

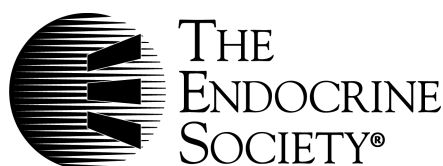
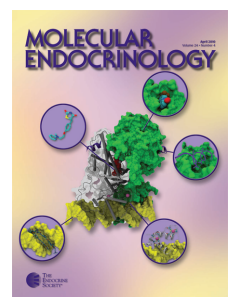
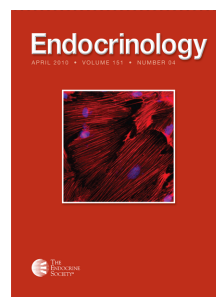
Endocrinology

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Peroxisomal Localization of the Proopiomelanocortin-Derived Peptides β -Lipotropin and β -Endorphin

Romana Höftberger,* Markus Kunze,* Till Voigtländer, Ursula Unterberger, Günther Regelsberger, Jan Bauer, Fahmy Aboul-Enein, Ferenc Garzuly, Sonja Forss-Petter, Hanno Bernheimer, Johannes Berger, and Herbert Budka

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The peptide hormones ACTH, MSHs, β -lipotropin (β -LPH), and β -endorphin are all derived from the precursor molecule proopiomelanocortin (POMC). Using confocal laser microscopy and immunoelectron microscopy in human pituitary gland, we demonstrate a peroxisomal localization of β -endorphin and β -LPH in cells expressing the peroxisomal ATP-binding cassette-transporter adrenoleukodystrophy protein (ALDP). The peroxisomal localization of β -LPH and β -endorphin was not restricted to the pituitary gland but was additionally found in other human tissues that express high levels of ALDP, such as dorsal root ganglia, adrenal cortex, distal tubules of kidney, and skin. In contrast to the peptide hormones β -LPH and β -endorphin, which are derived from the C terminus of POMC, the N-terminal peptides ACTH, α -MSH, and γ -MSH were never detected in peroxisomes. This novel peroxisomal localization of β -endorphin and β -LPH in ALDP-positive cells was confirmed by costaining with ALDP and the peroxisomal marker catalase. Moreover, peroxisomal sorting of β -LPH could be modeled in HeLa cells by ectopic expression of a POMC variant, modified to allow cleavage and release of β -LPH within the secretory pathway. Although β -LPH and β -endorphin were only associated with peroxisomes in cells that normally express ALDP, the transporter activity of ALDP is not necessary for the peroxisomal localization, as demonstrated in tissues of X-linked adrenoleukodystrophy patients lacking functional ALDP. It remains to be elucidated whether and how the peroxisomal localization of POMC-derived hormones has a role in the endocrine dysfunction of peroxisomal disease. (*Endocrinology* 151: 4801–4810, 2010)

Peroxisomes are ubiquitous, single membrane bound organelles, which exert a variety of important metabolic processes such as the degradation of pipecolic, phytanic, or very long-chain ($C \geq 22:0$) fatty acids (VLCFA), the synthesis of plasmalogens, bile acids, and docosa-hexaenoic acid or the detoxification of hydrogen peroxide (H_2O_2) (1). The importance of this organelle is stressed by severe inherited disorders, which can be caused by either general dysfunction of peroxisomes or defects in individual peroxisomal enzymes. The most common peroxisomal disorder is X-linked adrenoleukodystrophy (X-ALD), caused by mutations in the *ABCD1* gene encoding the peroxisomal ATP-binding cassette transporter, adreno-

leukodystrophy protein (ALDP) (2). We recently demonstrated that ALDP (*ABCD1*) is highly abundant in a subpopulation of anterior pituitary gland cells, producing the peptide hormone precursor molecule proopiomelanocortin (POMC). ALDP was also enriched in many other tissues of the human body that are known to express high levels of POMC, such as dorsal root ganglia, adrenal gland, distal tubules in kidney, liver, and skin (3, 4).

POMC is imported into the endoplasmic reticulum (ER) and then processed sequentially in the late secretory pathway resulting in various peptide hormones. First, prohormone convertase 1 (PC1/3) cleaves POMC into the N-terminal pro-ACTH and the C-terminal β -lipotropin

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Abbreviations: ALDP, Adrenoleukodystrophy protein; ER, endoplasmic reticulum; β -LPH, β -lipotropin; PC, prohormone convertase; PMP70, peroxisomal membrane protein 70 kDa; POMC, proopiomelanocortin; VLCFA, very long-chain fatty acids; X-ALD, X-linked adrenoleukodystrophy.

(β -LPH). Subsequently, PC2 can generate further bioactive peptides such as α -MSH, γ -MSH, γ -LPH, and β -endorphin (5, 6). These peptide hormones exert diverse auto-, para-, and endocrine functions in a variety of tissues. The most prominent peptide hormones of the pituitary gland are ACTH, regulating steroidogenesis in the adrenal gland (7), and β -endorphin, modulating pain sensation (8). In contrast, the biological role of β -LPH, which is generated concomitantly with ACTH, is still not fully understood, although a stimulatory action on adrenal steroidogenesis and lipolytic activity has been demonstrated (9, 10).

In the present study, we analyzed the subcellular distribution of β -LPH, β -endorphin, α -MSH, γ -MSH, and ACTH in the pituitary gland as well as in other tissues of the human body, which express high levels of ALDP.

Materials and Methods

Tissues

Cellular and subcellular distribution of POMC-derived peptide hormones was examined in pituitary gland, central nervous system, dorsal root ganglia, adrenal gland, testis, skin, kidney, and liver. For that purpose, formalin-fixed and paraffin-embedded human autopsy and biopsy material including tissues from two X-ALD patients (Table 1) (4) deceased with the severe childhood form at the age of 11 and 14 yr, respectively. Both patients were diagnosed by elevated VLCFA in plasma; in addition, genetic analysis was performed in one patient (ALD-7 1679, C>T, P560L). Although no detectable ALDP was present in cultured fibroblasts of both patients, some residual ALDP was still immunohistochemically detectable in tissues strongly expressing ALDP, such as the pituitary gland.

