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

I. TISSUE DISTRIBUTION AND SUBCELLULAR LOCALIZATION*

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Our group previously described a new type of G protein, the 78-kDa XLαs (extra large αs) (Kehlenbach, R. H., Matthey, J., and Huttner, W. B. (1994) Nature 372, 804–809 and (1995) Nature 375, 253). Upon subcellular fractionation, XLαs labeled by ADP-ribosylation with cholera toxin was previously mainly detected in the bottom fractions of a velocity sucrose gradient that contained trans-Golgi network and was differentially distributed to Golgi, which also peaked in the top fractions containing plasma membrane. Here, we investigate, using a new antibody specific for the XL domain, the tissue distribution and subcellular localization of XLαs and novel splice variants referred to as XLN1. Upon immunoblotting and immunofluorescence analysis of various adult rat tissues, XLαs and XLN1 were found to be enriched in neuroendocrine tissues, with a particularly high level of expression in the pituitary. By both immunofluorescence and immunogold electron microscopy, endogenous as well as transfected XLαs and XLN1 were found to be predominantly associated with the plasma membrane, with only little immunoreactivity on internal, perinuclear membranes. Upon subcellular fractionation, immunoreactive XLαs behaved similarly to Golgi but was differentially distributed to ADP-ribosylated XLαs. Moreover, the bottom fractions of the velocity sucrose gradient were found to contain not only trans-Golgi network membranes but also certain subdomains of the plasma membrane, which reconciles the present with the previous observations. To further investigate the molecular basis of the association of XLαs with the plasma membrane, chimeric proteins consisting of the XL domain or portions thereof fused to green fluorescent protein were analyzed by fluorescence and subcellular fractionation. In both neuroendocrine and non-neuroendocrine cells, a fusion protein containing the entire XL domain, in contrast to one containing only the proline-rich and cysteine-rich regions, was exclusively localized at the plasma membrane. We conclude that the physiological role of XLαs is at the plasma membrane, where it presumably is involved in signal transduction processes characteristic of neuroendocrine cells.

Heterotrimeric G proteins, which consist of an α-subunit and a βγ complex, transduce extracellular signals detected by heptahelical receptors to intracellular effectors (1–5). Our laboratory previously identified a new type of G protein, XLαs (extra large αs), that is characterized by a bipartite structure (6). The C-terminal half of XLαs is encoded by exons 2-13 of the Gas gene and, hence, contains the entire Gas sequence except for the N-terminal 47 amino acid residues encoded by exon 1 of the Gas gene. In XLαs, the latter residues are replaced by a novel sequence, referred to as the XL domain, yielding a protein with a molecular mass of 78 kDa and an electrophoretic mobility corresponding to 94,000 (rat XLαs) (6, 7). Thus, XLαs is the largest known variant of a G protein α-subunit.

Little is known about the tissue distribution and subcellular localization of XLαs, and no studies on the possible interaction of XLαs with βγ subunits, heptahelical receptors, and G protein effectors have been reported. Resolving these issues is important for elucidating the as yet unknown function of XLαs. Using in situ hybridization, the XLαs mRNA was previously detected in neuroendocrine but not other tissues (6), but it remains to be established whether or not a neuroendocrine-specific expression holds true for the XLαs protein. Using subcellular fractionation, the ADP-ribosylated form of XLαs has mainly been found in fractions containing trans-Golgi network (TGN) (6), but it is unclear whether this reflects the subcellular localization of XLαs under physiological conditions.

In the present study, we have generated antibodies specific to the XL domain of XLαs. We have used these antibodies to investigate by immunocytochemistry and immunoblotting of adult rat tissues the cellular and tissue distribution of XLαs and novel splice variants referred to as XLN1. In addition, we have studied (immuno)fluorescence, immunoelectron microscopy, and subcellular fractionation the subcellular localization of immunoreactive XLαs and XLN1 and green fluorescent protein (GFP) fusion proteins containing all or portions of the XL domain. In the accompanying paper (32), we investigate the possible interaction of XLαs with βγ subunits, heptahelical receptors, and the classical effector of Gas, adenyl cyclase.

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MATERIALS AND METHODS

Antibodies—The peptide NH₂-EPAEPAAEPAAEA-P-ConH₂, corresponding to amino acid residues 45–59 of the XLαs sequence (corrected translational start, see Kehlenbach et al. (7); amino acid residues 176–190 of the originally published sequence (6)), was coupled to keyhole limpet hemocyanin via glutaraldehyde and used to raise the rabbit antiserum RK5. RK5 antibodies were affinity-purified using the above peptide coupled to Affi-Gel 15 (Bio-Rad). The rabbit antiserum against the C-terminal decapetide of Gas and XLαs was the same as described previously (6). Other antibodies used were monoclonal anti-insulin antibody (mouse ascites fluid clone K38aC10), monoclonal anti-β-tubulin antibody (clone TUB 2.1), both purchased from Sigma, and rabbit polyclonal anti-GFP affinity-purified antibody (CLONTECH).

cDNAs—The plasmid CDM8-XLαs, originally called CDM8-XL (6), contains a ~2.6-kilobase insert starting at nucleotide position 380 of the originally published sequence (6) and encodes the entire XLαs protein sequence (see correction of translational start (7)) under the control of the cytomegalovirus promoter.

