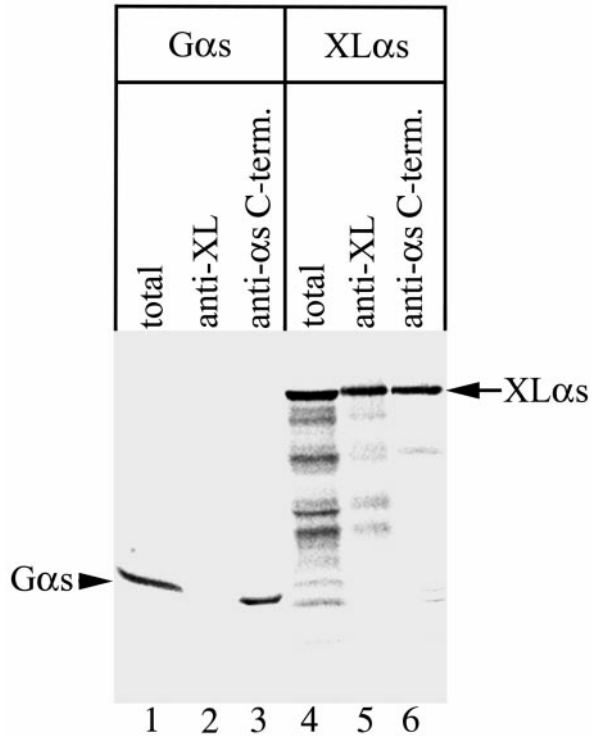


FIG. 1. **Characterization of the translation products obtained from Gαs and XLαs mRNAs.** Transcription products were generated from linearized plasmids (*lanes 1–3*, CDM8-Gαs; *lanes 4–6*, CDM8-XLαs-wt) and translated in the reticulocyte lysate in the presence of [^{35}S]methionine/cysteine. The ^{35}S-labeled translation products were subjected to SDS-PAGE either before (total) or after immunoprecipitation using either the anti-XL antiserum or the antiserum against the common C-terminal decapeptide of Gαs and XLαs (*anti-αs C-term.*) and visualized by phosphoimaging. *Arrow*, full-length XLαs; *arrowhead*, Gαs. The results shown are representative of three independent experiments.

Effect of βγ Subunits on the ADP-ribosylation of XLαs by Cholera Toxin—The ability of activated cholera toxin to catalyze the ADP-ribosylation of purified (*i.e.* monomeric), native Gαs (13) as well as of *in vitro* translated Gαs (3) is very poor. However, in either case, the addition of βγ subunits before the addition of activated cholera toxin greatly increases the extent of ADP-ribosylation (3, 13). ADP-ribosylation by cholera toxin is an irreversible modification and is therefore a very sensitive method for detecting even weak or transient interactions of *in vitro* translated Gαs and XLαs with βγ subunits. In the absence of βγ subunits, *in vitro* translated XLαs was indeed found to be a poor substrate for cholera toxin catalyzed ADP-ribosylation (Fig. 4, *lane 4*), whereas the addition of exogenous βγ subunits resulted in a >2.2-fold increase in the labeling of both the *in vitro* translated XLαs (Fig. 4, *lane 5*) as well as the endogenous Gαs present in the reticulocyte lysate (Fig. 4, *lanes 2* and *5*). The labeling of XLαs and Gαs in the absence of added βγ subunits (Fig. 4, *lanes 1* and *4*) is most likely due to the presence of some endogenous βγ in the reticulocyte lysate (14).

Effect of βγ Subunits on the Sedimentation Behavior of XLαs—To look for stable heterotrimer formation, we examined the effects of purified βγ subunits on the sedimentation behavior of *in vitro* translated XLαs and, for comparison, Gαs, using sucrose density gradients. In the absence of βγ subunits, XLαs (Fig. 5*B*, *open circles*) and Gαs (Fig. 5*A*, *open circles*) sedimented at a rate corresponding to a calculated molecular mass of 78 and 46 kDa, respectively, and hence a monomeric state, as revealed by comparison to the 68-kDa hemoglobin tetramer (Fig. 5, *bars*). In the presence of βγ subunits, XLαs (Fig. 5*B*, *filled circles*) and confirming previous results (5), Gαs (Fig. 5*A*, *filled circles*), sedimented at a faster rate. However, in the presence of βγ subunits, XLαs (Fig. 5*B*, *filled circles*) sedimented at a slower rate than Gαs (Fig. 5*A*, *filled circles*), although the total molecular mass of an XLαs-βγ heterotrimer is greater than that of the Gαs-βγ heterotrimer. This suggests that XLαs, upon contact with the βγ dimer, undergoes a conformational change, which alters its sedimentation behavior.