Primary antibodies

The following primary antibodies were used: 1) monoclonal (mouse) antibodies: anti-(α)-ALDP (1D6; Euromedex, Souffelweyersheim, France), α -ACTH (Dako, Carpinteria, CA), and α -non-phosphorylated neurofilament protein (SMI32, Sternberger Monoclonals Inc., Lutherville, MD); 2) polyclonal (rabbit) antibodies: α -chromogranin A (DakoCytomation, Glostrup, Denmark), α -peroxisomal membrane protein 70 kDa (PMP70; Affinity BioReagents, Golden, CO), α - α -MSH (Biomol, Hamburg, Germany), α - γ -MSH (Millipore, Bedford, MA), α - β -LPH (Fitzgerald Industries International, Concord, MA), and α - β -endorphin (Progen Biotechnik GmbH, Heidelberg, Germany); and 3) polyclonal (sheep) antibody: α -catalase (The Binding Site, Birmingham, UK).

Immunohistochemistry

Light microscopy

Immunohistochemistry for light microscopy followed previously described protocols (11). Briefly, 2- to 4- μ m-thick sections were deparaffinized and incubated in 3% hydrogen peroxide in methanol to block endogenous peroxidase activity. Slides were steamed in citrate buffer at pH 6.0 for antigen retrieval and incubated at 4 C overnight with primary antibodies. The Dako

EnVision detection kit, peroxidase/diaminobenzidine, rabbit/mouse (DakoCytomation) was used for visualization of the antibody reaction. Negative controls were performed by omitting the primary antibody and using universal negative control rabbit (Dako) for polyclonal rabbit antibodies or purified mouse myeloma IgG1 (Zymed Laboratories, San Francisco, CA) for monoclonal mouse antibodies.

Fluorescence labeling

Fluorescence labeling was performed as described in detail previously (11, 12). The following well-characterized fluorescent secondary antibodies were used: Alexa Fluor-488- and Alexa Fluor-633-coupled goat antimouse IgG (Molecular Probes, Eugene, OR), Cy3-coupled donkey antirabbit IgG (Jackson ImmunoResearch Laboratories, West Grove, PA), and Cy5-coupled donkey antigoat IgG (Jackson ImmunoResearch). For double/triple labeling, appropriate combinations of primary and fluorescent secondary antibodies were used. In case of labeling with two monoclonal antibodies, the Zenon Alexa Fluor-488 coupled mouse IgG1 labeling kit (Molecular Probes, Leiden, The Netherlands) was used. The slides were analyzed with a Zeiss LSM 510 motorized confocal laser scan microscope (Carl Zeiss, Jena, Germany) equipped with an argon-ion laser source (488 nm excitation) and two helium neon lasers (543 and 633 nm excitation). To eliminate bleed-through from either channel, an appropriate combination of excitation and barrier filters (bandpass filter 505–530 nm and long-pass filters 560 nm and 650 nm) was used.

Electron microscopy and immunogold labeling

Small blocks of 4% paraformaldehyde-fixed pituitary gland ($3 \times 3 \times 3$ mm) were dehydrated and immersed in Unicryl (British BioCell International, Cardiff, UK) according to the manufacturer's protocol. Polymerization was done at 60 C overnight. Ultrathin sections were cut and collected on nickel grids. After drying for 3 h at 56 C, grids were steamed for 20 min in citrate buffer (pH 6.0). Then grids were preincubated with Dako wash buffer (DakoCytomation) with 0.1% BSA for 10 min, followed by incubation of a mixture of primary antibodies (α -ALDP, 1:25, and α - β -LPH, 1:50, or α -ALDP and α -PMP70, 1:50) at 4 C overnight. After rinsing in Dako wash buffer, grids were incubated with a mixture of 20 nm gold-conjugated antirabbit and 10 nm gold-conjugated antimouse immunoglobulins, both 1:50 (British BioCell International) for 1 h at room temperature. Finally, grids were washed with double-distilled water, fixed with 2.5% glutaraldehyde (1 min), fixed with 2% osmium tetroxide (1 min), and contrasted with 0.2% lead citrate. We used a Zeiss electron microscope to evaluate the grids.

Plasmids

A cDNA comprising the open reading frame of human POMC was generated by PCR using PrimeSTAR HS DNA polymerase (Takara Bio Inc., Shiga, Japan), the oligonucleotides: 5'-GTCCGACATGCCGAGATCGTGCTGC-3' (1390, forward) and 5'-GGTACCTCACTCGCCCTTCTTG-3' (1100, reverse) and human pituitary cDNA as template, which was generated using the RNeasy minikit (QIAGEN, Valencia, CA) and the RNA-PCR core kit (Applied Biosystems, Foster City, CA) following the manufacturer's instructions. The PCR fragment was cloned into pCR2.1 (Invitrogen, Groningen, The Netherlands) and the nucleotide sequence confirmed (corresponding to GenBank accession no. NM 000939). The open reading frame was excised by the restriction

TABLE 1. Cellular localization of POMC derivatives in human tissues

Tissue	ALDP ^a		ACTH		β-Lipotropin		β-Endorphin	
	Amount	Localization	Amount	Localization	Amount	Localization	Amount	Localization
Adenohypophysis controls (n = 16), X-ALD (n = 2) CNS (n = 5)	<i>b</i>	Corticotroph cells	<i>b</i>	Corticotroph cells	<i>c</i>	Corticotroph cells	<i>b</i>	Corticotroph cells
	<i>d</i>	Hypothalamus, basal nucleus Meynert, periaqueductal gray matter, locus coeruleus, dorsal root ganglia	<i>e</i>		<i>d</i>	Hypothalamus, basal nucleus Meynert, periaqueductal gray matter, locus coeruleus, dorsal root ganglia	<i>d</i>	Hypothalamus, basal nucleus Meynert, periaqueductal gray matter, locus coeruleus, dorsal root ganglia
	<i>c</i>	Cortex	<i>e</i>		<i>c</i>	Cortex	<i>c</i>	Medulla, cortex
Adrenal gland controls (n = 8), X-ALD (n = 2) Testis (n = 4)	<i>d</i>	Leydig cells, Sertoli cells	<i>e</i>		<i>d</i>	Leydig cells, Sertoli cells	<i>d</i>	Leydig cells, Sertoli cells
	<i>c</i>	Hair follicle, excretory component of eccrine gland	<i>e</i>		<i>c</i>	Hair follicle, excretory and secretory component of eccrine gland, sebaceous gland	<i>f</i>	Hair follicle, excretory and secretory component of eccrine gland, sebaceous gland
Liver (n = 7)	<i>b</i>	Hepatocytes, macrophages	<i>e</i>		<i>b</i>	Hepatocytes, macrophages	<i>b</i>	Hepatocytes, macrophages
Kidney (n = 6)	<i>b</i>	Distal tubule	<i>e</i>		<i>d</i>	Distal tubule	<i>d</i>	Distal tubule