The isolation of the CDM8-XLα1a and CDM8-XLα1b cDNAs will be described elsewhere. The plasmid CDM8-XLα1a contains the XL-exon spliced to exon 2, exon 3, and exon N1 (8) and encodes a C-terminal-truncated version of the XLαs protein because of the presence of the stop codon-containing exon N1 (Fig. 1A). The plasmid CDM8-XLα1b is identical to CDM8-XLα1a except for a 95-base pair insertion between the XL-exon and exon 2, which in case of human XLαs has been shown to be due to the additional exon A20 in the XLαs/Gas gene (9).

To generate the GFP fusion proteins, the following regions of XLαs were amplified from pCDM8-XLαs by polymerase chain reaction introducing a 5′ Xhol and a 3′ BglII restriction site: 1) XL-GFP (amino acids 1–371); 2) XLβγ-GFP (amino acids 1–321); 3) XL(Δβγ+C)-GFP (amino acids 1–236); and 4) (P+C)-GFP (amino acids 201–312). The fragments were inserted into the Xhol/BamHI-digested eGFP-N2 vector (CLONTECH) upstream in-frame with the eGFP cDNA. All constructs were verified by DNA sequencing.

Cell Culture, Metabolic Labeling, and Transfections—PC12 cells were grown as described (10). [35S]Sulfate labeling and chase of PC12 cells were verified by DNA sequencing.

FIG. 1. Proteins containing the XL domain and XL-GFP fusion proteins. A, proteins containing the XL domain (cross-hatched boxes), which is encoded by a single exon of the rat XLαs/Gas gene (compare Refs. 29 and 30). Gray boxes, Gas domain of XLαs encoded by exons 2–13 of the XLαs/Gas gene; open box, C-terminal segment of XLα1a encoded by the stop codon-containing N1-exon; black box, portion in XLα1b encoded by the frameshift-inducing insertion (I). B, GFP fusion proteins containing either the entire XL domain or portions thereof. EAPA, region containing the EAPA repeats; AARA, 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 (7).

FIG. 2. Expression of rat XLαs mRNA and protein in neuroendocrine tissues. A, immunoblot analysis, using the anti-XL antibody, of a total membrane fraction of PC12 cell PNS and of whole cell lysate of HeLa cells either untransfected (wt) or transfected with XLα1a or XLα1b cDNA. B, immunoblot analysis of whole PC12 cell lysate using the anti-XL antibody in the absence (−) or presence (+) of EAPA peptide used as the antigen. Note the compression of the XLα bands due to the use of a minigel. C, Northern blot analysis of total RNA (10 μg) from various adult rat tissues probed with RNA corresponding to exons 2–9 of Gas. Only the region of the XLαs mRNA is shown. D, immunoblot analysis of total membranes (200 μg of protein) from various adult rat tissues using affinity-purified anti-XL antibody. Pituitary (A), anterior pituitary. Pituitary (T-N), pars intermedia plus neurohypophysis; this lane belongs to a different immunoblot (30 μg of protein) with a shorter exposure; the signal for XLαs and XLα1 is ~80-fold and ~120-fold greater than that in the adrenal gland.
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with fresh Met/Cys-free medium containing 1 mc/dish of [35S]Pro-mix (Amersham Pharmacia Biotech, >1000 Ci/mmol). After the pulse, cells were chased for 0, 15, or 120 min in normal growth medium (10). For transient transfection, PC12 cells harvested from a confluent 15-cm dish were transiently transfected using the calcium phosphate protocol (11) using 40 μg/dish of circular plasmid DNA. COS-7 cells were transfected in the same way (for the GFP constructs) or (in the case of XLas) by using the FuGENE transfection reagent (Roche Molecular Biochemicals) and 20 μg of circular plasmid DNA/15-cm dish.

Wild type and transfected cells were plated on coverslips (polylysine-coated) of the PC12 cells (for immunofluorescence) or on 15-cm dishes (for subcellular fractionation). Cells were used 2 days after transfection, with 10 mM sodium butyrate added in the case of XLas-transfected PC12 cells during the last 16 h to increase the expression of the transgene (12).

Immunofluorescence—Pancreas and pituitary of adult rats were fixed overnight in 4% paraformaldehyde in PBS at 4 °C, infiltrated with 32% sucrose in PBS at 4 °C, frozen in Tissue-Tek on liquid nitrogen and stored at −20 °C. Frozen sections (10 μm) were cut in a Leica Frigocut 2800N cryostat, air-dried on gelatin-coated slides, and processed for immunofluorescence as described (13), except that bovine serum albumin was used instead of fetal calf serum for blocking the sections, with the primary antibodies as follows: affinity-purified anti-XL antibody and XL at 1:100 dilution and XL at 1:800–1:1000 dilution, and [35S]protein A. For immunofluorescence, anti-GFP antibody at 1 μg/g of IgG/ml or anti-GFP antibody at 1 μg/g IgG/ml followed by horseradish peroxidase-conjugated secondary antibody and the ECL system (Amersham Pharmacia Biotech) or (ii) with anti-XL antisera (1:200 to 1:500 dilution) followed by 125I-protein A. To test the specificity of the anti-XL antibody, blotting of total membranes from various tissues and of velocity sucrose gradient centrifugation of selected fractions of the velocity sucrose gradient centrifugation were performed as described (10).