Activation of Adenylyl Cyclase by a GTPase-deficient Mutant of XLαs—To determine whether XLαs is capable of activating adenylyl cyclase, we constructed a GTPase-deficient mutant of XLαs, XLαsQ548L. This mutation corresponds to the Q227L

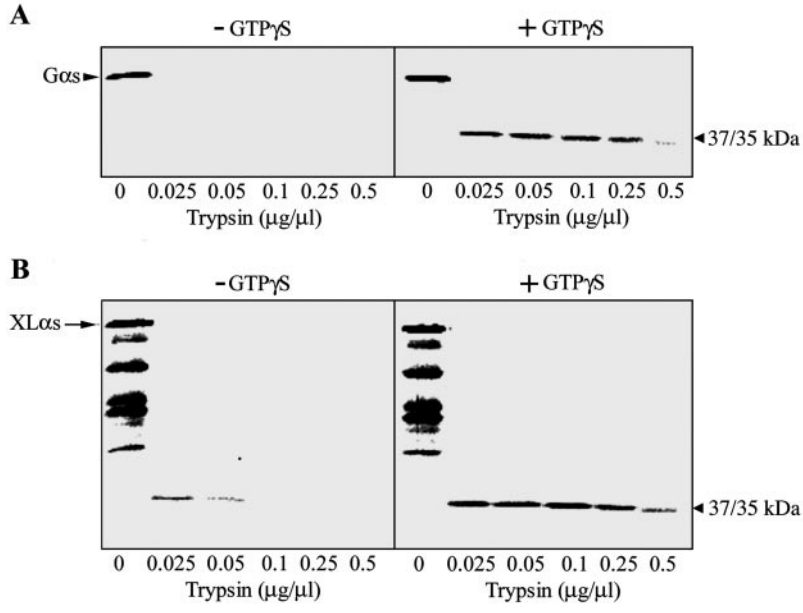


FIG. 2. **Differential sensitivity of XLαs to digestion by trypsin in the absence or presence of GTPγS.** Transcription products were generated from linearized plasmids (CDM8-Gαs or CDM8-XLαs-wt) and translated in the reticulocyte lysate in the presence of [^{35}S]methionine/cysteine. The translation products, either ^{35}S-labeled Gαs (A) or XLαs (B), were incubated for 10 min at 37 °C in the absence (−*GTPγS*) or presence (+*GTPγS*) of 100 μM GTPγS and then digested for 60 min at 30 °C with the indicated concentrations of trypsin. The ^{35}S-labeled products were subjected to SDS-PAGE and visualized by phosphoimaging. *Arrow*, full-length XLαs; *arrowhead*, full-length Gαs; *triangles* show the position of the tryptic fragments (37–35 kDa) that are protected from further digestion in the presence of GTPγS. The results shown are representative of three independent experiments.