^a Only in controls, not in X-ALD tissues; ^b marked; ^c moderate; and ^d slight positivity; ^e not detectable immunohistochemically; ^f most cells are negative or show very low amounts.

enzymes *Sall* and *KpnI* and ligated to the mammalian expression vector pCI (Promega, Madison, WI) cut with the same enzymes to generate pPOMC (P929). The expression plasmid encoding POMC with a C-terminal myc-tag was generated by PCR using the plasmid pPOMC as template and the oligonucleotides (1390) and 5'-GGATCCCTCGCCCTTCTGTAGGCGTTCTTAG-3' (1350, reverse). The PCR product was cloned into pCR2.1, sequenced, and excised from the plasmid by the restriction enzymes *Sall* and *BamHI*. This fragment was ligated with a 450-bp fragment encoding enhanced green fluorescent protein, excised from EGFP_N3 (CLONTECH, Palo Alto, CA) using the restriction enzymes *NdeI* and *Sall* and with a *BamHI*- and *NdeI*-restricted, modified variant of pcDNA3.1(zeo) that encodes the myc-epitope (GAATTCTGCAGTCGAGCAGAAGCTGATCTCTGAGGAGGACCTGTAAGAATTC) inserted in the *EcoRI* site (derived from the vector pBABE-puro-myc, kindly provided by Dr. Egon Ogris, Medical University of Vienna), resulting in the plasmid pPOMC-MYC (P912). A furin cleavage site was introduced at the ACTH/ β -LPH junction of POMC in both plasmids (pPOMC and pPOMC-MYC) by *in vitro* mutagenesis using the QuickChange site-directed mutagenesis kit (Stratagene, La Jolla, CA) according to the manufacturer's instructions and the oligonucleotides 5'-CCCCCTGGAGTTCagaAAGaggAGGGAGCTGACTGG-3' (forward) and 5'-CCAGTCAGCTCCCTcctCTTtctGAACTCCAGGGGG-3' (reverse), resulting in the plasmids pPOMC-(furin) (P949) and pPOMC-MYC(furin) (P942).

Cell culture

The human cervix carcinoma cell line HeLa was purchased from American Type Culture Collection (Manassas, VA). Cells were cultivated in DMEM supplemented with 10% fetal calf serum, 2 mM L-glutamine, 50 U/ml penicillin, and 100 μ g/ml streptomycin (Lonza BioWhittaker, Basel, Switzerland). Cells were transfected using Lipofectamine 2000 (Invitrogen, Paisley, UK) according to the manufacturer's instructions. Forty-eight hours after transfection, cells were fixed for 15 min with 4% paraformaldehyde in PBS. Cells were washed and permeabilized for 5 min with 0.1% Triton X-100 in PBS and blocked in blocking solution: PBS with 2% fetal calf serum, 5% BSA (Roche Diagnostics, Mannheim, Germany) and 0.1% fish skin gelatin (Sigma, St. Louis, MO). After incubation with primary antibodies from different species [rabbit polyclonal α - β -LPH (Neuromics, Northfield, MN; 1:300) and α -PMP70 (ABR, Golden, CO; 1:2000); mouse monoclonal α -ACTH (Santa Cruz Biotechnology, Santa Cruz, CA; 1:800), α -MYC (Upstate, Temecula, CA; 4A6), and α -ALDP (1D6, 1:600; Euromedex)], the slides were washed with PBS several times and exposed to compatible secondary antibodies (Cy2 and Cy3 labeled goat- α -rabbit IgG and goat- α -mouse IgG, 1:100; Jackson ImmunoResearch). Finally, cells were mounted in 3% 1,4-diazabicyclo (2, 2, 2)octane (Sigma) in PBS/glycerol (1:9).

Results

β -LPH and β -endorphin are compartmentalized to peroxisomes of POMC-producing cells of the pituitary gland

We have recently shown that in the anterior pituitary gland, only ACTH-positive cells harbor ALDP-positive

peroxisomes and that, on the subcellular level, these peroxisomes were either randomly distributed throughout the cytoplasm or arranged in ring-like structures (4). Now we immunohistochemically confirmed that these ACTH-positive cells of the anterior lobe also contain β -LPH and β -endorphin (Fig. 1, A–C) as well as α -MSH and γ -MSH (data not shown). On the subcellular level, the ALDP-positive ring-like structures colocalized with the peroxisomal matrix protein catalase (Fig. 1D) but not with chromogranin A-labeled secretory vesicles (Fig. 1E). The hormones β -LPH and β -endorphin showed a diffuse, granular staining pattern, consistent with secretory vesicles, but were, additionally, in a proportion of cells, arranged in ring-like structures resembling the pattern of peroxisomes. The β -LPH or β -endorphin signal of these ring-shaped structures and also of some other dot-like structures colocalized with ALDP, indicating that these peptide hormones are associated with peroxisomes (Fig. 1, F and G). In contrast, no colocalization of ACTH, α -MSH, or γ -MSH with peroxisomal marker proteins was detectable (Fig. 1, H–J).

In addition, we performed immunoelectron microscopy of the intermediate lobe of a pituitary gland to support these findings at the ultrastructural level. Thereby we detected vesicular structures that were labeled with the β -LPH antibody (Fig. 2A), indicating secretory vesicles, and numerous electron-dense, morphologically distinct vesicular structures that were labeled with ALDP and PMP70 antibodies (Fig. 2B), identifying them as peroxisomes. Interestingly, clusters of peroxisomes and secretory vesicles were often found in close proximity (Fig. 2C). Moreover, some of the dark, ALDP-positive peroxisomes were also positive for β -LPH (Fig. 2, C–E), confirming the luminal localization of β -LPH in ALDP-positive peroxisomes.

The peroxisomal localization of β -LPH and β -endorphin can be observed in many specific cells types of different organs throughout the human body

In a next step, we investigated whether the peroxisomal localization of β -LPH and β -endorphin is featured in other human tissues that express high levels of ALDP (Table 1). In general, the expression levels of β -LPH and β -endorphin were highly variable in different tissues of individual persons.