Transmission electron microscopy—Posterior lobes of adult rat pituitaries were dissected and fixed by immersion in 4% paraformaldehyde, 0.1 M glutaraldehyde in 0.1 M cacodylate buffer, dehydrated in ethanol and embedded in the acrylic resin LRWhite (London Resin Corp.). Semithin sections were obtained and stained with 1% toluidine blue, 1% sodium tetraborate to localize the pars intermedia. Ultrathin sections were labeled using affinity-purified anti-XL antibody at 1–5 μg/g of IgG/ml followed by protein A gold (9 nm). Sections were stained with saturated uranyl acetate in water.

Subcellular fractionation—PC12 and HeLa whole cell lysates were obtained by the addition of Laemmli sample buffer to the dishes followed by immediate boiling of the samples. Postnuclear supernatant (PNS) from PC12 cells was prepared as described (10). A total membrane fraction from the PNS was obtained by centrifugation at 100,000 × g for 1 h at 4 °C. Velocity sucrose gradient centrifugation of the PNS and equilibrium sucrose gradient centrifugation of selected fractions of the velocity sucrose gradient were performed as described (10).

For the analysis of membrane association of GFP fusion proteins transfected into PC12 cells, equal aliquots of the respective PNS were subjected to centrifugation at 100,000 × g for 1 h at 4 °C. Particulate and soluble fractions were analyzed by SDS-PAGE and immunoblotting.

For the analysis of the subcellular localization of newly synthesized Gas and XLas, velocity sucrose gradient fractions from PC12 cells pulse-labeled for 5 min with [35S]cysteine-methionine and chased for 15 or 120 min with 0.1 mM Hepes-KOH, pH 7.2. Membranes were collected by centrifugation (100,000 × g at 4 °C, 1 h). Gas and XLas were immunoprecipitated from the membranes using the antisera against the C-terminal decapetide of Gas and XLas at 1:100 dilution, as described (6).

For the analysis of membrane association of newly synthesized Gas and XLas, total membranes and cytosol were prepared by centrifugation (100,000 × g at 4 °C) of the PNS of PC12 cells pulse-labeled for 5 min with [35S]cysteine-methionine and chased for 0, 15, or 120 min. An aliquot of the total membrane preparation was subjected to carbonate stripping by incubation with 0.1 mM Na2CO3, 0.025% saponin, 2 mM EDTA, 0.25% sodium dodecyl sulfate, and 0.5 mM phenylmethylsulfonyl fluoride. Gas and XLas were immunoprecipitated from cytosol, total membranes, and carbonate-stripped membranes using the antisera against the C-terminal decapetide of Gas and XLas, as described (6).

Immunoblotting—Adult rat tissues were dissected and immediately frozen in liquid nitrogen followed by storage at −80 °C until use. Tissue was homogenized in 0.3 M sucrose, 10 mM Hepes-KOH, pH 7.2, 1 mM EDTA, 1 mM phenylmethylsulfonyl fluoride. In the case of the pancreas, homogenization was performed in the presence of a protease inhibitor mixture (pepsatin, leupeptin, chymostatin, antipain, soybean trypsin inhibitor). Homogenates were centrifuged twice for 5 min each at 1000 × g at 4 °C, the supernatants were collected and centrifuged at 100,000 × g for 1 h at 4 °C to obtain the total membranes. Membranes were resuspended in 10 mM Hepes-KOH, pH 7.2, and then boiled in Laemmli sample buffer.

Immunoblotting after SDS-PAGE was carried out according to standard procedures. For immunoblotting of velocity sucrose gradient fractions, we used 3% bovine serum albumin in PBS to block the nitrocellulose membranes, the antisera against the C-terminal decapetide of Gas and XLas at 1:800 to 1:1000 dilution, and 125I-protein A. For immunoblotting of total membranes from various tissues and of velocity sucrose gradient fractions from PC12 cells expressing various GFP fusion proteins, the nitrocellulose membranes were blocked with 5% lowfat milk powder in PBS and incubated (i) with either affinity-purified anti-XL antibody at 0.1 μg/g IgG/ml or anti-GFP antibody at 1 μg/g IgG/ml followed by horseradish peroxidase-conjugated secondary antibody and the ECL system (Amersham Pharmacia Biotech) or (ii) with anti-XL antisera (1:200 to 1:600 dilution) followed by 125I-protein A. To test the specificity of the anti-XL antibody, blots of whole...
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**RESULTS**

Expression of XLas and XLN1 in Neuroendocrine Tissues—Previous results obtained by *in situ* hybridization showed that the XLas mRNA occurs predominantly in certain endocrine cells and neurons (6). The C-terminal decapetides of XLas and Gas are identical, and hence, use of the corresponding antibody (6) against an epitope of the XL domain showed major immunoreactive bands very similar, if not identical, to two of the XLN1 proteins (8), and in the case of XLN1b, the frameshifting 95-base pair insertion (Fig. 1A), corroborating their identity as XLN1 proteins. In immunoblots of whole PC12 cell lysate, the immunoreactive bands corresponding to XLas and the XLN1 proteins were abolished when the anti-XL antibody was used in the presence of the peptide used as antigen (Fig. 2B), which further documents the specificity of this antibody. Essentially similar results were obtained when immunoblots were probed with an antibody raised against a glutathione S-transferase fusion protein containing the XL domain (data not shown).