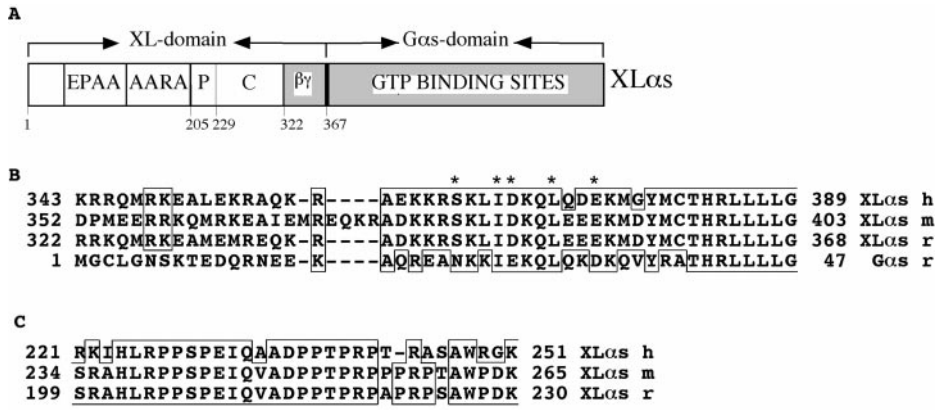


FIG. 3. **Sequence comparison of two highly conserved regions in the XL domain of human, mouse, and rat XLαs.** *A*, domain organization of rat XLαs (1). *EPAA*, region containing the EPAA repeats; *ARAA*, region containing the AARA repeats; *P*, proline-rich region; *C*, cysteine-rich region; *βγ*, region containing the putative βγ binding site; *numbers* refer to the corrected translational start of XLαs (2). *B* and *C*, comparison of the C-terminal amino acid sequence of the XL domain of human (*h*, Ref. 25), mouse (*m*, Ref. 31 and GenBank™ accession number AF116268), and rat (*r*, Refs. 1 and 2) XLαs with the corresponding N-terminal sequence of rat Gαs (*B*) and of the proline-rich region of XLαs across the three species (*C*). Boxes indicate conserved residues; *asterisks* in *B* indicate residues that are known to directly contact the βγ complex (11, 30).

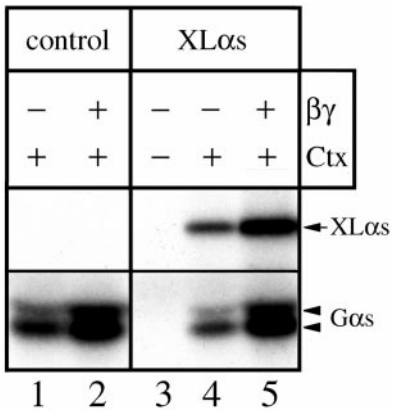


FIG. 4. **Effect of βγ subunits on the ADP-ribosylation of XLαs by cholera toxin.** Reticulocyte lysate either lacking (*lanes 1* and *2*) or containing (*lanes 3–5*) *in vitro* translated XLαs was incubated for 60 min at 30 °C with ^{32}P-NAD$^+$ in the absence (−) or presence (+) of purified $\beta_1\gamma_2$ subunits and cholera toxin (*Ctx*) as indicated, followed by immunoprecipitation with the antiserum against the C-terminal decapeptide of Gαs and XLαs. Immune complexes were analyzed by SDS-PAGE and autoradiography. The results shown are representative of three independent experiments.