Different neuronal cells of the central nervous system and dorsal root ganglia were evaluated by immunohistochemistry and confocal analysis. Two samples of dorsal root ganglia showed β -LPH and less pronounced β -endorphin in neuronal cells (Fig. 3, A and C). Moreover, β -LPH and, when present, β -endorphin colocalized with

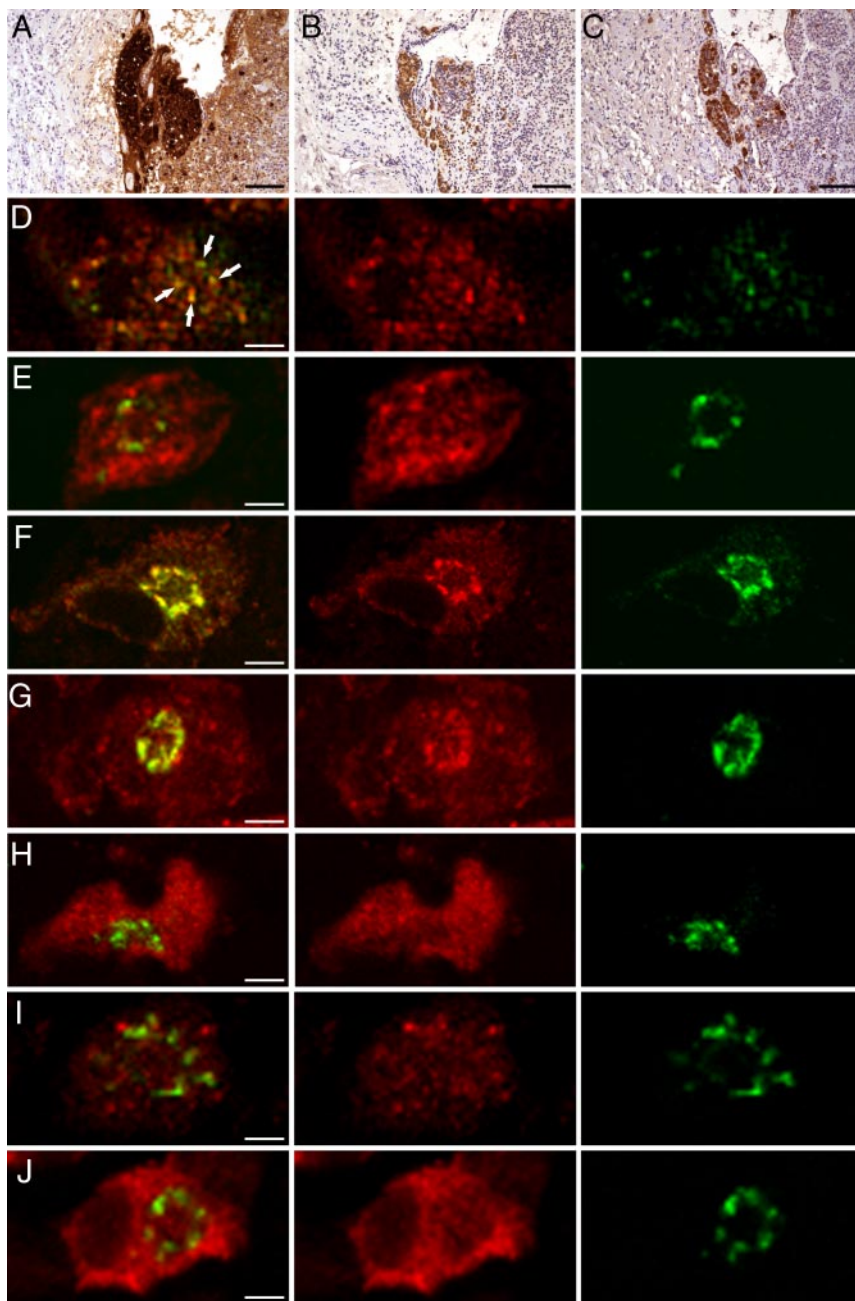


FIG. 1. Immunohistochemical detection of ACTH, β -LPH, and β -endorphin in human pituitary gland. By immunohistochemistry (A–C), POMC-producing cells of the pituitary gland are positive for ACTH (A), β -LPH (B), and β -endorphin (C). By double-immunofluorescence labeling (D–J), the peroxisomal marker catalase (red) colocalizes with ALDP (green, arrows) also arranged in ring-like structures (D). Secretory vesicles are positive for the specific marker chromogranin A (red) and do not colocalize with ALDP (green) (E). Double labeling of either β -LPH (red) (F) or β -endorphin (red) (G) with ALDP (green) reveals a peroxisomal localization of these peptide hormones. ACTH (H), α -MSH (I), and γ -MSH (J) (red) show a diffusely punctuated staining pattern, which does not colocalize with ALDP (green) in the overlay. Original magnification (A–C), $\times 100$, bar, 50 μ m; (D–J) $\times 1000$, bar, 5 μ m.

ALDP (Fig. 3, B and D). In the brain, β -LPH (five of five samples), and to a lesser extent β -endorphin (four of five samples; likely due to sensitivity of the immunohistochemistry), showed the same distribution as ALDP, with a granular staining pattern in hypothalamus, basal nucleus of Meynert, periaqueductal gray matter, dorsal nucleus of

vagus, and locus coeruleus (data not shown), whereas ACTH was not detectable immunohistochemically (Table 1).

Five skin samples were evaluated by immunohistochemistry and confocal laser microscopy. We found strong immunoreactivity for β -LPH in the secretory and excretory portions of the eccrine glands in four of five samples (Fig. 3E) and less reactivity in the hair follicles and the basal layer of sebaceous glands in three of five samples. β -Endorphin was visible in hair follicles in one sample and occasionally observed in the eccrine glands. In the excretory portion of the eccrine glands, in which high amounts of ALDP were found (Table 1), β -LPH and ALDP were largely colocalized (Fig. 3F). In the secretory portion of the eccrine gland, no ALDP was visible; however, larger, nonperoxisomal β -LPH-positive vesicles could be found. There was no colocalization of ALDP with β -endorphin in the eccrine glands (data not shown) and ACTH was not detectable.

Hepatocytes of the liver (seven of seven) and Sertoli and Leydig cells of the testis (three of four samples) showed granular staining of β -LPH and β -endorphin, which partly colocalized with ALDP (Table 1).