We used the anti-XL antibody to examine the tissue distribution of the XLas protein in comparison with that of the XLas mRNA. Northern blot analysis of various rat tissues revealed the presence of the XLas mRNA in the adrenal gland, brain, cerebellum, heart, and pituitary gland, with the levels in the brain and heart at the limit of detectability and the levels in the

PC12 cell lysate were incubated with the antibody solution containing 10 μg/ml of the EPAA peptide.

Northern Blot—Tissues from adult rats were directly homogenized in guanidine isothiocyanate, and total RNA was isolated as described (15, 16). Northern blot analysis (10 μg of total RNA) was carried out using a [32P]dCTP-labeled cDNA fragment corresponding to exons 2–9 of a Gas cDNA as probe.

**ADP-ribosylation**—ADP-ribosylation of PNS using cholera toxin and [35S]sulfate-labeled for 4.5 min and chased for 15 min (c) and PC12 cells [35S]sulfate-labeled for 10 min without further chase (d) was subjected to the same sequential velocity and equilibrium sucrose gradient analysis. Fractions from the equilibrium gradient were analyzed by autoradiography (c) or phosphoimaging (d). [32P]ADP-ribosylated (c) and [125I]labeled immunoreactive (d) XLas (filled circles) was quantitated and is expressed as percentage of total labeled XLas recovered per gradient. In parallel, PNS from PC12 cells [35S]sulfate-labeled for 10 min without further chase (d) was subjected to the same sequential velocity and equilibrium sucrose gradient analysis. Fractions 2–5 of velocity sucrose gradients containing [32P]ADP-ribosylated or unlabeled XLas were subjected to equilibrium sucrose gradient centrifugation (panels c and d, respectively). Fractions (1 = top of gradient) were analyzed by SDS-PAGE followed by autoradiography (c) or immunoblotting with the antibody against the C-terminal decapetide of GasXLas, 125I-protein A, and phosphoimaging (d). [35S]sulfate-labeled heparan sulfate proteoglycan (hsPG, filled triangles), a marker of constitutive secretory vesicles and the plasma membrane, and [32P]ADP-ribosylated secretogranin II (SgII, open squares), a marker of immature secretory granules (18, 20), were quantitated and are expressed as percentage of total labeled marker recovered per gradient.

**ADP-ribosylation** of XLas upon subcellular fractionation. Top panels, velocity sucrose gradient analysis. PC12 cell PNS after cholera toxin-catalyzed ADP-ribosylation with [32P]-NAD (a) or unlabeled PNS (b) were subjected to velocity sucrose gradient centrifugation. Fractions (1 = top of gradient) were analyzed by SDS-PAGE followed by autoradiography (a) or immunoblotting with the antibody against the C-terminal decapetide of GasXLas, 125I-protein A, and autoradiography (b). [32P]ADP-ribosylated (a) and [125I]labeled immunoreactive (b) XLas (filled circles) and Gas (open circles) were quantitated by densitometric scanning of the autoradiograms. The absorbance values obtained are given as arbitrary units (a.u.). To facilitate the comparison of XLas and Gas, the scales of the ordinates differ by a factor of 2 (a) and 5 (b). Fractions 13 (a) and 14 (b), which contained the pellet, are not shown. Bottom panels, equilibrium sucrose gradient analysis. Fractions 2–5 of velocity sucrose gradients containing [32P]ADP-ribosylated or unlabeled XLas were subjected to equilibrium sucrose gradient centrifugation (panels c and d, respectively). Fractions (1 = top of gradient) were analyzed by SDS-PAGE followed by autoradiography (c) or immunoblotting with the antibody against the C-terminal decapetide of GasXLas, 125I-protein A, and phosphoimaging (d). [32P]ADP-ribosylated (c) and [125I]labeled immunoreactive (d) XLas (filled circles) was quantitated and is expressed as percentage of total labeled XLas recovered per gradient. In parallel, PNS from PC12 cells [35S]sulfate-labeled for 4.5 min and chased for 15 min (c) and PC12 cells [35S]sulfate-labeled for 10 min without further chase (d) was subjected to the same sequential velocity and equilibrium sucrose gradient analysis.
pituitary much higher than in the other neuroendocrine tissues (Fig. 2C). No XLα mRNA was detected in kidney, liver, and spleen. These observations are consistent with the neuroendocrine-specific tissue distribution of the XLα mRNA previously observed by in situ hybridization (6). Immunoblot analysis revealed the presence of the XLα protein and the XLN1 proteins in rat brain, cerebellum, adrenal gland, pancreas (at the limit of detection) and, at very high levels, the pituitary gland but not in liver and kidney (Fig. 2D). Within the pituitary gland, XLα and XLN1 immunoreactivity per total protein was much higher in the posterior lobe (containing the pars intermedia plus neurohypophysis) than in the anterior lobe (see legend to Fig. 2D). The level of XLα and XLN1 immunoreactivity per total protein in the pituitary posterior lobe was \( \approx 80 \) times (XLα) to \( \approx 120 \) times (XLN1) higher than that in the adrenal.