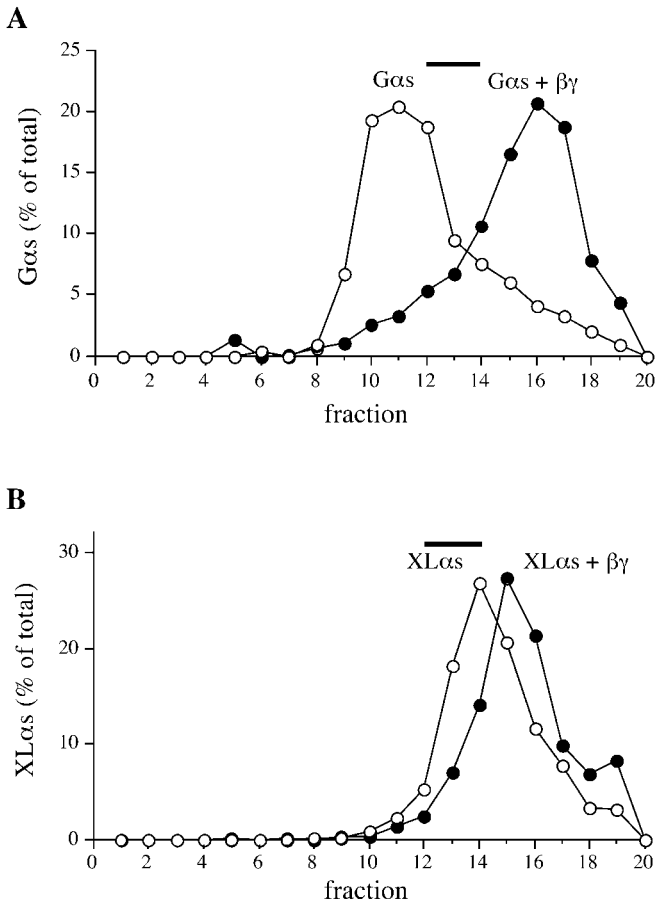


FIG. 5. **Effect of βγ subunits on the sedimentation behavior of XLαs.** Transcription products were generated from linearized plasmids (CDM8-Gαs or CDM8-XLαs-wt) and translated in the reticulocyte lysate in the presence of [^{35}S]methionine/cysteine. The translation products, either ^{35}S-labeled Gαs (*A*) or XLαs (*B*), were incubated in the presence of GDPβS and in the absence (*open circles*) or presence (*filled circles*) of purified unlabeled βγ subunits, followed by centrifugation on a linear 5–30% sucrose gradient. An aliquot of each fraction (fraction *1* = top of gradient) was subjected to SDS-PAGE, and the ^{35}S-labeled Gαs (*A*) or XLαs (*B*) was visualized by phosphoimaging and quantified. The amount of ^{35}S-labeled Gαs (*A*) or XLαs (*B*) recovered in each fraction is expressed as percent of the total (sum of the values of all fractions). The *bar* indicates the position of the hemoglobin tetramer (68 kDa), derived from the rabbit reticulocyte lysate, which served as an internal molecular mass standard. The results shown are representative of four independent experiments.

mutation in Gαs, which is analogous to the Q61L mutant of p21^{ras}. In Gαs, this mutation leads to a 100-fold reduction in the rate constant of GTP hydrolysis (15). Hence, XLαsQ548L should be constitutively activated.

Immunoblotting of PC12 cell membranes using the antibody against the common C-terminal decapeptide of Gαs and XLαs (Fig. 6, *bottom panel*) indicated that transient transfection with the cDNA for XLαs or XLαs-Q548L substantially increased the amount of XLαs above the endogenous level. Membranes of transfected and untransfected PC12 cells were then analyzed for adenylyl cyclase activity (Fig. 6, *top panel*). Cells transfected with the activated form of XLαs, XLαs-Q548L, showed a massive increase in adenylyl cyclase activity as compared with wild type or mock-transfected cells. Transfection of XLαs resulted in only a small increase in adenylyl cyclase activity, presumably because XLαs was predominantly in the GDP-bound state and therefore inactive toward adenylyl cyclase. The addition of forskolin to the membranes from wild type, mock-transfected, and XLαs-transfected PC12 cells increased adenylyl cyclase activity to the level observed with membranes from XLαs-Q548L-transfected cells (data not shown), showing that adenylyl cyclase in the former membranes could be acti-

vated. When HeLa (rather than PC12) cells were transiently transfected with XLαs-Q548L, they also showed an increase (4-fold) in adenylyl cyclase activity as compared with mock-transfected cells (data not shown).

Receptor Activation of Gαs, but Not XLαs, in Reconstituted S49cyc⁻ Membranes—S49cyc⁻ cells are deficient in Gαs (16–18) as well as XLαs, as revealed by immunoblotting (data not shown), but still express the β2-adrenergic receptor, βγ subunits, and the adenylyl cyclase. Given that XLαs can activate adenylyl cyclase (Fig. 6), we used S49cyc⁻ cells to study the signal transduction properties of XLαs. As reported previously (5, 8), adenylyl cyclase activity of S49cyc⁻ membranes could be stimulated upon the addition of isoproterenol, a β2-adrenergic receptor agonist, when the membranes had been reconstituted with *in vitro* translated Gαs (Fig. 7*A*). Compared with the addition of GTP alone, adenylyl cyclase activation upon receptor stimulation was increased to about half of the maximal value obtained in the presence of GTPγS (Fig. 7*A*).

Because Gαs and XLαs contain the same C-terminal domain and may therefore couple to the same type of receptor (19–21), we investigated whether XLαs, like Gαs, was able to restore receptor stimulation of adenylyl cyclase activation in reconstituted S49cyc⁻ membranes. In contrast to Gαs (Fig. 7*A*), XLαs mediated only a small, barely significant increase in adenylyl cyclase activation upon receptor stimulation, as compared with the addition of GTP or GTPγS alone (Fig. 7*B*). We therefore conclude that, in this *in vitro* system, XLαs couples to the β2-adrenergic receptor much less efficiently than Gαs, if at all. Consistent with these *in vitro* findings, we observed, in comparison with untransfected PC12 cells, an increased adenylyl cyclase activity upon the addition of 100 μM CGS 21680 (adenosine 2A receptor agonist) in membranes of Gαs-transfected, but not XLαs-transfected, PC12 cells (data not shown).

