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

II. SIGNAL TRANSDUCTION PROPERTIES*

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) succrose 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-de-}

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Characterization of XLas

For construction of CDM8-XLas in vitro, the plasmid pVL-1393-Gas-Q227L (kindly provided by Dr. Peter Gierschik, University of Ulm), which encodes the entire human Gas protein sequence, was cut with Eco47III and NasiI. The resulting 667-nucleotide fragment, corresponding to amino acid residues 165–386 of Gas, was cloned into the Eco47III and NasiI sites of CDM8-XLas, resulting in a predicted XLas 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-Gas-Q227L and the rat CDM8-XLas do not cause any amino acid sequence variation between the two species (3).

**In Vitro Transcription**

After linearization by Ndel, 10 μg of each plasmid (CDM8-Gas and CDM8-XLas-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 nuclelease-free distilled H2O. 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 nucleic-acid-treated reticulocyte lysate following the manufacturer's instructions. Briefly, a typical translation mixture contained 35 μl of the reticulocyte lysate, 7 μl of nuclelease-free distilled H2O, 1 μl of RNase Inhibitor (40 units/μl), 1 μl of the amino acid mixture containing, without methionine, 4 μl of the 1-[35S]Met/Cys-ProMixTM (Amersham Pharmacia Biotech, 1000 Ci mmol−1), and 2 μl of a 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 S49cyc2 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 Gas and XLas 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), 3 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 rpm in a Heraeus Megafuge at 4 °C and washed once in ice-cold phosphate-buffered saline containing 0.5 mM PMSF. The supernatant was mixed with two volumes of immunoprecipitation buffer (MBI Fermentas), 3 μl of the rabbit antiserum against the C-terminal 47III and NsiI containing 20 μg of circular plasmid DNA. Transfected cells were plated on a 15-cm cell culture dish and used 2 days after transfection, with 10 μg sodium butyrate added during the last 16 h to increase the expression of the transgene (6). S49cyc2 cells were grown in flasks to a density of 1 x 10^6 – 2 x 10^6 cells/ml in Dulbecco's modified Eagle's medium (4.5 mg/ml glucose) supplemented with 10% fetal calf serum at 5% CO2 at 37°C.

**Tryptic Digestion**

Tryptic digestion of Gas and XLas was performed as described previously (4). Briefly, in vitro translated 35S-labeled proteins were incubated for 10 min at 37 °C in TMED (25 mM Tris-Cl, pH 8.0, 10 mM MgCl2, 1 mM EDTA, 1 mM DTT) in the absence or presence of 100 μM GTP[S] and then digested for 1 h at 30 °C in the presence of various concentrations (0.05–0.5 μg/μl) of trypsin (as 1:1-tyrosylamido-2-phenyl-

1 The abbreviations used are: PMSF, phenylmethylsulfonyl fluoride; DTT, dithiothreitol; AA-GTP, [32P]GTP-azidoanilide; PNS, post-nuclear supernatant; PAGE, polyacrylamide gel electrophoresis; GDS, guanosine 5′-O-(2-thiodiphosphate); GTPγS, guanosine 5′-3′-O-(thio)-triphosphate; CHAPS, 3-(3-cholamidopropyl)dimethylammonio)-1-propanesulfonic acid; PACAP, pituitary adenyl cyclase-activating polypeptide; CGS 21860, 2,4-(4-carboxyethyl)-phenethylamine-adenosine-5′-N-ethylcholomethane; 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 XLas was performed by a modification of the procedure of Audiger (3). In vitro translated α-subunit (20 μl) was mixed with 30 μl of 20 mM HEPES-KOH, pH 7.2, 2 mM MgCl2, 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 MgCl2, 1 mM EDTA, 2 mM DTT, and 11 mM CHAPS) either lacking or containing 43 ng βγ subunits was added to the mixture, followed by the immediate addition of 1.75 μl of 0.5 mM NaPO4, pH 7.2, 50 mM KCl, 1 mM MgCl2, 5 mM GTP, 43 ng βγ 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 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.