Adult rat pituitary cryosections were examined by immunofluorescence using the anti-XL antibody. Strong immunostaining for XLα/XLN1 was observed in most, if not all, cells of the pars intermedia, the melanotrophs (Fig. 3a and b). The level of immunostaining was lower in the anterior pituitary and restricted to a subset of cells (Fig. 3, a and c). The neurohypophysis was not immunostained above background (Fig. 3a). In both anterior pituitary and pars intermedia, the staining was predominantly observed at the cell periphery, delineating the shape of the cells.

Immunofluorescence of cryosections of adult rat pancreas showed that the immunoreactivity for XLα/XLN1 was present in the islets of Langerhans, the endocrine part of the organ, but not in the surrounding exocrine part (Fig. 3d). Comparison of the staining with that for insulin by double immunofluorescence revealed that XLα/XLN1 was expressed in most (Fig. 3, d and e, larger arrows) but not all (Fig. 3, d and e, arrowheads) β cells. XLα/XLN1 expression was not restricted to β cells but also observed in other endocrine cells of the pancreas, as indicated by the presence of cells immunoreactive for XLα/XLN1 but negative for insulin (Fig. 3, d and e, small arrows). Taken together, XLα and XLN1 are neuroendocrine-specific proteins expressed in many, but not all, neuroendocrine cells in the adult. Essentially similar results were obtained when immunofluorescence of pituitary and endocrine pancreas was performed using an antibody raised against a glutathione S-transferase fusion protein containing the XL domain (data not shown).

**Subcellular Localization of XLα: Subcellular Fractionation.—** Upon subcellular fractionation of PC12 cells, we previously observed a differential distribution of XLα and Gas, \( ^{32} \text{P} \)ADP-ribosylated by cholera toxin, across a velocity sucrose gradient (6). The majority of the \( ^{32} \text{P} \)ADP-ribosylated Gas, but at most a quarter of the \( ^{32} \text{P} \)ADP-ribosylated XLα, was recovered in the top fractions of the gradient (6), which are known to contain plasma membrane (17). By contrast, only about one-third of the \( ^{32} \text{P} \)ADP-ribosylated Gas, but almost two-third of the \( ^{32} \text{P} \)ADP-ribosylated XLα, was recovered in the bottom fractions of the gradient (6), which are known to contain TGN membranes (10). Fig. 4a shows the results of another such subcellular fractionation experiment, which confirms the previously observed differential distribution of
ADP-ribosylated G\(\alpha\)s and XL\(\alpha\)s upon velocity sucrose gradient centrifugation.

Surprisingly, however, no significant difference in the distribution upon velocity sucrose gradient centrifugation was observed when immunoreactive rather than \([^{32}\text{P}]\)ADP-ribosylated G\(\alpha\)s and XL\(\alpha\)s were compared (Fig. 4b). Immunoblotting using the antibody against the C-terminal decapeptide of G\(\alpha\)s/XL\(\alpha\)s showed that for either G protein, the majority was found in the top fractions of the gradient and, at most, one-third in the bottom fractions. Equilibrium sucrose gradient analysis provided further evidence that the XL\(\alpha\)s found in the top fractions of the velocity sucrose gradient, i.e. the majority of the immunoreactive XL\(\alpha\)s (Fig. 4b) but only about one-third of the \([^{32}\text{P}]\)ADP-ribosylated XL\(\alpha\)s (Fig. 4a), was indeed associated with the plasma membrane. Both the \([^{32}\text{P}]\)ADP-ribosylated (Fig. 4c) and the immunoreactive (Fig. 4d) XL\(\alpha\)s of fractions 2–5 of the velocity sucrose gradient cofractionated upon equilibrium sucrose gradient centrifugation with \([^{35}\text{S}]\)sulfate-labeled heparan sulfate proteoglycan chased from the TGN to constitutive secretory vesicles and the plasma membrane (18, 19) and exhibited a distinct distribution from \([^{35}\text{S}]\)sulfate-labeled secretogranin II chased into immature secretory granules (18, 20).

Immunoblotting of velocity sucrose gradient fractions using the anti-XL-antibody showed that the distribution of XLN1 was very similar to that of XL\(\alpha\)s, with the majority found in the top fractions containing plasma membrane (Fig. 5, A and B). Remarkably, the distribution of XL\(\alpha\)s and XLN1 in the bottom half of the gradient was distinct from that of secretogranin II pulse-labeled with \([^{35}\text{S}]\)sulfate in the TGN (Fig. 5B). This suggests that even the XL\(\alpha\)s and XLN1 found in the bottom half of the velocity gradient is associated with membranes other than the TGN.