Receptor Activation of Gαs, but not XLαs, in Pituitary Membranes—Given that XLαs couples only weakly to the β2 adrenergic receptor in reconstituted S49cyc⁻ membranes, we investigated whether or not XLαs is capable of coupling to a G protein-coupled receptor *in vivo*. For this purpose, we used photoaffinity labeling of G protein α subunits with AA-GTP (10). When combined with immunoprecipitation using antibodies specific for a given G protein α subunit, such photoaffinity labeling is a powerful tool to identify the specific G protein α subunit activated by a given receptor.

First, we investigated whether XLαs binds AA-GTP with the same affinity as Gαs. PC12 cell membranes were incubated in the presence of AA-GTP with increasing concentrations of either unlabeled GTP or ATP. As shown in Fig. 8*A*, binding of

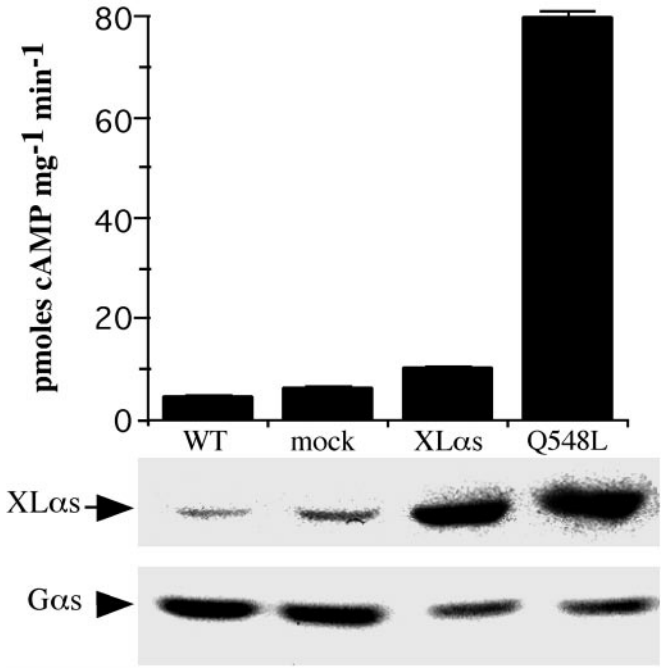


FIG. 6. **Activation of adenylyl cyclase by a GTPase-deficient mutant of XLαs (XLαs-Q548L).** Wild type PC12 cells (*WT*) or PC12 cells transfected with the CDM8 vector without insert (*mock*), with CDM8-XLαs (*XLαs*), or with CDM8-XLαs-Q548L (*Q548L*) were used. *Top*, adenylyl cyclase activity in total membranes (15 μg of protein/assay). The mean of duplicate determinations is shown. *Error bars* indicate the variation of the individual values from the mean; for some conditions, these are too small to be seen. *Bottom*, immunoblot of a similar membrane preparation (30 μg of protein/*lane*) using the antiserum against the common C-terminal decapeptide of Gαs and XLαs. The results shown are representative of three independent experiments.