The peroxisomal localization of β -LPH is restricted to ALDP-positive cells

We recently observed a specific expression pattern of ALDP in the kidney (4). Whereas catalase-positive peroxisomes were found in variable amounts in all tubules (Fig. 3K), peroxisomes colabeled for both catalase and ALDP were confined to the distal tubules (Fig. 3L). Consequently, we investigated whether POMC-derived peptides are similarly distributed in this organ. In fact, a granular staining of β -LPH and β -endorphin was found in four of six

samples and was restricted to the ALDP-positive distal tubules, whereas the proximal tubules remained negative (Fig. 3, G and I). Focally, also γ -MSH (Fig. 3M) and α -MSH (data not shown) stained some of the distal tubule cells (Fig. 3M). As illustrated by the double-immunoflu-

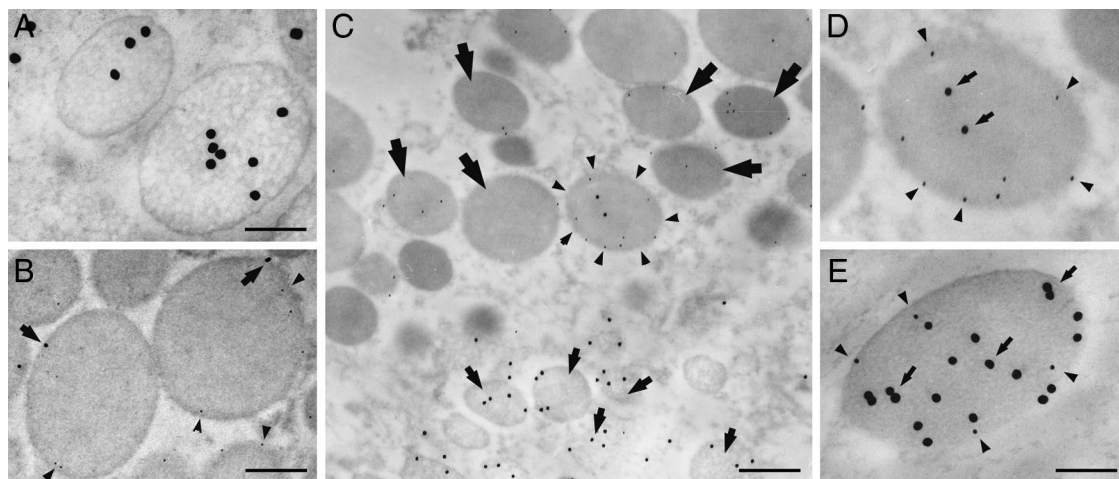


FIG. 2. Immunoelectron microscopy in human pituitary cells. Pituitary corticotroph cells contain numerous secretory granules immunoreactive for β -LPH (A, 20 nm gold particles) as well as peroxisomes that are characterized by more intense electron density (B; ALDP, 10 nm gold particles, arrowheads; PMP70, 20 nm gold particles, arrows). Double-immunogold technique shows clusters of β -LPH-positive secretory granules (20 nm gold particles, small arrows) that are localized next to clusters of ALDP-positive peroxisomes (C; 10 nm gold particles, large arrows). Moreover, some peroxisomes whose membranes are immunoreactive for ALDP (10 nm gold particles, arrowheads) show the presence of β -LPH (20 nm gold particles, arrows) within their vesicle (C–E). Magnification (A and E), $\times 85,000$, bar, 90 nm; (B) $\times 30,000$, bar, 250 nm; (C) $\times 20,000$, bar, 400 nm.

orescence images, β -LPH (Fig. 3H) and β -endorphin (Fig. 3J) colocalized with ALDP, but γ -MSH did not (Fig. 3N).

In the same way, we investigated the adrenal gland, in which only the cortex shows ALDP expression, whereas the cells of the adrenal medulla remained ALDP negative (4). Again, β -LPH was restricted to the ALDP-positive cortical cells (Fig. 4A), whereas the adrenal medulla remained β -LPH negative. Conversely, β -endorphin was mainly found in the adrenal medulla (Fig. 4E) but only in low amounts in the cortex (Fig. 4C). α -MSH and γ -MSH were found in variable amounts in cortex as well as in the medulla (data not shown). Both β -LPH (Fig. 4B) and β -endorphin (Fig. 4D) colocalized with ALDP-positive peroxisomes in the cortex (Fig. 4B, D). However, β -endorphin did not colocalize with catalase-positive/ALDP-negative peroxisomes in the medulla (Fig. 4F).

The peroxisomal targeting of β -LPH can be modeled in a cell culture system

To test whether peroxisomal β -LPH can be generated from the normal POMC protein, this precursor was ectopically expressed in HeLa cells. However, even after overexpression of POMC, β -LPH was hardly detectable, probably due to a lack of the processing PC1 activity. Therefore, we slightly modified the PC1 cleavage site to generate a cleavage site (RKRR↓) for the ubiquitous endopeptidase furin (13) in the POMC protein between proACTH and β -LPH (Fig. 5A). Furin was originally found in the ER but appears predominantly in the trans-Golgi-network (14, 15), in which the initial PC1 cleavage of POMC also normally occurs. When we transiently expressed this modified POMC variant in HeLa cells, β -LPH was well detectable in a subfraction of transfected cells and colocalized with ALDP (Fig. 5B) but not with

the N terminally derived peptide ACTH (Fig. 5C). This result indicates that β -LPH derived from POMC within the secretory apparatus can be targeted to peroxisomes with a different intracellular fate than ACTH. Moreover, we tagged this modified POMC variant with a C-terminal myc-epitope, which allows the detection of all POMC-derived peptides containing the C terminus of the protein. When this construct was transiently expressed in HeLa cells, we found that in some cells the myc-epitope partly colocalized with the peroxisomal marker PMP70 (Fig. 5D), whereas in other cells the myc-tag was solely found in other structures resembling the ER and the Golgi apparatus but not in peroxisomes (Fig. 5E). These results are in good agreement with the observation that only in a fraction of transfected cells, the level of β -LPH that is generated and transported to peroxisomes, is sufficient for visualization.

The peroxisomal targeting of β -LPH and β -endorphin appears to be ALDP independent

Because the peroxisomal localization of β -LPH and β -endorphin occurred only in ALDP-positive cells, we investigated the subcellular localization of β -LPH and β -endorphin in tissues derived from patients lacking functional ALDP due to mutations in the *ABCD1* gene. We found that in pituitary and adrenal gland cells of these patients, β -LPH and β -endorphin colocalized with the peroxisomal marker catalase, indicating that functional ALDP is not required for peroxisomal targeting of these peptide hormones (Fig. 6, A and B).