Subcellular Localization of XL\(\alpha\)s: Immunofluorescence and Immunoelectron Microscopy—Immunofluorescence analysis of PC12 cells stained with the anti-XL antibody showed that most immunoreactivity was associated with the plasma membrane, with only occasional staining in the perinuclear region of the cells (Fig. 6, a–c). The plasma membrane-associated immunoreactivity extended into plasmalemmal protrusions and processes emerging from the cell body (Fig. 7). PC12 cells overexpressing XL\(\alpha\)s upon transfection showed an increased immunoreactivity, the pattern of which was similar to that of mock-transfected PC12 cells (Fig. 6, d–f). Preincubation of the anti-XL antibody with the EPAA peptide used as antigen abol-
ished the immunostaining (Fig. 6, g and h).

XLas was transfected into HeLa and COS-7 cells, two non-neuroendocrine cell types that normally do not show XL-immunoreactivity (see the untransfected cell indicated by the arrow in Fig. 8a, which is a counterstaining for β-tubulin, and data not shown). Immunofluorescence using the anti XL antibody showed that transfected XLas was exclusively localized at the plasma membrane, with clear labeling of membrane protrusions (Fig. 8b). The same was observed for HeLa cells transfected with XLN1 (data not shown). An exclusive plasma membrane localization of XLas was also observed for the vast majority of transfected COS-7 cells (Fig. 8c). In a minority of transfected COS-7 cells (<10%), which showed very high levels of XLas expression, XL immunoreactivity was not only seen on the plasma membrane but also in a perinuclear location (Fig. 8d, arrowhead). These observations show that transfected XLas is primarily targeted to the plasma membrane in non-neuroendocrine cells and imply that at least part of the plasma membrane-associated endogenous immunoreactivity observed with the anti-XL antibody in PC12 cells was due to XLas (rather than XLN1 only). Essentially similar results were obtained when immunofluorescence of transfected HeLa cells was performed using the antibody raised against a glutathione S-transferase fusion protein containing the XL domain (data not shown).

Immunogold electron microscopy using the anti-XL antibody was performed on ultrathin sections of LRWhite-embedded posterior lobe of adult rat pituitary to examine the subcellular localization of XLas/XLN1 in the pars intermedia, a tissue with a high content of XLas as shown by immunoblotting (Fig. 2D). Gold particles were exclusively detected over the plasma membrane of melanotrophs (Fig. 9). No immunogold labeling was detected in the axon terminals of the neurohypophysis present in the same sections (data not shown). We conclude that by subcellular fractionation, immunofluorescence, and immunoelectron microscopy, the vast majority of XLas is associated with the plasma membrane.

The XL Domain Contains a Plasma Membrane-targeting Signal—The finding that both XLas and XLN1 are localized on the plasma membrane, as observed by immunofluorescence and subcellular fractionation, suggested that the targeting signal responsible for this localization resides within the XL domain. To investigate this directly, we constructed chimeric proteins containing the XL domain and portions thereof fused to GFP (Fig. 1B). Although the GFP reporter was soluble in the cytosol of transfected PC12 cells (Figs. 10 and 11, b–d), the XL domain indeed mediated the association of GFP with the plasma membrane (Figs. 10 and 11, f–h). The same was the case for an XL domain lacking the putative βγ binding region (32) (Figs. 10 and 11, j–l), indicating that the plasma membrane association of XLas does not depend on βγ interaction (as has been reported for Goas (21)) but is mediated by another region within the XL domain. In fact, this region appears to be the cysteine-rich region because GFP fused to an XL domain lacking both the putative βγ binding region and the cysteine-rich region (6) was found to be soluble in the cytosol (Figs. 10 and 11, n–p), as is wild type GFP. A portion of the XL domain comprising only the proline-rich region and the cysteine-rich region (6) was sufficient to mediate the association of GFP with membranes (Fig. 10) that, remarkably, were not only plasma membrane but also some internal membranes of PC12 cells (Fig. 11, r–t).

Transfection of the various GFP fusion constructs (Fig. 1B) into COS-7 cells gave essentially the same results (Fig. 11, a, e, i, and m), except that in the case of GFP fused to the proline-rich and cysteine-rich regions of the XL domain, the staining of the internal membranes that was observed in addition to the plasma membrane fluorescence was characterized by a distinct perinuclear pattern (Fig. 11, q, arrow).