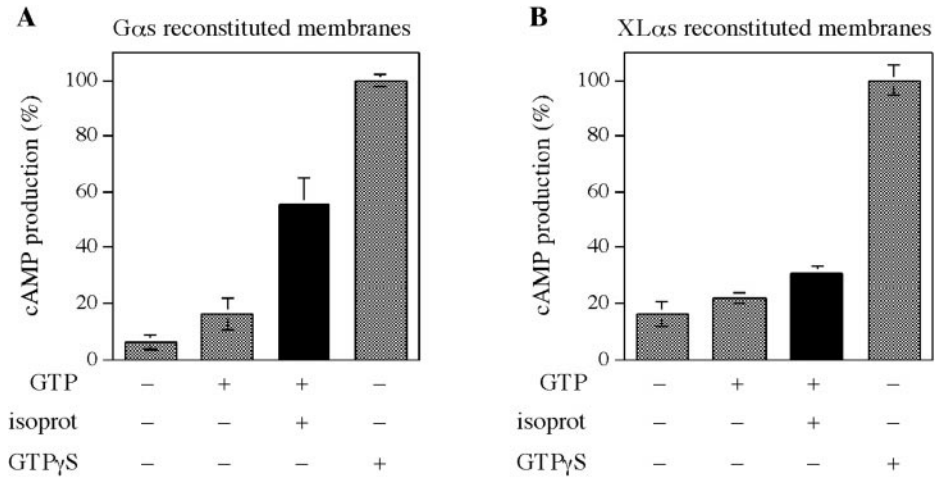


FIG. 7. **Differential effects of Gαs and XLαs on the receptor-mediated adenylyl cyclase activation in S49cyc⁻ membranes.** Transcription products were generated from linearized plasmids (CDM8-Gαs or CDM8-XLαs-wt) and translated in the reticulocyte lysate. S49cyc⁻ membranes were reconstituted either with reticulocyte lysate alone or with reticulocyte lysate containing *in vitro* translated Gαs (*A*) or XLαs (*B*). Reconstituted membranes were incubated in the presence of 100 μM GTP, 10 μM (−)isoproterenol (*isoprot*), or 10 μM GTPγS, as indicated. cAMP formation was determined in triplicate. Note that the mean values obtained with membranes that had been reconstituted with the reticulocyte lysate only (0.86 ± 0.28 pmol of cAMP mg^{-1} protein min^{-1}) were subtracted from the mean values obtained with membranes that had been reconstituted with reticulocyte lysate containing *in vitro* translated Gαs or XLαs. The mean value of the GTPγS condition (*A*, 8.8 ± 0.2 pmol of cAMP mg^{-1} protein min^{-1}; *B*, 6.2 ± 0.3 pmol of cAMP mg^{-1} protein min^{-1}) is arbitrarily set to 100, and the other mean values are expressed relative to this. *Bars* indicate the error of the final value. The results shown are representative of three independent experiments.

AA-GTP to XLαs could be competed with increasing concentrations of GTP but not ATP. The comparison of the competition profiles obtained for XLαs and Gαs shows that XLαs binds to AA-GTP with virtually the same affinity as Gαs.

Second, we determined whether AA-GTP binding to XLαs could be enhanced by the activation of known Gαs-coupled receptors. For this purpose, we used rat pituitary membranes because the expression level of XLαs was highest in this tissue (32). Fig. 7*B* shows that the receptor for pituitary adenylyl cyclase-activating polypeptide (PACAP) was capable of activating Gαs, as indicated by an increased incorporation of AA-GTP in the presence of PACAP but not XLαs.

Other Gαs-coupled receptors in the pituitary, *i.e.* that for vasoactive intestinal polypeptide (VIP, used at 1–10 μM) and corticotropin releasing factor (CRF, used at 10 μM) as well as the adenosine 2A receptor of PC12 cells (22, 23) (stimulated by 10 μM CGS 21680), were also found to activate Gαs but not XLαs (data not shown). Changing the experimental conditions, *e.g.* by the addition of various concentrations of GDP to suppress the basal rate of guanine nucleotide exchange, or using different labeling times also did not reveal any significant receptor stimulation of guanine nucleotide exchange on XLαs (data not shown).