Discussion

In our present study, we demonstrate for the first time that the POMC-derived peptide hormones, β -LPH and β -en-

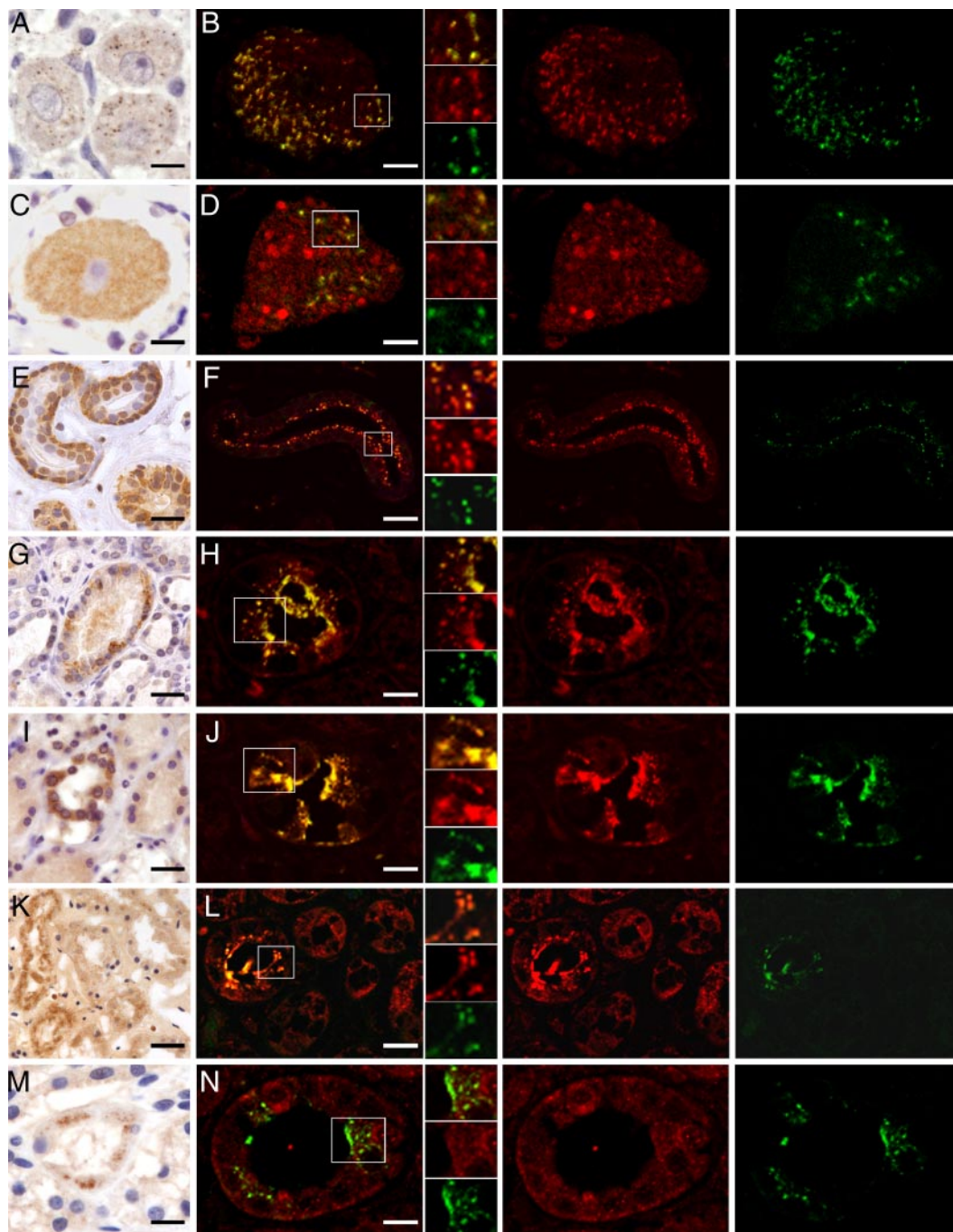


FIG. 3. Immunohistochemical detection of β -LPH and β -endorphin in human dorsal root ganglia, skin, and kidney. By light microscopy (A, C, E, G, I, and K), neurons of the dorsal root ganglia (A) are positive for β -LPH (A) and β -endorphin (C). By confocal laser microscopy (B, D, F, H, J, and L), colocalization of β -LPH (red) (B) and β -endorphin (red) (D) with ALDP (green) is seen in the dorsal root ganglia. High amounts of β -LPH are detectable in the excretory and secretory components of the eccrine gland in the skin (E), colocalization of β -LPH (red) with ALDP (green) is detectable only in the excretory component (F). In the kidney, β -LPH (G) and β -endorphin (I) can be found in distal tubule cells and colocalize with ALDP (green) (H and J). Whereas the peroxisomal marker catalase (K) is ubiquitously found in the tubules of the kidney, ALDP (green) is confined to the distal tubules (L, catalase, red). γ -MSH is found in distal tubule cells (M) but does not colocalize with ALDP (green) (N, γ -MSH red). Original magnification (A and C), $\times 600$; (B, D, E, and G–M), $\times 400$; (F, and N), $\times 630$. Bar (A–D), 12 μ m; (E–G, I, and K–L), 25 μ m; (H, J, and M–N), 17 μ m.

dorphin, can be localized in peroxisomes of various cell types of the human body, which have in common that they normally express high levels of ALDP. These observations indicate that β -LPH and probably also β -endorphin (no data for maturation) can be actively diverted from the

secretory apparatus and transported to peroxisomes, suggesting a functional link between peroxisomes and β -LPH or β -endorphin.