We further investigated the differential localization of the GFP fusion proteins containing either the entire XL domain (XL-GFP) or only its proline-rich and cysteine-rich regions ((P+C)-GFP) by subcellular fractionation. Immunoblotting of velocity sucrose gradient fractions with anti-GFP antibody showed that XL-GFP was exclusively found in the top fractions

![Fig. 8. Immunofluorescence analysis of HeLa and COS-7 cells transfected with XLas.](image)

HeLa cells and COS-7 cells were transfected with the CDM8-XLas vector. a and b, double immunofluorescence of HeLa cells using a monoclonal anti-β-tubulin antibody (a) and affinity-purified anti-XL antibody (b) followed by conventional fluorescence microscopy. Immunoreactivity for XLas in the transfected cell is located to plasma membrane protrusions (b). Tubulin staining reveals the presence of an additional, untransfected cell that lacks endogenous XLas (a, arrow). c and d, immunofluorescence of COS-7 cells using affinity-purified anti-XL antibody followed by confocal microscopy. The pattern of XLas immunoreactivity shown in panel c, which is characteristic of its localization on the plasma membrane, is representative of the vast majority of transfected cells, whereas the pattern shown in panel d, which shows staining of both the plasma membrane and internal membranes (arrowhead), is observed only in a minority of transfected cells with a very high level of XLas expression.

![Fig. 9. Ultrastructural localization of XLas in the pars intermedia of the pituitary.](image)

Ultrastructural section of LRWhite-embedded posterior lobe of adult rat pituitary stained with affinity-purified anti-XL antibody followed by 9-nm protein A-gold. Gold particles (arrows) are found at the plasma membrane of melanotrophs, the endocrine cell of the pars intermedia. Bar, 0.5 μm.
were analyzed by SDS-PAGE followed by immunoblotting using an anti-GFP antibody (A). For each fusion protein, the amount in the pellet is expressed as a percentage of total (sum of supernatant and pellet) in panel B.

of the gradient known to contain plasma membrane (Fig. 12A), whereas (P+C)-GFP was found not only in these fractions but also in the bottom half of the gradient with a peak in fraction 9 (Fig. 12B), which also contains the peak of TGN membranes (compare Fig. 5B, open squares).

Dynamics of Membrane Association of XLas—We used pulse-chase in conjunction with subcellular fractionation of PC12 cells to investigate the kinetics of membrane association of XLas in comparison with Gαs. PC12 cells were pulse-labeled with [35S]cysteine-methionine and chased for either 15 min or 2 h. After 15 min, more than half and, after 2 h, nearly all of XLas were found to be membrane-associated (Fig. 13, filled squares). At either chase time, the majority of the membrane-associated XLas was resistant to carbonate extraction (Fig. 13, open squares). The kinetics of membrane association of XLas was similar to that of Gαs (Fig. 13, circles).

The membranes to which newly synthesized XLas becomes associated were fractionated on the velocity sucrose gradient (compare Figs. 4, a and b, 5, and 12). After 15 min of chase, the majority of newly synthesized and membrane-associated XLas was found in the top fractions, and only a minor proportion was found in the bottom fractions of the gradient (Fig. 14A, filled circles). The latter proportion of XLas decreased slightly after 2 h of chase (Fig. 14A, open circles). Similar observations were made for Gαs (Fig. 14B), with the change in its distribution between top and bottom fractions upon long chase more obvious than in the case of XLas.

DISCUSSION

Tissue Distribution—The present study shows that the XLas and XLN1 proteins are specifically expressed in neuroendocrine tissues. For XLas, these findings are consistent with the previous observation (6) confirmed here that the XLas mRNA is present in neuroendocrine but not other tissues. Within the neuroendocrine system, however, XL- immunoreactivity (which reflects XLas and XLN1) was not detected in all (neuro)peptide- and peptide hormone-producing cells but, rather, in certain subpopulations of these cells. For example, in the adult rat, XL immunoreactivity was found at high levels in virtually all cells of the intermediate lobe of the pituitary but at lower levels in, and not in all cells of, the anterior pituitary. In the endocrine pancreas, XL immunoreactivity was detected in most but not all insulin-producing β-cells and also in other islet cells. From our data, it appears that there is no straightforward correlation between the expression of XL immunoreactivity in a given neuroendocrine cell and the peptide hormone/neuropeptide produced or the extracellular input received by this cell. If any such correlation is to be speculated upon, it would be with the pituitary adenyl cyclase-activating polypeptide (PACAP) receptor, whose tissue distribution shows some resemblance to that of XLas and XLN1 (22–24).

The neuroendocrine-specific tissue distribution of XLas may have implications for the pathophysiological mechanisms underlying the phenotype of patients afflicted by mutations in exons 2-13 of the GNAS1 gene (25–28). Since these exons encode not only most of the Gαs sequence but also half of the XLas sequence (6, 9, 29, 30), any phenotype confined to the neuroendocrine system of these patients may reflect the dysfunction of XLas rather than that of the ubiquitously expressed Gαs.

Subcellular Localization—An unexpected finding of the present study was that in both tissues and isolated cells in culture, XL immunoreactivity was almost exclusively localized to the plasma membrane, as observed in immunofluorescence and immunoelectron microscopy. It cannot be argued that the XL immunoreactivity at the plasma membrane reflects solely the subcellular localization of XLN1 rather than that of XLas for the following reasons. First, upon subcellular fractionation of PC12 cells, XLas and XLN1 show the same distribution (Fig. 5B). Second, upon expression from cloned cDNAs, almost exclusive plasma membrane localization of XL immunoreactivity was observed for either XLas (Figs. 6, d–f, and 8, b–d) and XLN1 (data not shown).