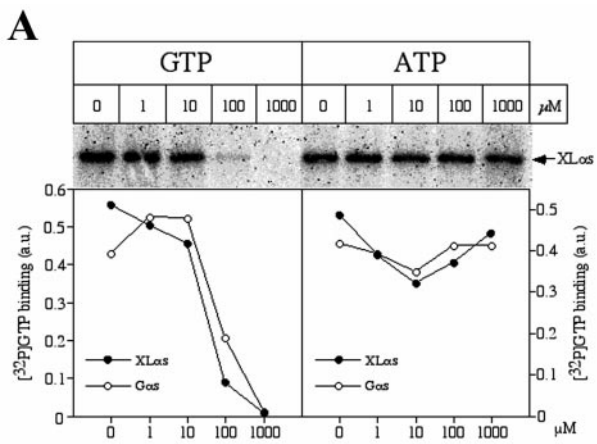
DISCUSSION

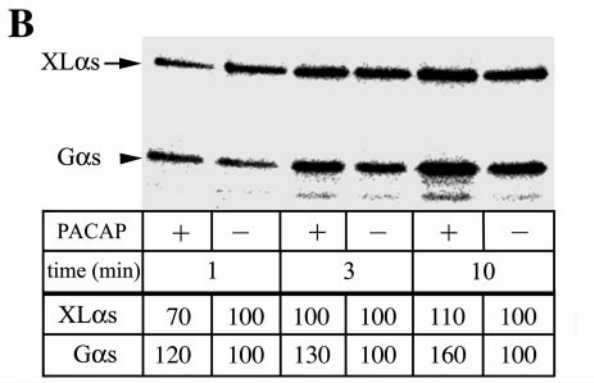
Our study shows that XLαs shares many, but not all, functional properties of Gαs. XLαs (i) forms a heterotrimer with βγ subunits, (ii) binds GTP and undergoes a conformational change upon GTP binding, and (iii) activates, when in the GTP state, adenylyl cyclase. However, XLαs does not appear to be activated by known Gαs-coupled receptors.

Binding to the βγ Dimer—We used two methods to demonstrate the ability of XLαs to interact with βγ subunits: (i) ADP-ribosylation by cholera toxin, which in the case of Gαs is greatly promoted by its heterotrimeric state (3, 13, 24), and (ii) sucrose density gradient centrifugation. The addition of βγ subunits to *in vitro* translated XLαs increased its ADP-ribosylation as well as its sedimentation rate in sucrose density gradients, indicating that XLαs forms heterotrimers with βγ subunits. Remarkably, however, comparison of the sedimentation behavior of Gαs and XLαs in the presence of βγ dimers revealed that XLαs sedimented more slowly than Gαs, although the molecular mass of an XLαs-βγ heterotrimer is greater than that of a Gαs-βγ heterotrimer. The observations that (i) heterotrimeric rather than monomeric Gαs (3, 13, 24) and XLαs (Fig. 4) is a substrate for cholera toxin-catalyzed ADP-ribosylation and (ii) XLαs, like Gαs, undergoes ADP-ribosylation by cholera toxin *in vivo* (1) strongly suggest that XLαs exists in the heterotrimeric state *in vivo*. It is therefore likely that the sedimentation of XLαs observed in the presence of βγ subunits reflected that of heterotrimeric XLαs rather than that of a monomeric XLαs that had adopted a different conformation upon transient contact with the βγ dimer. This in turn suggests that the heterotrimerization of XLαs with the βγ dimer is associated with a stable conformational change of XLαs toward a more rod-like state, resulting in a slower sedimentation of the XLαs-βγ heterotrimer than the Gαs-βγ heterotrimer. The proline-rich region of the XL domain of XLαs (1) (Fig. 3, *A* and *C*) could serve as a hinge for this conformational change. It is worth noting that this region, like the C-terminal region of the XL domain implicated in βγ binding (1) (Fig. 3, *A* and *B*) is more highly conserved in XLαs of various species (Fig. 3*C*) than other regions of the XL domain (for a comparison of human and rat XLαs, see Hayward *et al.* (25)).

The ability of XLαs to form a heterotrimeric complex with βγ subunits also has implications for the observations reported in the preceding paper (32) that immunoreactive and ADP-ribosylatable XLαs show a distinct distribution upon subcellular fractionation, whereas this is not the case for Gαs. Specifically, the subpopulation of XLαs molecules that were poorly, if at all, ADP-ribosylated by cholera toxin were preferentially recovered in fractions containing plasma membrane, whereas the subpopulation that was well ADP-ribosylated was preferentially recovered in fractions containing Golgi membranes and certain subdomains of the plasma membrane (see preceding paper (32)). This raises the possibility of an interplay between βγ binding to XLαs and its subcellular localization, which does not take place for Gαs.