In general, maturation of peptide hormones from a precursor molecule in the late secretory apparatus is con-

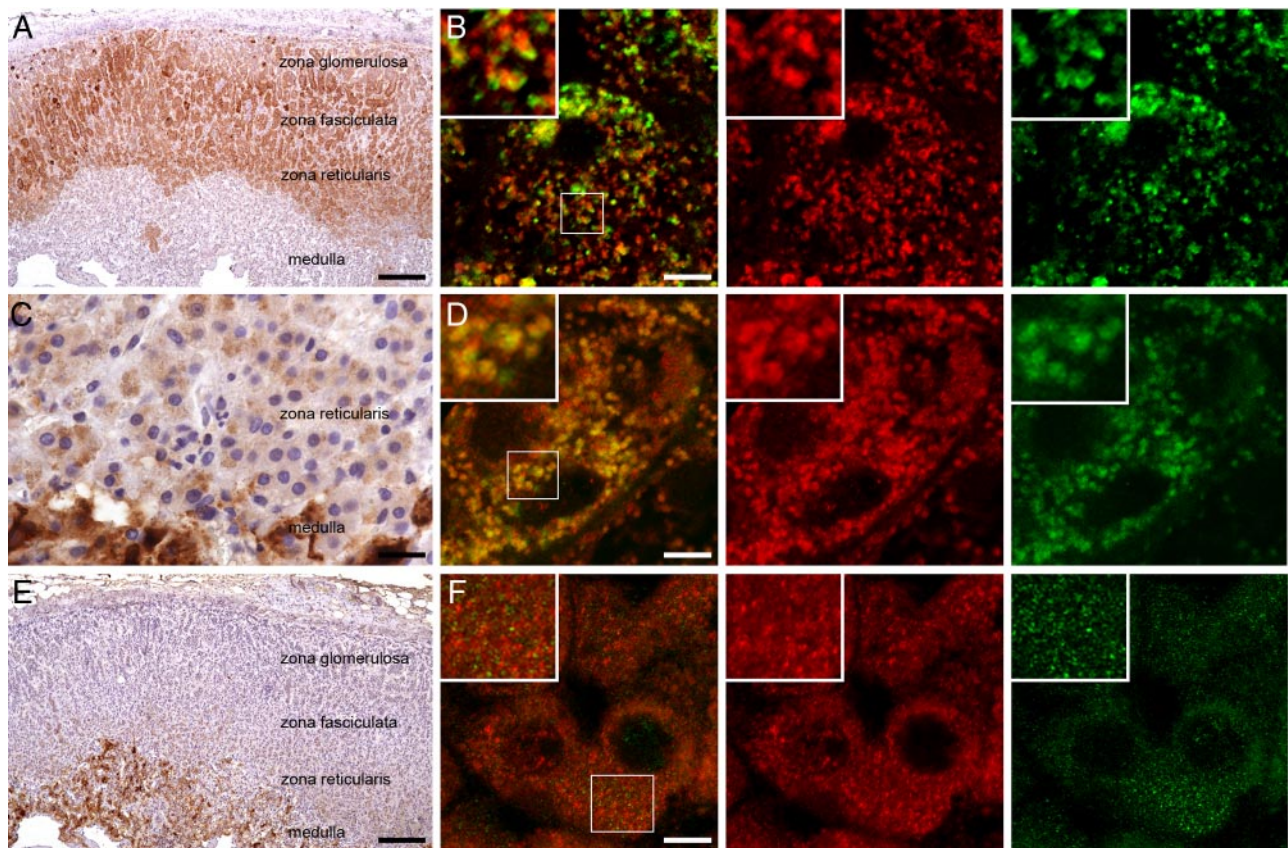


FIG. 4. Immunohistochemical detection of β -LPH and β -endorphin in the human adrenal gland. By immunohistochemistry (A and C), the adrenal cortex shows strong immunoreactivity for β -LPH (A), whereas adrenal medulla is virtually negative. By double-immunofluorescence labeling, β -LPH (red) partly colocalizes with ALDP (green) (B). In contrast, β -endorphin is weakly stained in the cortex (C) and mainly expressed in the medulla (C and E). β -endorphin (red) colocalizes with ALDP (green) in the cortex (D). No colocalization of β -endorphin (red) with the peroxisomal marker catalase (green) is visible in the medulla (D). Original magnification (A and E), $\times 40$, bar, 240 μm ; (B, D), $\times 630$, bar, 6 μm ; (C), $\times 400$, bar, 25 μm ; (F), $\times 1000$, bar, 6 μm .

nected to the regulated secretion of these hormones on demand. However, the peroxisomal localization of β -LPH and β -endorphin suggests that a subfraction of the mature, POMC-derived peptides can be rerouted within the cell and thus escapes from secretion. Until now, the localization of peptide hormones in peroxisomes has not been described. It remains to be investigated whether these peptides serve as intracellular messengers addressed to the peroxisome or are primarily excluded from secretion and destined for degradation. Interestingly, the peptidase insulin degrading enzyme can be detected in peroxisomes (16). Insulin degrading enzyme is a multifunctional protease in cellular regulation and a possible role in the degradation of intraperoxisomal peptide-hormones might be considered.

In the adrenal gland, we observe different expression patterns of C-terminally derived hormones, which originate in the different processing of the precursor POMC by either PC1/3 alone in the cortex or PC1/3 and PC2 in the medulla. Whereas β -LPH was detected only in the cortex, β -endorphin was mainly found in the medulla. The divergent occurrence of POMC-derived peptide hormones in

adrenal cortex and medulla reflects very well the different physiological functions. Whereas endocrine secretion of medullary β -endorphin mediates stress-induced analgesia, paracrine and autocrine cortical ACTH and γ -MSH regulate steroidogenesis. Interestingly, in all tissues and cell types that secrete peptide hormones derived from the N-terminal part of POMC, such as γ -MSH in the adrenal cortex or kidney, α -MSH in the skin, and ACTH in the pituitary gland (8, 17, 18), β -LPH and β -endorphin are associated with peroxisomes. In contrast, in tissues, that secrete peptide hormones derived from the C-terminal part of POMC, such as β -endorphin in the adrenal medulla, no localization of the peptide hormone in peroxisomes was observed. Thus, it could be considered that whenever β -LPH or β -endorphin arises as by-products during maturation and secretion of the N-terminal part of POMC, they are transported to the peroxisome to prevent them from secretion.