The almost exclusive plasma membrane localization of immunoreactive XLas is in contrast to the previous conclusion (6) that XLas is predominantly associated with the TGN, which was based on the distribution of ADP-ribosylated XLas in comparison with ADP-ribosylated Gαs upon subcellular fractionation of PC12 cells using an established velocity sucrose gradient centrifugation protocol (18). Specifically, the majority of ADP-ribosylated XLas, but only the minority of Gαs, was recovered in the bottom half of the velocity gradient (6), an observation confirmed here (Fig. 4a). The bottom half of the velocity gradient is known to contain the bulk of the TGN membranes of PC12 cells (18) (Fig. 5B). However, unexpectedly, the distribution within the bottom half of the velocity gradient of immunoreactive XLas and XLN1 was distinct from that of the TGN (defined by [35S]sulfate pulse-labeled secretogranin II) (Fig. 5B). This finding together with the almost exclusive plasma membrane localization of XLas and XLN1 observed in immunofluorescence leads us to conclude that the fractions in the bottom half of the velocity gradient that contain the peak of XLas and XLN1 (but not the peak of TGN membranes) are enriched in plasma membrane fragments that represent a specialized domain of the plasma membrane (see below).

The reason why the ADP-ribosylated XLas is differentially
distributed across the velocity gradient to the immunoreactive XLαs is unclear. This difference does not appear to be caused by ADP-ribosylation of XLαs. Upon immunoblotting of velocity gradient fractions from PC12 cells incubated overnight in the presence of cholera toxin, which is likely to result in stoichiometric ADP-ribosylation of XLαs (6), the majority of the immunoreactive XLαs was found in the top fractions of the gradient (data not shown), as was the case for XLαs from control PC12.
Rather, the XLs associated with the plasma membrane fragments recovered in the upper half of the velocity sucrose gradient appears to undergo in vitro ADP-ribosylation by cholera toxin to a lesser extent than the XLs associated with the plasma membrane fragments sedimenting to the lower half of the gradient. Possible explanations for the lesser extent of ADP-ribosylation of the XLs recovered in the upper half of the velocity sucrose gradient include (i) a lower probability to be in the trimeric state (see also the accompanying paper by Klemke et al. (32), (ii) a reduced ability to interact with ADP-ribosylation factor, and (iii) an interaction with a hypothetical protein that inhibits ADP-ribosylation. Whatever the explanation, the plasma membrane fragments obtained after homogenization of PC12 cells that contain the majority of the ADP-ribosylatable XLs but relatively little ADP-ribosylatable Gs must be relatively large because they sediment to the bottom half of the velocity gradient. These fragments may well be derived from thin cell surface protrusions, a specialized domain of the plasma membrane, which contain XLs (Figs. 6 and 7).

The almost exclusive localization of both XLs and XLN1 on the plasma membrane is mediated by the XL domain, as shown by the targeting of a GFP fusion protein (Figs. 11, c–h, and 12A). In contrast to the GFP fusion protein containing the entire XL domain, a fusion protein containing only the proline-rich and cysteine-rich regions of the XL domain ((P+C)-GFP) was not only targeted to the plasma membrane but also to internal membranes in the perinuclear region (Fig. 11, q–t), most likely Golgi membranes, as suggested by subcellular fractionation (Fig. 12B). The subcellular localization of (P+C)-GFP in COS-7 cells (Fig. 11q) is very similar to that observed by Ugur and Jones for an essentially identical fusion protein and reported (31) after submission of the original version of this paper. Our finding that deletion of the cysteine-rich domain abolishes membrane association (Figs. 10 and 11, m–p) also is consistent with the conclusion of these authors that the cysteine residues in this domain, which are palmitoylated, are of critical importance for membrane binding of XLs (31). However, the present study clearly demonstrates that the vast majority of XLs is not associated with the Golgi complex, as claimed by Ugur and Jones (31), but with the plasma membrane. The discrepancy in conclusion between this and the present study may in part be explained by the fact that Ugur...
and Jones (31) did not use an antibody specific to the XL domain as we have done (Fig. 2, A and B) but rather an antibody to the C terminus of Gas, which also recognizes XLs; this poses a certain risk when attributing immunoprecipitated using the antibody against the C-terminal decapeptide of Gas/XL, followed by SDS-PAGE and fluorography. [35S]XLs (A) and [35S]Gas (B) in the gradient fractions were quantified and are expressed as percent of total (sum of the gradient fractions).

A virtually exclusive recovery in subcellular fractions enriched in plasma membrane (rather than TGN, compare Fig. 14A with Fig. 5B) was observed for newly synthesized, metabolically labeled XLs. Tight association of newly synthesized XLs with the plasma membrane occurred rapidly, since already after 15 min of chase, the majority of the membrane-associated XLs was resistant to extraction at pH 11 (Fig. 13). Hence, in conclusion, our results point to a role of XLs at the plasma membrane in neuroendocrine cells, most likely in signal transduction. Consequently, the possible interaction of XLs with βγ subunits and receptor/effecter systems known to couple to Gas are addressed in the subsequent paper (Klemke et al. (32)).

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