GTP Binding and Functional Units—Photoaffinity labeling showed that XLαs binds GTP. The αs domain of XLαs is likely folded in the same way as Gαs and, upon GTP binding, undergoes the same conformational change as Gαs, since the pattern





PACAP	+	−	+	−	+	−
time (min)	1		3		10	
XLαs	70	100	100	100	110	100
Gαs	120	100	130	100	160	100

FIG. 8. **Differential effect of PACAP receptor stimulation on GTP binding to XLαs and Gαs.** *A*, PC12 cell membranes were incubated for 10 min with [α-^{32}P]GTP-azidoanilide in the presence of the indicated concentrations of unlabeled GTP or ATP, followed by photocross-linking. XLαs and Gαs were immunoprecipitated from the solubilized membranes using an antiserum against the common C-terminal decapeptide, and immunecomplexes were analyzed by SDS-PAGE followed by autoradiography. *Top*, XLαs region of the autoradiogram. *Bottom*, quantitation of the ^{32}P-labeled XLαs (*filled circles*) and Gαs (*open circles*) by densitometric scanning; *a.u.*, arbitrary units. To facilitate comparison with XLαs, the Gαs values (sum of two Gαs bands) were divided by 2.5 (GTP) and 2.2 (ATP). *B*, rat pituitary membranes were incubated for the indicated times with [α-^{32}P]GTP-azidoanilide in the presence of 10 μM GDP in the absence (−) or presence (+) of 1 μM PACAP, followed by photocross-linking. XLαs and Gαs were immunoprecipitated from the solubilized membranes using an antiserum against the common C-terminal decapeptide, and immune complexes were analyzed by SDS-PAGE and phosphoimaging (*top*). *Bottom*, quantitation of the ^{32}P-labeled XLαs and Gαs. For each period of incubation, the value obtained in the absence (−) of PACAP was arbitrarily set to 100, and the value obtained in the presence (+) of PACAP was expressed relative to this. The results shown are representative of five independent experiments.

of the trypsin-resistant fragments was identical for Gαs and XLαs. This implies that the XL domain does not significantly affect the folding of the αs domain of XLαs and its conformational change upon GTP binding.

Together, our data suggest that XLαs is composed of two independent units that are connected by the proline-rich region of the XL domain (Fig. 3*A*), a putative hinge (see above). Although encoded by the XL exon, the cysteine-rich region of the XL domain (Fig. 3*A*) (1), which is palmitoylated and hence contributes to membrane attachment of XLαs (see Ugur and Jones (26) and the preceding paper (32)) and the C-terminal region of the XL domain, which is implicated in βγ binding (Fig. 3*A*), are suggested to belong, together with the αs domain, to one unit that exerts all Gαs-type functions of XLαs. The other unit, whose function remains to be elucidated, comprises the alanine-rich, repetitive region of the XL domain (Fig. 3*A*) (1), which does not show a high sequence conservation between rat, mouse, and human XLαs (data not shown; for a comparison of human and rat XLαs, see Hayward *et al.* (25)).

Activation of Adenylyl Cyclase—Overexpression of a constitutively active mutant of XLαs in PC12 cells led to a massive increase in cAMP production, showing that XLαs-GTP, via its Gαs-type unit, is capable of activating adenylyl cyclase. Hence, in neuroendocrine cells, two distinct G protein α-subunits, Gαs and XLαs, converge onto the same effector system, adenylyl cyclase.

Receptor Coupling—The C-terminal domain of a Gα subunit is not only critical for the interaction with a receptor (27–29) but also a determinant of the specificity of G protein-receptor interaction (19–21). Given that (i) the C-terminal domain of XLαs is identical to Gαs and (ii) XLαs is able to form a heterotrimeric complex with βγ subunits, which in the case of typical Gα subunits is a prerequisite for their interaction with a receptor, one might have expected XLαs to become activated by Gαs-coupled receptors. Remarkably, however, using various experimental approaches including reconstitution of S49cyc$^-$ membranes, AA-GTP photoaffinity labeling, and transfection of cells, all of which allowed the detection of receptor-mediated activation of Gαs, we have been unable to obtain conclusive evidence for receptor-mediated activation of XLαs.

It is possible that XLαs is not subject to activation by any receptor. However, the apparent lack of receptor-mediated activation of XLαs may also reflect a novel, as yet not understood, mode of activation (rather than the wrong choice of receptors examined in this study). If so, it is tempting to speculate that the presence of the alanine-rich, repetitive region of the XL domain, which is located N-terminal to the Gαs-type unit, provides another level of regulation, for example by sterically hindering in a reversible manner the coupling of the αs domain of XLαs to a receptor.

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