**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.

S49cyc2 Membranes—S49cyc2 cells (50 μl – 10^6 cells/ml) were pelleted at 8000 g 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 μl of homogenization buffer (0.25 M sucrose, 1 mM EDTA, 1 mM magnesium acetate, 1 mM DTT, 0.5 mM PMSF, and 10 μM 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 followed by a 1-ml syringe followed by a 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 TL-A45 rotor at 43,000 rpm at 4 °C. The membrane pellet was resuspended in 10 μl 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.
Reconstitution of S49cyc Membranes with in Vitro Translated Gas and XLas

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 TLS45 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 Tris-Cl, pH 7.4, 1 mM DTT. The reconstituted membranes were kept on ice and immediately used for the adenyl cyclase assay.

Adenyl Cyclase Assay

The activity of adenyl cyclase was determined by the method of Solomon (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 MgCl₂, 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-32P]ATP, and GTP, GTP-β-S, and isoprotrenol as indicated in the figure legend. Reactions were carried out for 30 min at 30 °C.

The adenyl cyclase activity in PC12 membranes was assayed in the presence of 165 μM ATP, 5 mM MgCl₂, 10 mM creatine phosphate, 0.5 mM/ml creatine kinase, 0.5 mM DTT, 3 μg GTP, 1 mg/ml bovine serum albumin, 3 mM HEPES-KOH, pH 7.2, and 1 μCi [α-32P]ATP. Reactions were carried out for 30 min at 30 °C.

Photoaffinity Labeling of α-Subunits with [32P]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 MgCl₂, 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-32P]AA-GTP (1.4 × 10⁶ cpm/μl), and were further incubated for 1–10 min at 30 °C. The translations 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 MgCl₂, 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 decapetide of Gas and XLas. 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-32P]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 MgCl₂ and 50 mM NaCl, and GDP was omitted; (ii) no receptor agonist was added; (iii) incubation was for 10 min.

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 mM sucrose, 1 mM MgCl₂, 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 10,000 rpm. The homogenate was centrifuged for 10 min 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.
exon 1-encoded portion of Goa and other types of Goa 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 XLas (Fig. 3B) but also between XLas and the various types of Goa subunits (1). It is therefore possible that XLas, like Goa, binds to the βγ complex. To investigate this issue, we used (i) ADP-ribosylation by cholera toxin to detect even weak or transient XLas-βγ interactions and (ii) sucrose density gradient centrifugation to search for stable heterotrimer formation.

Effect of βγ Subunits on the ADP-ribosylation of XLas by Cholera Toxin—The ability of activated cholera toxin to catalyze the ADP-ribosylation of purified (i.e., monomeric), native Goa (13) as well as of in vitro translated Goa (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 Goa and XLas with βγ subunits. In the absence of βγ subunits, in vitro translated XLas 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 XLas (Fig. 4, lane 5) as well as the endogenous Goa present in the reticulocyte lysate (Fig. 4, lanes 2 and 5). The labeling of XLas and Goa 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 XLas—To look for stable heterotrimer formation, we examined the effects of purified βγ subunits on the sedimentation behavior of in vitro translated XLas and, for comparison, Goa, using sucrose density gradients. In the absence of βγ subunits, XLas (Fig. 5B, open circles) and Goa (Fig. 5A, 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, XLas (Fig. 5B, filled circles) and confirming previous results (5), Goa (Fig. 5A, filled circles), sedimented at a slower rate than Goa (Fig. 5A, filled circles), although the total molecular mass of an XLas-βγ heterotrimer is greater than that of the Goa-βγ heterotrimer. This suggests that XLas, upon contact with the βγ dimer, undergoes a conformational change, which alters its sedimentation behavior.

Activation of Adenyl Cyclase by a GTPase-deficient Mutant of XLas—To determine whether XLas is capable of activating adenyl cyclase, we constructed a GTPase-deficient mutant of XLas, XLasQ548L. This mutation corresponds to the Q227L
mutation in \( G_{\alpha} \), which is analogous to the Q61L mutant of \( p21 \) \( \text{ras} \). In \( G_{\alpha} \), this mutation leads to a 100-fold reduction in the rate constant of GTP hydrolysis (15). Hence, XL\( \alpha \)sQ548L should be constitutively activated.