The observation that large amounts of β -LPH occurred selectively in tissues and cell types that harbor high levels of the peroxisomal ATP-binding cassette transporter protein ALDP, suggests a link between this protein and

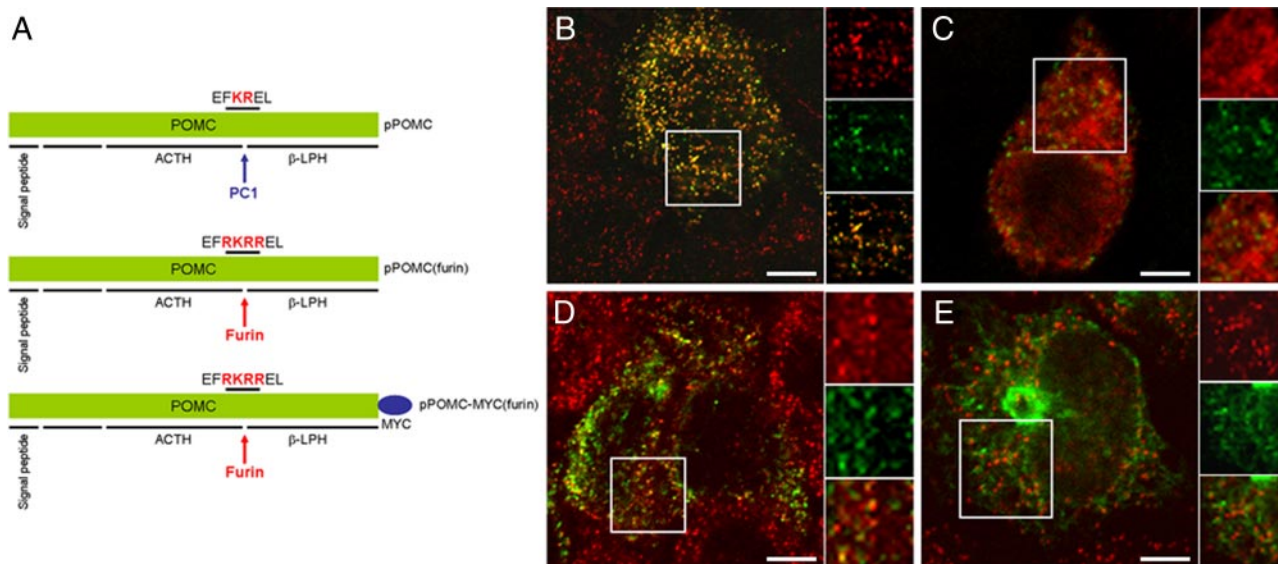


FIG. 5. Ectopically expressed β -LPH colocalizes with the peroxisomal marker ALDP but not with ACTH in HeLa cells. Schematic drawing of the modified variants of human POMC (A): POMC(furin), which encodes a furin cleavage site between ACTH and β -LPH instead of the PC1 cleavage site, and POMC-MYC(furin) with a C-terminal myc-tag as indicated. By double-immunofluorescence labeling (B–E), HeLa cells transfected with the plasmid encoding POMC(furin) show colocalization of β -LPH (green) with ALDP (red) (B) but not of β -LPH (green) with ACTH (red) (C). Expression of POMC-MYC(furin) results in colocalization of the C-terminal myc-epitope (green) with the peroxisomal membrane protein PMP70 (red) in a subset of cells (D) but not in others (E). Original magnification (B–E), $\times 1000$, bar, 4.5 μ m.

β -LPH. In contrast, β -endorphin was also well detectable in tissues lacking ALDP (*e.g.* adrenal medulla), but the peroxisomal targeting of β -endorphin correlates well with the presence of ALDP because in the adrenal medulla, this peptide hormone was found only in extraperoxisomal vesicular structures. However, ALDP is probably not directly required for the transport of β -LPH and β -endorphin into peroxisomes because the peptides seem to be also colocalized with peroxisomes of tissues from patients lacking functional ALDP due to a mutation in the *ABCD1* gene.

The disease associated with ALDP dysfunction, X-ALD, is characterized by an accumulation of VLCFA in tissues and body fluids and presents with various pathological signs such as the demyelination of the central

nervous system (with differing severity), peripheral neuropathy with atrophy of the dorsal root ganglia, and endocrinological abnormalities including atrophy of the adrenal cortex and hypergonadotropic hypogonadism. This overlap of the sites of pathology with the peroxisomal localization of β -LPH further corroborates the hypothesis that this localization of β -LPH is specifically linked to ALDP function. It remains to be investigated whether cellular changes that are caused by the lack of functional ALDP in X-ALD patients influence synthesis rate, processing, or secretion of POMC-derived peptides. Previous descriptions are mostly limited to the altered regulation of ACTH secretion due to the decreased feedback signaling via the hypothalamus-pituitary-adrenal axis, which is often associated with the adrenocortical insufficiency in

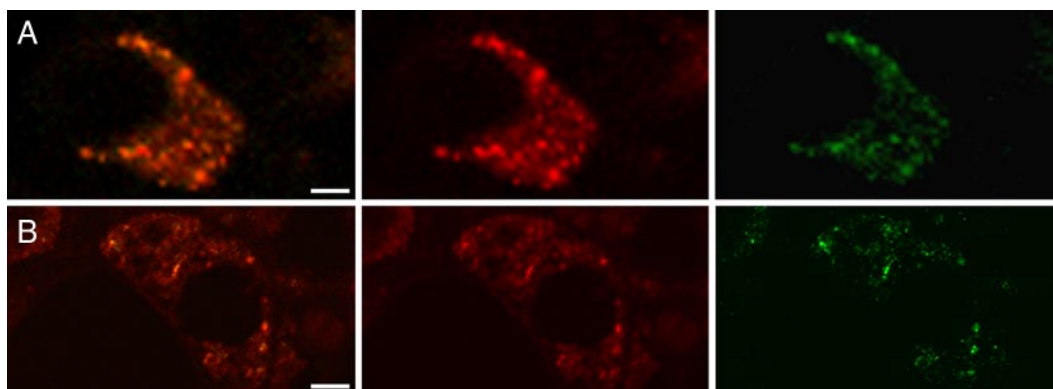


FIG. 6. Targeting of β -LPH and β -endorphin to peroxisomes is ALDP independent. Colocalization of β -endorphin (A, red) and β -LPH (B, red) with catalase (green) can also be detected in pituitary gland (A) and adrenal gland (B) of X-ALD patients lacking functional ALDP. Original magnification, $\times 1000$, bar, 4 μ m.

X-ALD. Our current observations could add a further level of complexity to the endocrinological aspects of X-ALD pathology, which are usually attributed to local toxicity of an accumulation of VLCFAs.

In summary, our data provide evidence for a novel subcellular localization of the POMC-derived peptide hormones, β -LPH and β -endorphin, in peroxisomes, which are not recognized as part of the secretory system. This could represent a hitherto unknown intracellular function of these peptide hormones, which complements the well-characterized auto-, para-, and endocrine roles of these hormones. It remains to be elucidated whether and how this links peroxisomal disorders with endocrinal dysfunction.

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