Immunoblotting of PC12 cell membranes using the antibody against the common C-terminal decapeptide of \( G_{\alpha} \) and XL\( \alpha \)s (Fig. 6, bottom panel) indicated that transient transfection with the cDNA for XL\( \alpha \)s or XL\( \alpha \)s-Q548L substantially increased the amount of XL\( \alpha \)s 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 XL\( \alpha \)s, XL\( \alpha \)s-Q548L, showed a massive increase in adenylyl cyclase activity as compared with wild type or mock-transfected cells. Transfection of XL\( \alpha \)s resulted in only a small increase in adenylyl cyclase activity, presumably because XL\( \alpha \)s 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 XL\( \alpha \)s-transfected PC12 cells increased adenylyl cyclase activity to the level observed with membranes from XL\( \alpha \)s-Q548L-transfected cells (data not shown), showing that adenylyl cyclase in the former membranes could be activated by cholera toxin.

![Figure 3](image.png) **Fig. 3.** Sequence comparison of two highly conserved regions in the XL domain of human, mouse, and rat XL\( \alpha \)s. A, domain organization of rat XL\( \alpha \)s (1). EPAA, region containing the EPAA repeats; ARAA, region containing the AARA repeats; \( P \), proline-rich region; \( C \), cysteine-rich region; \( \beta \gamma \), region containing the putative \( \beta \gamma \) binding site; numbers refer to the corrected translational start of XL\( \alpha \)s (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\textsuperscript{TM} accession number AF116268), and rat (\( r \), Refs. 1 and 2) XL\( \alpha \)s with the corresponding N-terminal sequence of rat \( G_{\alpha} \) (\( B \)) and of the proline-rich region of XL\( \alpha \)s across the three species (\( C \)). Boxes indicate conserved residues; asterisks in \( B \) indicate residues that are known to directly contact the \( \beta \gamma \) complex (11, 30).

![Figure 4](image.png) **Fig. 4.** Effect of \( \beta \gamma \) subunits on the ADP-ribosylation of XL\( \alpha \)s by cholera toxin. Reticulocyte lysate either lacking (lanes 1 and 2) or containing (lanes 3–5) in vitro translated XL\( \alpha \)s was incubated for 60 min at 30 °C with \( ^{32}\text{P}-\text{NAD} \) in the absence (−) or presence (+) of purified \( \beta \gamma \) subunits and cholera toxin (Ctx) as indicated, followed by immunoprecipitation with the antiserum against the C-terminal decapeptide of \( G_{\alpha} \) and XL\( \alpha \)s. Immune complexes were analyzed by SDS-PAGE and autoradiography. The results shown are representative of three independent experiments.

![Figure 5](image.png) **Fig. 5.** Effect of \( \beta \gamma \) subunits on the sedimentation behavior of XL\( \alpha \)s. Transcription products were generated from linearized plasmids (CDM8-\( G_{\alpha} \) or CDM8-XL\( \alpha \)s-wt) and translated in the reticulocyte lysate in the presence of \( [\text{S}] \)methionine/cysteine. The translation products, either \( ^{35}\text{S} \)-labeled \( G_{\alpha} \) or XL\( \alpha \)s, were incubated in the presence of GDP\( \beta \text{S} \) and in the absence (open circles) or presence (filled circles) of purified unlabeled \( \beta \gamma \) 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 \( ^{35}\text{S} \)-labeled \( G_{\alpha} \) or XL\( \alpha \)s was visualized by phosphoimaging and quantified. The amount of \( ^{35}\text{S} \)-labeled \( G_{\alpha} \) (\( A \)) or XL\( \alpha \)s (\( B \)) 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.
vated. When HeLa (rather than PC12) cells were transiently transfected with XLs-Q548L, they also showed an increase (4-fold) in adenylyl cyclase activity as compared with mock-transfected cells (data not shown).

Receptor activation of Gas, but not XLs, in reconstituted S49cyc- membranes—S49cyc- cells are deficient in Gas (16–18) as well as XLs, as revealed by immunoblotting (data not shown), but still express the β2-adrenergic receptor, βγ subunits, and the adenylyl cyclase. Given that XLs can activate adenylyl cyclase (Fig. 6), we used S49cyc- cells to study the signal transduction properties of XLs. 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 Gas (Fig. 7A). 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. 7A).

Because Gas and XLs contain the same C-terminal domain and may therefore couple to the same type of receptor (19–21), we investigated whether XLs, like Gas, was able to restore receptor stimulation of adenylyl cyclase activation in reconstituted S49cyc- membranes. In contrast to Gas (Fig. 7A), XLs 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. 7B). We therefore conclude that, in this in vitro system, XLs couples to the β2-adrenergic receptor much less efficiently than Gas, 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 Gas-transfected, but not XLs-transfected, PC12 cells (data not shown).

Receptor activation of Gas, but not XLs, in pituitary membranes—Given that XLs couples only weakly to the β2 adrenergic receptor in reconstituted S49cyc- membranes, we investigated whether or not XLs 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 XLs binds AA-GTP with the same affinity as Gas. PC12 cell membranes were incubated in the presence of AA-GTP with increasing concentrations of either unlabeled GTP or ATP. As shown in Fig. 8A, binding of

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Fig. 6. Activation of adenylyl cyclase by a GTPase-deficient mutant of XLs (XLs-Q548L). Wild type PC12 cells (WT) or PC12 cells transfected with the CDM8 vector without insert (mock), with CDM8-XLs (XLs), or with CDM8-XLs-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 antisera against the common C-terminal decapeptide of Gas and XLs. The results shown are representative of three independent experiments.

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Fig. 7. Differential effects of Gas and XLs on the receptor-mediated adenylyl cyclase activation in S49cyc- membranes. Transcription products were generated from linearized plasmids (CDM8-Gas or CDM8-XLs-wt) and translated in the reticulocyte lysate. S49cyc- membranes were reconstituted either with reticulocyte lysate alone or with reticulocyte lysate containing in vitro translated Gas (A) or XLs (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 Gas or XLs. 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 XLs could be competed with increasing concentrations of GTP but not ATP. The comparison of the competition profiles obtained for XLs and Gas shows that XLs binds to AA-GTP with virtually the same affinity as Gas.

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

Other Gas-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 Gas but not XLs (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 XLs (data not shown).

**DISCUSSION**

Our study shows that XLs shares many, but not all, functional properties of Gas. XLs 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, adenyl cyclase. However, XLs does not appear to be activated by known Gas-coupled receptors.

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

The ability of XLs 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 XLs show a distinct distribution upon subcellular fractionation, whereas this is not the case for Gas. Specifically, the subpopulation of XLs 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 XLs and its subcellular localization, which does not take place for Gas.

**GTP Binding and Functional Units—Photoaffinity labeling** showed that XLs binds GTP. The αs domain of XLs is likely folded in the same way as Gas and, upon GTP binding, undergoes the same conformational change as Gas, since the pattern...
of the trypsin-resistant fragments was identical for Gβ and XLαs. This implies that the XL domain does not significantly affect the folding of the Gα 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. 3A), a putative hinge (see above). Although encoded by the XL exon, the cysteine-rich region of the XL domain (Fig. 3A) (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. 3A), are suggested to belong, together with the Gα domain, to one unit that exerts all Gα-type functions of XLαs. The other unit, whose function remains to be elucidated, comprises the alanine-rich, repetitive region of the XL domain (Fig. 3A) (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 Adenyl 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 Gα-type unit, is capable of activating adenyl cyclase. Hence, in neuroendocrine cells, two distinct G protein α-subunits, Gαs and XLαs, converge onto the same effector system, adenyl 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 Gα domain of XLαs to a receptor.

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